Are *Stemphylium* spp. Seed Borne Pathogens of Pea (*Pisum sativum* L.)?

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The effects of *Stemphylium* spp. on pea seeds and plants were studied in four controlled environment and laboratory experiments. Laboratory seed health testing of commercial pea seed lots established that *Stemphylium* spp. can be seed borne in peas, with infection levels varying from 0 to 46% depending on production season and cultivar. *Stemphylium* spp. were isolated from naturally infected marrowfat pea seeds, grown on artificial culture media, and their characteristics described. Molecular tests performed by the Plant Pathology Laboratory, USDA/ARS (USA), confirmed the five main species isolated were: *S. loti* Graham, *S. vesicarium* (Wallr) Simmons, *S. herbarum* Simmons, *S. astragali* Yoshii and *S. sarciniforme* (Cavara) Wiltshire. *Stemphylium* spp. did not kill seeds or affect laboratory seed germination. The species of *Stemphylium* isolated from seeds are likely to vary in their pathogenicity and ability to reduce viability.

Of the isolates, an isolate of *Stemphylium herbarum* produced the most conidia. This isolate was tested in a third experiment in order to determine its pathogenicity on pea plants. Plants at three different stages of development were inoculated with sterile distilled water, sterile distilled water plus Tween, *S. herbarum* conidial suspension and *S. herbarum* conidial suspension plus

Tween. Under the conditions (mean temperature of 19°C and 100% relative humidity) *S. herbarum* caused lesions on pea leaves, especially in the early stages of plant development (seedlings at the 3- 5 node stage). Symptoms observed were brown, irregular to oval shaped lesions, approximately 6mm in diameter. On a scale of 0-5 seedlings inoculated with conidial suspension of the fungus had a mean disease score of 2 while untreated plants had a disease score of 0. The mean leaf diseased tissue area was also higher for seedlings inoculated with *S. herbarum* conidial suspension than for untreated seedlings (29 mm^2 cf. 0 mm^2, respectively). Scanning electron microscopy revealed that *S. herbarum* penetrated the leaf tissue via the epidermis as well as the stomata. Infection was more successful on seedlings than adult plants. *Stemphylium herbarum* was re-isolated from infected tissue, thus fulfilling Koch's Postulates. Movement of the fungus from infected leaf tissue to seeds was not demonstrated.

Infected seeds may be the first source of inoculum of *Stemphylium* spp., ensuring the perpetuation of the fungi and their spread to new areas. Experiment four tested the effectiveness of registered seed treatments for peas (Aliette super, Apron XL and Wakil XL) and a hot water soak treatment (50 ± 0.5°C for 30 minutes) for control of *Stemphylium* spp. Chemicals had limited success in controlling *Stemphylium* spp. infection. Hot water treatment was the most effective method, eliminating 100% of *Stemphylium* spp. from the seed lots tested, but it was harmful to the seeds, reducing germination by approximately 34 %.

Keywords: *Stemphylium*, seed, peas, seed borne, pathogenicity, seed treatment.

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1 General introduction

1.1 Overview

New Zealand is internationally recognized as an important pea growing country. High quality standards for pea seeds are achieved through top technology, careful processing and a favourable climate during harvest. Peas have been grown in New Zealand since the beginning of the 20th century and the main producing areas are Canterbury and Hawke's Bay (White 1987). In 2004 around 10,000 hectares of dry peas were harvested with a total production of 31,000 tonnes (FAO 2004). This product is mainly exported for human consumption as cooked canned peas or for snack food. Processed peas are worth NZ$ 96 million each year, NZ$ 51 million for export and NZ$ 45 million for the domestic market, while seeds and peas for consumption are estimated to be worth approximately NZ$ 50 million annually (J. G. Hampton, personal communication, 15 October 2003).

The traditional and most common dried pea for the majority of farmers and processors is the marrowfat type. Marrowfat peas have large drum-shaped seeds with excellent texture and flavour. They are grown to the fully mature stage, at which point they are either used for seed production or canned after rehydration.

Quality and yield are the key points to ensure profitability in pea production. The export market requires a high quality product, which basically involves cooking properties associated with attractive appearance (shape, size and colour). For seed production the relevant characteristics are high seed germination, high vigour and good health.

Pea plants are subjected to a wide range of diseases which may affect seed/grain quality and yield. Fungi play an important role among the microorganisms that attack peas during seed production. Fungi cause damage at several stages of crop development and on different parts of the plant - from roots to leaves and pods, and consequently seeds. The occurrence of fungi in pea seeds may affect the visual appearance of the grain (discolouration or spots) as well as the weight of seeds. Both characteristics are undesirable for the food or seed industry.

Common disease problems occurring in pea growing areas in Canterbury include the Ascochyta disease complex (*Phoma medicaginis* var. *pinodella* (L.K. Jones), *Ascochyta pisi* (Lib.) and *Mycosphaerella pinodes* (Berk. et Blox.) Vestergren), powdery (*Erysiphe pisi* Syd.) and downy mildew (*Peronospora viciae* (Berk)). These diseases lead to decreased yields and generally lower product quality with poor colour and shrivelling problems. Fungi that form the Aschochyta complex are transmitted by seeds, and seed health testing is necessary to determine whether seed lots carry the pathogens. The use of disease free seeds is important in controlling the multiplication of the pathogens and raising better crops.

Seed infected or contaminated with fungi may have low germination and perform poorly in the field. The importance of fungal diseases in pea crops varies considerably according to the weather or crop management. Most fungi need specific levels of humidity and temperature to develop. Under optimum conditions, other fungi considered as "weak pathogens" might become a problem in pea crops. They can occur in combination with other important fungal types and contribute to reducing seed yield and quality.

Stemphylium spp. are considered one of these "weak" pathogens. Some species of this genus are important pathogens causing leaf lesions in a number of crops. Multiple species of *Stemphylium* affect asparagus (*Asparagus officinalis* L.) and clover (*Trifolium* spp.) (Bradley et al. 2003). *Stemphylium solani* G. F. Weber is a main pathogen of tomato (*Lycopersicon lycopersicum* L.) (Basset et al. 1978) and a group of *Stemphylium* spp. (*S. botryosum* Wallr, *S. alfalfae* Simmons, *S. globuliferum* Vestergr, *S. herbarum* and *S. vesicarium*) are responsible for leaf spot on lucerne (*Medicago sativa* L.) and are considered seed borne in lucerne seeds (Lamprecht & Knox-Davies 1984; Hoffman et al. 1998). More recently, *S. botryosum* has been associated with foliar lesions and poor performance of spinach (*Spinacia oleracea* L.) seeds in the United States (Koike et al. 2001; du Toit & Derie 2003).

In Australia *S. radicinum* (Meier) Drechsler & Eddy and *S. botryosum* have recently been recognized as causing poor germination in carrot (*Daucus carota* L.) seeds (Coles & Wicks 2001). In Taiwan *S. vesicarium* was negatively correlated with the seedling emergence rate of ornamental plants such as pot marigold (*Calendula officinalis* L.) (Wu 2001; Wu et al. 2001).

Stemphylium spp. are present in New Zealand. The first record of these fungi in Canterbury was made in 1986 in lucerne plants (Anonymous 2001). In 2001-2002 some pea seeds harvested in Canterbury and tested at Lincoln University by a commercial laboratory (New Zealand Seed Technology Institute Plant Diagnostic Laboratory - BioLinc) revealed the presence of *Stemphylium* spp. associated with fungi of the Ascochyta complex. Infected seeds had a "bruised" seed coat, which depreciates their commercial value. In addition, the germination

of seeds carrying *Stemphylium* fungi was considerably lower than that of healthy seeds.

Some seed lots were reported to be highly infected with *Stemphylium* spp. (up to 70% of seeds) (K.D.R. Wadia and R.G. Bakker, personal communication, 10 February 2003) suggesting that this fungus may be an important causal agent of low germination of pea seeds. There are few studies on the pathogenicity of *Stemphylium* species in pea plants in the literature although, *Stemphylium sarciniforme* (Cavara) Wiltshire has been reported as being non pathogenic in peas (Thanutong et al. 1982) . However, *Stemphylium* species have been associated with the Ascochyta complex and *Alternaria* spp. Nees ex Wallr on pea seeds (Faris Mokaiesh et al. 1995; Marcinkowska 1997).There are no detailed reports relating to *Stemphylium* spp. contamination or infection on pea plants. Additionally, there is no information in the literature about *Stemphylium* spp. interfering with pea seed quality. Research to determine the pathogenicity of *Stemphylium* spp. to pea seeds and plants is required. Also, if proven to be pathogens, methods to reduce their incidence through seed treatment will be required.

1.2 Objectives

The presence of fungi and their effects on seed germination were observed in several pea seed lots examined in the New Zealand Seed Technology Institute Plant Diagnostic Laboratory - BioLinc during 2001, 2002 and 2003. Poor germination, abnormal seedlings and dead seeds were often associated with the presence of fungi in the samples. Some of these seed lots were tested at the BioLinc laboratory for fungal infection (Ascochyta complex assessment). The results indicated that some seed lots were infected with *Stemphylium* spp., which may be related to the poor germination performance of those seed lots. In

addition, *Stemphylium* spp. may also cause visual symptoms on pea plants. This project aimed to:

(i) investigate the extent of *Stemphylium* spp. infection in selected pea seed lots;

(ii) determine if the presence of *Stemphylium* spp. would affect pea seed performance ;

(iii) determine the pathogenicity to pea of *Stemphylium* strains isolated;

(iv) evaluate the effectiveness of registered pea seed treatments for the control of *Stemphylium* spp. infection.

1.3 Thesis structure

The thesis is organized into eight chapters. Chapter 1 brings an overview of the subject (General introduction), chapter 2 is the Literature review, chapter 7 consists of a general discussion and chapter 8 contains the conclusions. The investigation consisted of four experiments, which follow a sequence and complement each other. The thesis is subdivided according to the experiments in four main result chapters: chapters 3, 4, 5 and 6. Each chapter contains the sections: introduction, material and methods, results, discussion and a summary with main conclusions. Figure 1.1 shows a flow diagram of the thesis structure.

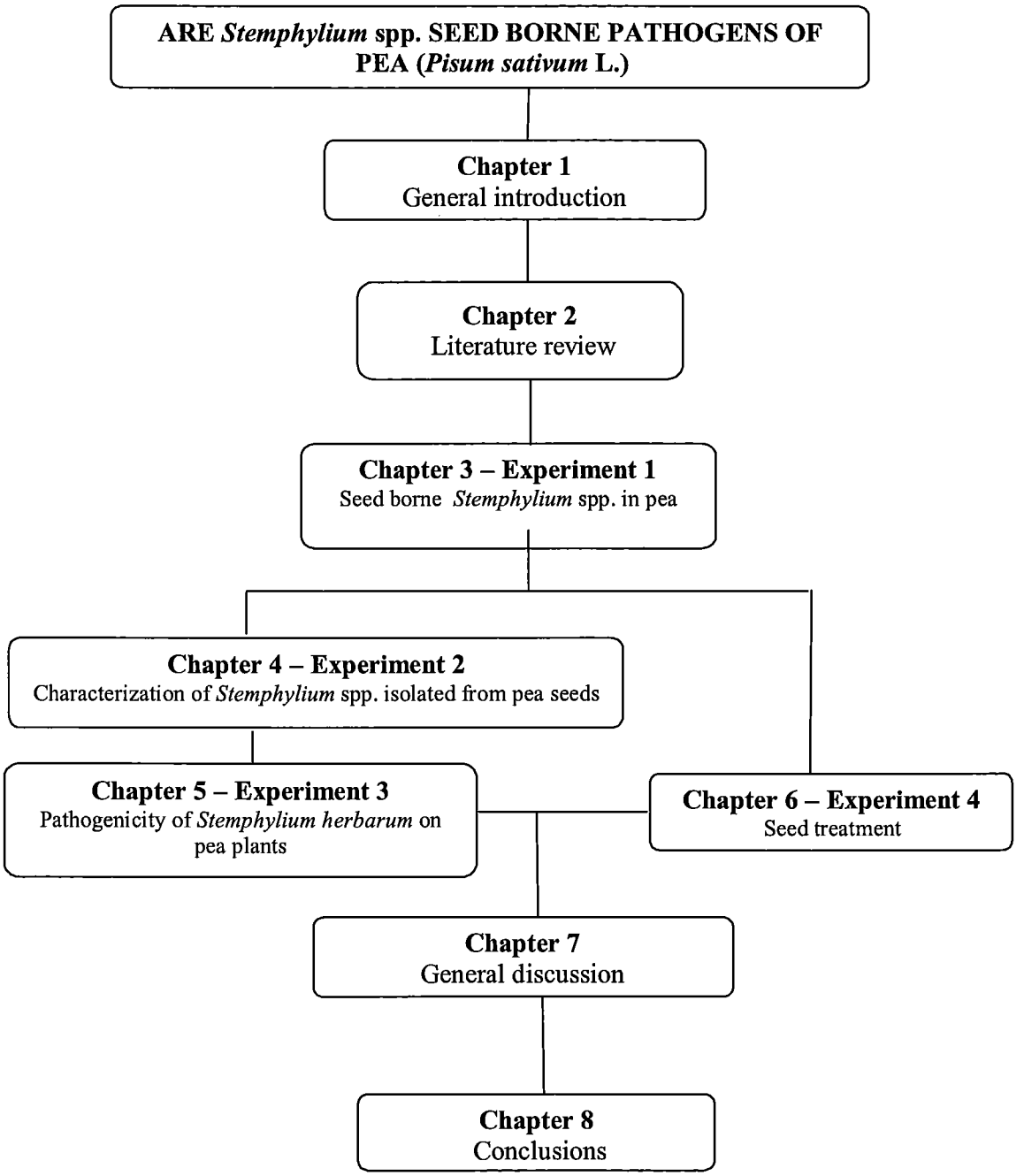

Figure 1.1 Graphic representation of the relationship of each chapter to the main objectives of the research and to other chapters.

2 Literature review

2.1 Peas

2.1.1 Pea origin and distribution

Pea (*Pisum sativum* L.) is native to the Eastern Mediterranean and to Western Asia (Davies et al. 1985). Modern cultivars have been introduced into Australasia, Africa, China, Europe, North America and India (Allen & Allen 1981). Pea crops are widely cultivated in temperate zones and as a cool season crop in tropical areas at high altitudes. In temperate climates most peas are spring sown crops, usually planted late August – November in New Zealand (or February-March in the Northern hemisphere) (Davies & Casey 1993).

2.1.2 Pea Production

Peas are an important source of protein for human consumption as well as for animal feeding (Lough 1987; White 1987). Farmers grow peas for cash return, to improve the levels of nitrogen in the soil, and as an option for cultural rotation, especially to break cereal disease cycles (White 1987).

Canada and France rank as the main producers of dried peas with a total production of 2.1 and 1.6 million tonnes per year, respectively. Annual production of green peas is headed by India (3.2 million tonnes annually) and China (2.0 million tonnes) (FAO 2003). In 2004 the production of peas in New Zealand was 31,000 and 55,000 tonnes of dry and green peas, respectively (FAO, 2004).

2.1.3 Pea Systematic

Pea plants are both wild and cultivated (Allen & Allen 1981) and belong to the Fabaceae family and Faboideae subfamily, a group of plants commonly known as pulses (Allen & Allen 1981; Sewell 1986). *Pisum sativum* is the type most widely used for human consumption. The subspecies *arvense* is used for human consumption or animal feed, whereas the subspecies *axiphium* (or sugar pea) is used for eating both pod and seed as a green vegetable (Gane 1985).

2.1.4 Morphology

Peas are annual and herbaceous plants with only one dominant shoot. The first two and three nodes bear trifid scale leaves. The next few nodes bear foliage leaves with a single pair of leaflets. Nodes above these have larger leaves with a greater number of pairs of leaflets (Pate 1975). Upper leaves and subterminal leaflets are modified as tendrils (Pate 1975; Trawally 1984). The leaves and tendrils are the principal means of plant photosynthesis, but the large green stipules and green pods also contribute to the process (Davies & Casey 1993). Leaf size usually increases up to the node of the first flower and then decreases thereafter (Davies et al. 1985).The root system consists of a main primary root and first and secondary lateral roots. In the presence of *Rhizobium* bacteria the roots have nodules responsible for nitrogen fixation. In standard genotypes, the inflorescences are axilary, each with one or two flowers on a peduncle (Davies et al. 1985). All peas are self-pollinated, diploid and the seeds may be wrinkled, dimpled or round depending on cultivar (Davies & Casey 1993).

2.1.4.1 Vegetative development

The vegetative development of a pea crop is dependent on suitable conditions of temperature and water. Peas require mild temperatures to grow and the production is largely concentrated in temperate zones, where mean temperatures range from 10°C to 30°C or in tropical areas at high altitude. Germination will

occur at temperatures as low as 4.4°C with a basal temperature of 1.4°C (Angus et al. 1981) although more suitable temperatures for germination of pea seeds are between 15.5 and 21.1°C. Emergence occurs between 5 to 14 days according to the cultivar, seedbed conditions and environment (Kennell 2003).

The vegetative development starts just after the appearance of the first true leaf and extends until the appearance of the first flower for determinate genotypes (Trawally 1984). The rate each new leaf or node appears varies according to the ambient temperature and cultivar. Experiments conducted in New Zealand have shown that the phyllochron (the period between the appearance of two successive nodes) with a basal temperature of 4.5°C ranged from 37° degree days (Cd) for cultivar Whero and 27°Cd for cultivar Massey. For cultivar Trounce the vegetative phase ceased when the plant accumulated an average 383°Cd (Wilson & Robson 1996).

2.1.4.2 Reproductive Development

The reproductive phase starts with the formation of small buds of approximately 6mm enclosed in the terminal shoot. However, it does not necessarily mark the end of the vegetative phase, as both can run concurrently, especially for more indeterminate genotypes (Knott 1987). A simplified description of the reproductive stages in garden peas is presented in Table 2.1 according to Gane et al. (1984) and Castillo (1992).

Peas are particularly sensitive to high temperatures in the reproductive phase. Temperatures above 26°C during blooming and fruit filling are negatively correlated with yields (Davies et al. 1985; Guilioni et al. 2003). There is no photoperiod sensitivity for floral induction or initiation for most genotypes

(Davies et al. 1985; Wilson & Robson 1996). However, short days may cause abortion of flowers in some genotypes (Davies et al. 1985).

Table 2.1 Reproductive stages (R) of peas (Gane et al. 1984; Castillo 1992).

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2.1.5 Pea seed development

Once fertilization of flowers has occurred seed development begins. Pea seed development consists of three main stages according to the water status.

- Phase 1: development of the embryo axis and cellular structures that accumulate reserve materials. There is a rapid increase in whole seed fresh weight, and moisture content is high and stable at 85 % (Bewley & Black 1994).

- Phase 2: seed filling. The seeds start depositing storage reserves after the pods have attained their maximum fresh weight (Desai et al. 1997).

Proteins accumulate in the embryo from carbon supplied mainly by pods and adjacent leaflets. As seed development progresses carbon is also mobilised from senescing tissues of the shoot and roots. The major pathway for this long distance transport from the vegetative parts is via the phloem (Bewley & Black 1994). At this phase the seed moisture content declines until it reaches approximately 55%. Physiological maturity is reached in the end of this phase (Desai et al. 1997; J. G. Hampton, personal communication, 10 June 2004).

- Phase 3: Desiccation phase. Seed moisture decreases from approximately 55% to 25%-15%. All plant tissues start to senesce and become brown. Fresh weight of all fruit parts and dry matter of the pod wall decrease (Knott 1987; Ney & Turc 1993).

There are differences in maturity between lower and upper pods and these are more evident for indeterminate cultivars. The maturity of all pods and seed evens up until all the seed is dry (Knott 1987). Maximum germination of pea seeds occurs a few days after physiological maturity. Seed maturity period (from anthesis to harvest) will vary according to genotype and the prevailing environmental conditions (Bewley & Black 1994). It may require 40 to 45 days in temperate areas like Australia, Canada, England and only 30 to 35 in hot areas such India (Davies et al. 1985).From pollination to the later stages of maturity and during harvest and processing seeds may be attacked by various microorganisms. Fungi in particular can contaminate or infect pea seeds under suitable environmental conditions, causing detrimental effects in terms of seed yield and quality (Agarwal & Sinclair 1997; Hampton 2003).

2.1.6 Seed structure

Mature legume seeds have three main components: the seed coat, the cotyledons and the embryonic axis which constitute 8, 90 and 2% of the seed, respectively. The seed coat or testa is the outer layer of the seed. Usually legumes have a moderately thick seed coat. The endosperm is short lived and at maturity it is reduced to a thin layer surrounding the cotyledons or embryo. After soaking and removing the seed coat the endosperm comes off and the reminder is composed of the embryonic structure, which includes the shoot (two cotyledons) and the radicle or embryonic root (Chakraverty et al. 2003).

2.1.7 Pea types

2.1.7.1 Classification according to growth habit

Peas can be classified according to their growth habit into dwarf and climbing types. Indeterminate plants can grow 1.5 m high or more if supported. As they are also indeterminate in their pattern of flowering the harvest of fresh pods is possible over an extended period of time (Pate 1975; Jermyn 1986).

However, to more suit modern agricultural practices and mechanical harvesting the plant was modified. New cultivars differ from the traditional indeterminate pea. They are at maximum 70 cm tall and with more uniform pod and seed maturation. In addition, semi leafless pea cultivars were created aiming to achieve better yields and less harvest losses (Gent 1977; Davies & Casey 1993). According to Gent (1977) in the UK, semi leafless varieties (such as Progreta, Countess, Solara and Dryden) produce a better-ventilated crop, faster drying and additional standing ability.

2.1.7.2 Classification according to the purpose of use

Depending on their intended use, peas can be categorized as field or vegetable peas. Cultivars specifically created for forage and animal feeding are called field peas. Some field peas are grown for the production of grain (known as dried peas) used for processing. Immature peas destined for canning or freezing are called vegetable peas (Taweekul 1999). Each of these uses requires cultivars with specific characters including suitability for sowing, seed size, colour, time of maturity and cooking properties (Savage et al. 2001). Snoad (1985) classified the pea crop into vining, dried (or combining) or forage pea. Vining peas are harvested at the green stage with high sugar content. The dried peas, as the name suggests, are harvested at the dry stage and used for packeting and canning post rehydration. Finally, the forage pea is harvested as a whole plant for hay or silage. Among the cultivated peas *P. sativum* ssp. *sativum* are usually the horticultural types, and *P. arvense* are the fodder and winter types (Muehlbauer 1993). In New Zealand, peas are grown mainly for export and dried peas constitute the most important fraction of the sales (White 1987) .

2.1.7.3 Marrowfat Peas

Marrowfat is a popular dry pea type grown in England and New Zealand and used in the canning industry (Muehlbauer 1993). They are large drum shaped seeds (300 to 350 g per thousand seeds) with excellent texture and flavour. They are grown to the fully mature stage, as if for seed, and then canned after rehydration (Gent 1977) . In Japan and other Asian countries (such as Malaysia and Indonesia) marrowfat peas are consumed as a snack food. They are imbibed in water and then fried in oil. In Japan gritted peas are used to prepare extruded snacks known as "fried beans" or "green nuts" similar to roasted peanuts. This market wants dark green grain, a large even size, and with no staining or dirt. Japan is the major market for premium grade and does not purchase any other

quality grade (Sewell 1986; Savage et al. 2001). In the UK, dried peas are imbibed and then cooked and canned. For this use, large pea grains that remain firm after cooking are required (Savage et al. 2001). According to Muehlbauer (1993) marrowfat types tend to be late maturing and often are severely attacked by infestations of powdery mildew. Marrowfats often bleach and are of poor quality when wet conditions coincide with crop maturity. Seeds of acceptable colour can be grown if the crop is swathed at about 18 to 23% seed moisture content, threshed as soon as possible, and dried artificially. Important marrowfat peas are cultivars Maro and Progreta, developed during the ' 60s and '70s in Europe, respectively (Sewell 1986; White 1987) and Midichi in the '90s (White & Russel 2001a).

2.1.8 Production of marrowfat peas in New Zealand

Marrowfat peas grown in New Zealand are exported for different purposes. According to Lough (1987) exports of peas for human feeding (especially blues and marrowfat) have been more valuable than for livestock consumption. Therefore physical aspects such as colour and shape are important for the export market as well as cooking characteristics.

The production is destined for the European, Indian and Japanese markets due to the favourable characteristics of shape, size and cooking properties (Sewell 1986; White 1987; White & Russell 2001b) There are several commercial cultivars of peas available. Table 2.2 shows a summary of some characteristics of growth, and origin of some of the peas cultivated in Canterbury.

Table 2.2 Characteristics of marrowfat pea cultivars.
(Jermyn 1986; White & Russel 2001b; Short et al. 2002)

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2.1.9 Pea crop establishment and management

Peas require mild and moist conditions to grow. As already mentioned they do not grow well in hot weather. At suitable temperature (between 15 and 21°C) emergence may occur in a period of 1 or 2weeks (Kennell, 2003). A brief and general description of the management of spring sown pea crops in Canterbury as suggested by Jermyn (1986) is presented below:

Site selection: peas are sensitive to impeded drainage soils and soil borne diseases. Soil testing and rotation (five years) is recommended to avoid build up of diseases.

Cultivar, seed quality and treatment: will vary according to the contracts available, purpose of sale and potential yield and disease resistance. Quality of seed can be checked with germination and vigour tests for garden pea seeds. Fungicide seed treatments are recommended.

Sowing rate: a range of 80 - 100 plants m^{-2} is a standard. Sowing rates (kg seeds ha^{-1}) may be adjusted according to the TSW (thousand seed weight). Cultivar Whero for example, should be sown at 280 kg seeds ha^{-1}. For Maro seeds the quantity is 400 kg ha^{-1}. For field peas such as the cultivar Crusader a recommended sowing rate is 220 kg ha^{-1} (White & Russell 2001b).

Seedbed preparation: peas are sensitive to soil compaction. The seedbed needs to be firm and without a tillage pan above 15 cm. Sub soiling may be necessary on heavy soils. Cultivation must be minimal to achieve a level, rubbly seedbed. Drill slowly at 40 – 70 mm depth and ensure that large seeded cultivars are not being damaged in some drills. Checking plant population (plants m^{-2}) after emergence is essential.

Fertilisers and weed control: adjusted according to the soil fertility and weed occurrence. Pre-plant and pre-emergence herbicides are available.

Irrigation: Greenwood and McNamara (1987) reported seed yield increases of 35 % in field peas (cultivar Rovar) irrigated during the flowering period compared with dryland pea crops. Irrigation was not required in the vegetative phases of crop development but during flowering and pod swelling, irrigation increased the number of pods per plant by 28% and the quantity of seeds per pod by 20%. However, over-watering can depress yields and favour disease incidence.

Disease control: disease occurrence varies according to the environment, cultivar susceptibility and pathogen presence. More detailed descriptions of diseases in peas and their control are presented in sections 2.2.

2.2 Diseases of peas

Pea plants are subjected to a wide range of diseases caused by nematodes, bacteria, viruses and fungi which can significantly decrease both yield and quality (Kulik 1995; Kraft et al. 1998). Fungi, particularly, play an important role in pea crops, especially if weather conditions are favourable for their occurrence (Howard et al. 1994) . The quality of seed can also be affected by some pathogens (Maude 1996).

The negative effects of fungal presence on or in seeds are related to losses in seed quality. Visual appearance, poor germination and establishment as well as the possibility of the seed introducing and disseminating disease to new areas are the main problems (Lamprecht & Knox-Davies 1984; Howard et al. 1994; Maude 1996).

Common problems occurring in pea growing areas in Canterbury include Ascochyta blight (or Ascochyta complex) and powdery and downy mildew, especially if suitable environmental conditions such as rain are likely to occur. These lead to decreased yields and generally lower product quality with poor colour and shrivelling problems (Kraft et al. 1998; Anonymous 2000).

2.2.1 Diseases caused by fungi

Of all infectious pathogens, fungi are known to cause the most serious damage on plants (Agrios 1988; Oku 1994a). There are a wide range of fungi affecting plants and consequently seeds. Some cause minimal damage to the host (known as biotrophs). The majority of the fungi obtain organic material from nonliving tissues living saprophytically on them (Atkinson et al. 1956; Maude 1996). These are called necrotrophs. In general, the biotrophs establish a parasitic relationship with the host, while the necrotroph attacks are non host specific. They are more common and difficult to control due to the possibility of survival

in an area over several seasons through volunteer hosts (i.e. weeds) or in plant remains (Stackman & Harrar 1975).

Fungi can affect pea crops at any stage of plant development and cause damage in different parts of the plant - from roots to leaves and pods, and consequently seeds. The main diseases of pea crops caused by fungi are summarized in Table 2.3.

In Canterbury, mildew (*Peronospora viciae* and *Erysiphe pisi*) may be problematic in early sown crops subject to wet spring weather and rapid growth. Late sown crops with less growth tend to harbour less disease but are only possible to grow under irrigation (Freeman 1987). Although irrigation has been used to increase the yield of pea (grain and seed), this moisture also increases the chances of incidence of fungal diseases such as the Ascochyta complex and powdery and downy mildew (Greenwood & McNamara 1987). An account of their importance in peas growing in Canterbury follows.

Table 2.3 Main diseases of peas caused by fungi

Disease	Causal agent	Symptoms
Ascochyta blight	*Phoma medicaginis* var. *pinodella*, *Ascochyta pisi* and *Mycosphaerella pinodes*	Small purple to black spots on leaves and stems; blackening or death of seedlings.
Powdery mildew	*Erysiphe pisi*	White to off coloured spots on upper surface of the leaves.
Downy mildew	*Peronospora viciae*	Grey-brown lesions on underside of pea leaflets.
Sclerotinia white mould	*Sclerotinia sclerotiorum* (Lib.) de Bary	Fluffy white mycelium develops and later dense mycelial mats form at the soil surface on vines, pods and leaves.
Pythium rot	*Pythium* spp. (Pringsh)	Damping off and seed and seedling rot of peas.
Rhizoctonia seedling blight	*Rhizoctonia solani* J.G. Kuhn	Seed and seedling rot, mainly infecting hypo and epicotyls as water soaked appearance; redish or brown lesions near cotyledonary node.
Aphanomyces root rot	*Aphanomyces euteiches* Drechsler	Soft water soaked lesions on the surface of the lower stem and root.
Fusarium root rot	*Fusarium solani* (Mart.) Appel & Wr. f. sp. *pisi* (F.R. Jones) Snyd.& Hans.	Redish to blackish-brown lesion in the hypocotyl, epicotyl and cotyledonary attachment.
Fusarium Wilt	Several races of *Fusarium oxysporum* Schltdl	Vascular discoloration of the root and stem.
Rust	*Uromyces fabae* (Grev.) Fuckel	Rust colored, blister –like pustules develop on leaves and stem.
Septoria leaf blotch	*Septoria pisi* Westend	Older leaves with a yellow straw-coloured blotches with ill defined margins surrounded by chlorotic halos.

Adapted from (Allen & Allen 1981; Hagerdon 1984; Harvey 1986; Jermyn 1986; Howard et al. 1994).

- **Ascochyta blight**

The most common seed borne fungi of pea are those known as the Ascochyta blight complex. This is caused by three pathogens: *Phoma medicaginis* var. *pinodella*, *Ascochyta pisi* and *Mycosphaerella pinodes*. They cause leaf, stem and pod lesions. Also, they are likely to cause discoloration of cotyledons, hypocotyls and root areas. Even low levels of infection may represent significant losses. Few areas of crops are completely free from Ascochyta. The disease is widespread in most important temperate pea growing areas, including New Zealand (Davies & Casey 1993; Kraft & Pfleger 2001).

According to Kraft et al. (1998) distinction between the three pathogens is difficult under field conditions and it is practical to consider the three as causing a single disease. *Mycosphaerella pinodes* is considered the most aggressive and causes most of the economic losses which may be as high as 50% in processing peas (Howard et al. 1994).

All three pathogens are seed borne. Seed is the most important means of transmission of *Ascochyta pisi*, which does not produce a soil borne resting stage. *M. pinodes* and *P. medicaginis* are vigorous saprophytes and colonize pea residues forming resting structures (sclerotia, chlamydospores, pycnidia and pseudothecia) that survive as infectious agents for disease establishment (Howard et al. 1994; Kraft et al. 1998).

From those structures ascospores (produced from pseudothecia and chlamydospores) and conidia (produced from pycnidia) are forcibly ejected by wind and thus are able to be disseminated over large areas. Ascospores require dry conditions for release but need high humidity for germination. New crops of ascospores are produced in the same season on diseased foliage at intervals of

about 13 days. The secondary inocula are the conidia that are extruded in a gelatinous matrix from the pycnidia and depend on rain splash and wind for dispersal. Successive infections occur under moist conditions once the spores produce a germ tube and penetrate into the host directly through the cuticle and cell walls (Agrios 1988; Howard et al. 1994).

Symptoms vary depending upon the causal agent. Lesions caused by *M. pinodes* and *P. medicaginis* appear in two to four days, whereas symptoms of *A. pisi* require six to eight days. Circular lesions characterize Ascochyta leaf and pod spot (Figure 2.1). Lesions caused by *A. pisi* are slightly sunken, tan to brown with a distinct dark border (Howard et al. 1994).

Figure 2.1 Symptoms caused by Ascochyta complex in different parts of pea plants (Hagerdon 1984).

- **Downy mildew**

This disease is common in wet and cold seasons reducing plant populations, seed quality and yield, especially of vining peas. Young leaves just emerged are highly susceptible to infection, becoming more resistant as they mature (Ashby et al. 1987). The disease can come into a crop from two sources: resting spores produced on infected pods, which persist in the soil for many years, or from airborne spores produced from other infected plants (Harvey 1986).

Infection can be systemic, occurring before flowering and causes stunting and distortion. Growth of seedlings infected systemically is limited. Later systemic infections may be restricted to growing points with infection later spreading to lower leaves. Pods formed on infected plants are flattened, yellow, distorted and rarely set seed. Internal as well as external fungal growth on the pod prevents the seed from maturing (Ashby et al. 1987).

Localized symptoms are the result of wind blown spore infections. Young lesions range from 0.2 – 2 cm in diameter. They are light green with brown spots on the upper surfaces. The lesions may also appear as yellow to brown spots on the leaf surface with areas of fluffy white to bluish cottony mycelium on the under surface (Hagerdorn 1984; Ashby et al. 1987). As the lesions develop, chlorotic patches appear on leaves and stems. Symptoms start on the lowest leaves and progress up the plant. Pod infection occurs due to high humidity and appears as yellow lesions on the surface.

- **Powdery mildew**

Powdery mildew is caused by *Erysiphe pisi* and it is found wherever peas are grown. The disease is serious in warm and dry conditions where nights are sufficiently cool for dew formation. The disease is also likely to occur late season or in wet areas which allows the plant to remain in the vegetative stage. Under sprinkler irrigation or frequent rainfall powdery mildew is not important (Kraft et al. 1998).

Infection occurs through the penetration of *E. pisi* haustoria, that lie outside the cell cytoplasm. Conidia can germinate and penetrate the plant surface at variable and rather low humidities (Hagerdon 1984; Howard et al. 1994). Severe infection results in early crop senescence and reduced quality as well as decreased green pea and seed yields. The pathogen can overwinter on infected plant debris, on

alternative hosts and is also seed borne (Hagerdon 1984; Agrios 1988; Howard et al. 1994; MacNab 2004).

Symptoms of powdery mildew on peas are white off-coloured spots on the upper surface of the lowest and oldest leaves. These spots increase in size and appear as white powdery areas. The disease progresses quickly in susceptible cultivars until the entire plant is covered with a powdery mycelial growth. Tissues beneath the infected areas may turn purplish in colour (Kraft et al. 1998; Kraft & Pfleger 2001).

2.2.2 Diseases caused by bacteria

There are two main diseases caused by bacteria: bacterial blight and brown spot caused by *Pseudomonas pisi* and *Pseudomonas syringae* pv. *syringae*, respectively (Hagerdon 1984; Harvey 1986; Kraft et al. 1998). The first is an important seed borne disease and for trade of seeds in New Zealand and abroad seed health testing is required. Due to the economic importance of this disease in New Zealand pea seeds it will be described.

- **Bacterial Blight (*Pseudomonas pisi*)**

The only bacterial disease of economic importance to peas in New Zealand peas causing considerable losses, mainly in wet cold conditions such as the autumn sowing period. The bacteria can be present internally and externally on seeds. Lesions are dark green or brown occurring on the nodes and on the stipules. Initially the infection appears on the underside of the leaves as water soaked lesions. Pods may be attacked and the lesions are roughly circular, watery and sunken in appearance. The control of this disease is mainly done by the use of non infected seeds. As the bacteria do not survive in the soil a suitable crop rotation and incorporation of plant debris can assist the control of the disease (Ashby et al. 1987) .

2.2.3 Diseases caused by viruses

Viruses in pea crops are transmitted by aphids. The aphids overwinter in lucerne, clover or weeds and the population increases in spring. They fly to emerging pea plants and carry with them viruses which were present in the overwintered crop. A number of viral diseases of pea have been reported and they are listed below. Two of them (alfalfa mosaic virus and pea seed borne mosaic virus) deserve more attention because of the effects on seeds (Ashby et al. 1987). Main viral diseases of peas according to several authors (Hagerdon 1984; Harvey 1986; Kraft et al. 1998; Kraft & Pfleger 2001) are :

Alfalfa mosaic virus
Pea seed borne mosaic virus
Aster yellows mycoplasma
Bean yellow mosaic virus
Pea enation mosaic virus
Pea streak virus
Pea stunt virus

- Alfalfa mosaic virus (AMV)

The symptoms of AMV in peas are a purpling of areas on the surface of the pod which later become sunken and blackened. Stem and veins in the upper leaves may appear necrotic. The yield is reduced and seeds produced from diseased plants may show brown discoloration of the seed coat (Ashby et al. 1987).

- Pea seed borne mosaic virus

This virus has constituted a problem since the 1970s. It is transmitted by seed and therefore the movement of pea seeds is regulated by field inspections, seed testing and quarantine requirements. Symptoms in pea plants are downward rolling of the leaf margin and a slight clearing of the veins of the youngest leaves. Usually the lesions are very mild and difficult to recognize in the field

(Hagerdon 1984; Harvey 1986; Ashby et al. 1987; Howard et al. 1994; Kraft et al. 1998; Kraft & Pfleger 2001).

2.2.4 Pathogenicity

The ability of an organism to invade plants and interfere with their normal function is called pathogenicity (Oku 1994a). Three events determine fungal pathogenicity : the ability to penetrate into plants (infection), to overcome host resistance and to induce disease (symptoms) (Agrios 1988; Oku 1994a).

The majority of plant pathogens have specific host plants, but some fungi can attack a wide range of plants. Fungi are known to penetrate the host by puncturing epidermal cell walls and via natural wounds. Several pathogenic fungi may enter plants by directly penetrating an unbroken surface, through the cuticle. In this process, the germ tube arising from a spore becomes flattened or forms an appressorium when contacting the cuticle surface (Guest & Brown 1997). The hyphae penetrate through the epidermal wall, increasing in size or forming haustoria. Usually young seedlings or plants with a weakly developed epidermis are the subject of direct penetration (Oku 1994a). After a successful entrance the pathogen may overcome the host defence mechanisms. This can be done by killing the host cells and living on them saprophytically (Oku 1994a). Most necrotrophs have limited effect on the overall physiology of their hosts. However, they may produce toxins that are transported throughout the plant, causing extensive damage (Guest & Brown 1997).

Recognition of a microorganism's pathogenicity is achieved by following Koch's postulates ("proof" of pathogenicity) which involves:

1. Association of the pathogen with all diseased plants examined;

2. Isolation of the pathogen (non obligate parasites), growth in pure culture on nutrient media and description of characteristics.

3. Inoculation of the pathogen from pure cultures onto healthy plants of the same species on which the disease appears and production of the same visual symptoms. Inoculation is the process when spores or infective propagules of a pathogen come into contact with the potential host (Guest & Brown 1997). For *Stemphylium* spp. the most common infective propagules are the conidia (Neergaard 1945; Barash et al. 1975; Basset et al. 1978; Stuville & Erwin 1990; Aveling 1992; Anonymous 2003c).

4. Re-isolation of the pathogen and confirmation of the same colony characteristics (Kulik 1995).

2.2.5 *Stemphylium* spp. and peas

There are few reports in the literature about the occurrence of *Stemphylium* on peas. Thanutong et al. (1982) investigated the pathogenicity of *S. sarciniforme* and concluded that this species was non pathogenic on pea plants. Further studies on the occurrence of *Ascochyta* spp. in peas revealed that saprophytic fungi were associated with lesions on leaf and pods. Fungi isolated from diseased pea plants included *Stemphylium botryosum*, *Cladosporium cladosporioide* (Fresen.) G.A. de Vries, *Epicoccum purpurascens* Ehrenb. ex Schlecht and *Sordari fimicola* (Roberge) Cesati & de Notaris (Thanutong et al. 1982; Wegrzycka 1990; Wegrzycka 1991).The perfect state of *S. herbarum* (*Pleospora herbarum*) (Simmons 1969; Camara et al. 2002) have been found in peas (Anonymous 2004b).

In general, *Stemphylium* spp. are often associated with other fungi, especially with *Alternaria* spp. (Neergaard 1945; Faris Mokaiesh et al. 1995). Marcinkowska (1997) isolated *Alternaria alternata* (Fr.) Keissl. and *Stemphylium botryosum* from winter pea seed samples (cv. Arvense) harvested in Poland. Additionally, 20 pathogenic fungal species were isolated from dry pea seeds (cv. Ramir), with the most frequent occurrence being *Alternaria alternata* and *Stemphylium botryosum* (Marcinkowska 1997).

There are several species of *Stemphylium* found in New Zealand (section 2.3.7) that could potentially affect pea crops, causing foliar lesions and infecting seeds. *Stemphylium* species were reported from pea seeds harvested in Canterbury (K.D.R. Wadia and R.G. Bakker, personal communication, 10 February 2003) and it was suggested that high infection by the fungus may be an important causal agent of low germination of pea seeds

2.3 The genus *Stemphylium*

The genus *Stemphylium* Wallr. comprises filamentous and saprophytic imperfect fungi of the Hyphomycetes group which are distributed worldwide on decomposing vegetation (Basset et al. 1978; du Toit & Derie 2002). In humans, members of the genus *Stemphylium* may cause eye and skin infections known as phaeohyphomycosis (Ellis 1971; Camara et al. 2002). In agriculture *Stemphylium* spp. are responsible for diseases in many crops, with damage to the foliage of the plants and movement of the fungi into seeds (Aveling 1992; Christensen & Wysong 1997; Coles & Wicks 2001).

Some species of *Stemphylium* have sexual states. For instance, *Stemphylium vesicarium* is the imperfect (or conidial) stage of *Pleospora allii* (Rabenh.) Ces. & De Not.(Bradley et al. 2003). The perfect stage is briefly described in 2.3.4.

The following is a list of the *Stemphylium* species described by Ellis (1971) and Camara et al. (2002):

S. alfalfae Simmons
S. astragali Yoshii
S. botryosum Wallr.
S. callistephi K. Baker
S. globuliferum Vestergr.
S. gracilariae Schatz & Simmons
S. herbarum Simmons
S. lancipes (Hill) Simmons
S. loti Graham
S. lycopersici (Pryor) Simmons
S. majusculum Simmons
S. radicinum (Meier) Drechsler & Eddy
S. sarciniforme (Cav.) Wiltsh
S. solani Weber
S. trifolii Graham
S. triglochinicola Simmons
S. vesicarium (Wallr.) Simmons
S. xanthosomatis Huguenin

Stempylium species have been reported as pathogens of many crops. The sequence of some characterist symptoms of *Stemphylium* spp. infection in some cultivated plants will be described.

2.3.1 Symptoms caused by *Stemphylium* spp. in forage legume crops

2.3.1.1 Lucerne and clover

Stemphylium spp. are important fungal pathogens of cultivated lucerne and clover (*Trifolium* spp.), responsible for foliar necrosis, leaf spots and early senescence (Stuville & Erwin 1990; Bradley et al. 2003).

In lucerne the main symptoms are circular or irregularly shaped lesions on leaves. Lesions may be tan, brown, or black, and may have yellow halos (Figure

2.2). Severe symptoms result in defoliation and losses can be severe if wet conditions coincide with active lucerne growth (Christensen & Wysong 1997; Anonymous 2003b). The foliar lesions are approximately 2-5 mm in diameter. Visual symptoms appear about one week after exposure to the pathogen. As the infection progresses, lesions expand and coalesce, covering larger portions of tissue. Seven to ten days after initial appearance, diseased foliar tissues turn light tan to brown, and become papery in texture (Stuville & Erwin 1990; Christensen & Wysong 1997).

In clovers, leaf spot caused by multiple *Stemphylium* species can be important. The species involved are: *S. sarciniforme* or a complex formed by *S. botryosum*, *S. alfalfae*, *S. globuliferum*, *S. trifolii* and *S. vesicarium* (Bradley et al. 2003; Anonymous 2003c). The lesions are initially small and brown. They expand, gradually becoming predominantly brown (Figure 2.2) about 5-10 mm in diameter (Anonymous 2003c).

2.3.1.2 Chinese milk vetch (*Astragalus sinicus* L.)

Symptoms caused by *S. loti* appear on leaves, petioles and stems. They appear first as light brown lesions which enlarge and become round, about 5 mm in diameter. A clear zonation appears in the lesion and the surroundings become water soaked and faint green. Defoliation of leaves is common. In the stem, the lesions are long, spindly and copper coloured (Anonymous 2003c).

2.3.2 Vegetable crops

2.3.3 Spinach and asparagus

In spinach *S. botryosum* has been reported as the main cause of grey to brown spots on the foliage (Koike et al. 2001; du Toit & Derie 2002; du Toit & Derie 2003). *Stemphylium vesicarium* is the causal agent of purple spots in asparagus (Elmer 2001; Hausbeck 2003) (Figure 2.2).

Figure 2.2 Symptoms of *Stemphylium* spp. on asparagus (a), spinach (b), lucerne (c) and clover (d) (Christensen & Wysong 1997; Koike et al. 2001; Anonymous 2003b; Anonymous 2003c).

2.3.3.1 Onion (*Allium cepa* L.) and Garlic (*Allium sativum* L.)

Symptoms of disease caused by *S. vesicarium* on garlic leaves are white small oval lesions (Ligero et al. 1998). In onion, *S. vesicarium* initially causes numerous white irregular spots which enlarge and become dark purple brown lesions (Aveling 1992; Ligero et al. 1998).

2.3.3.2 Solanaceae crops

Stemphylium solani is the causal agent of grey leaf spot on tomato leaves whereas *S. lycopersici* causes leaf blight. The symptoms of both diseases are similar: small spots with a yellow halo eventually become necrotic, they dry out and crack across. Grey leaf spot occurs only on leaves. When the disease is severe it leads to defoliation and conspicuous yellowing. *Stemphylium lycopersici* however, may also attack floral parts on which conidia are borne in later stages of infection (Blancard 1992; Barreto & Scaloppi 1999).

In pepper (*Capsicum annuum*) premature defoliation and lesions are caused by *S. solani* and *S. lycopersici.* Symptoms are light brown spots on young leaves, which expand, developing red brown margins with a distinct white centre as leaves mature (Cho et al. 2001).

This species also causes potato leaf scorch and leaf spots in scarlet eggplant (*Solanum aethiopicum* L.) (Howard et al. 1994; Syndir & Lacoste 1994).

2.3.3.3 Lettuce (*Lactuca sativa*)

Leaf spot in lettuce caused by *S. botryosum* begins as water soaked specks which are visible 3 days after infection begins. Later the lesions become brown spots, 7-9 mm in diameter (Barash et al. 1978).

2.3.3.4 Pears

Stemphylium vesicarium is the causal agent of brown spot, a serious disease in Mediterranean pear growing areas of Europe (Llorente et al. 2000). Infection and necrosis occur on leaves, fruits and twigs with maximum disease incidence occurring just prior to harvest (Singh et al. 2002). On fruits the lesions reach 6 mm in diameter (Laidou & Thanassoulopoulos 2000)

2.3.4 The life cycle and environmental requirements for *Stemphylium* spp.

Epidemiology of foliar fungal diseases depends on leaf surface moisture. Many foliar pathogens require extended periods in free water for spore germination, germ tube growth and host penetration (Bradley et al. 2003). The extent of disease development depends on environmental factors such as temperature and humidity. Different species and strains may have different temperature requirements for development (Vincelli 2003).

Source of inoculum: The fungus overwinters as mycelia (*Stemphylium* spp.) or resting bodies (teleomorph *Pleospora* spp.) on dead stems, leaves and on seeds. In warmer climates the fungus survives without becoming dormant during the winter and conidia are produced throughout the year. Conidia serve as the main source of primary inoculum and in these circumstances the role of the sexual stage may be diminished. Conversely, in colder climates, the fungus overwinters in fruiting bodies (pseudothecia), which produce ascospores, the main source of primary inoculum in the spring (Stuville & Erwin 1990; Christensen & Wysong 1997; Suheri & Price 2000; Vincelli 2003).

Environmental factors: Ligero et al. (1998) observed that mean temperatures ranging from 5 to 10°C and relative humidity higher than 96% are optimum for pseudothecia development of *S. vesicarium* on garlic debris. These structures matured 1 – 4 months after deposition of debris on the soil surface. These authors noted that the pseudothecia degenerated when temperatures reached 15°C or above, along with the degradation of the plant debris. Sporulation, infection and disease severity increase with longer wetness periods (Bradley et al. 2003). Gilchrist et al. (1982) reported that for *S. botryosum*, continuous light for 72 hours after inoculation produced a lower disease severity compared with the most favourable light regime (12 hour period).

Inoculum dispersal and host invasion: Air currents and rain splash disseminate the primary inoculum (ascospores or conidia) to susceptible tissue. Rainfall or dew is required for ascospore release (Ligero et al. 1998). Spores germinate when free moisture is present on the leaf surface (Bradley et al. 2003; Ligero et al. 2003). The pathogen invades the host tissue primarily through natural openings such as stomata or through wounded tissue, but some penetration may occur directly through the cuticle between epidermal cells (Oku 1994a; Bradley

et al. 2003). Ligero et al. (1998) reported that *S. vesicarium* penetrated the plant tissue producing appressoria when temperatures ranged from 18 to 25°C and there was a minimum leaf wetness period of 16 hours.

2.3.5 Effects of *Stemphylium* spp. on crop yield

The main problems caused by pathogens of cultivated plants are a decrease in yield and deterioration of quality. These deleterious effects may be caused by deleterious enzymes, toxins produced by pathogens and abnormal metabolites resulting from host pathogen interactions (Oku 1994a). Additionally, pathogens affect the source-sink patterns in plants due to direct damage and nutrient depravation (Guest & Brown 1997).

In lucerne, losses caused by Stemphylium leaf spot can be severe, reducing dry matter production by at least 50% (Anonymous, 2003a). In the mid 1990s in Brazil severe epidemics of *S. solani* in cotton (*Gossypium hirsutum* L.) caused the total loss of crops (Mehta & Brogin 2000; Mehta 2001).

Guest and Brown (1997) stated that pathogens may interfere with the respiration rate, transpiration, hormonal balance, translocation and photosynthesis of plants. Certainly, foliar diseases have a negative effect on photosynthesis. The action of fungi on leaves leads to a reduction in radiation interception because of a decrease in green leaf area, and consequently interruption of normal plant development and growth. Facultative parasites negatively affect the photosynthetic process primarily by degeneration of the chloroplasts. As a result there is a losses in chlorophyll content and less carbon fixation (Goodman et al. 1986a). Leaf lesions caused by fungi enlarge causing early yellowing and premature senescence of leaves. When infections are heavy the results may be

severe defoliation, retarded growth or necrosis and death of leaves (Nicoletti et al. 2003).

Fungal damage however, is not restricted to foliar tissues and can also extend to seeds. In some circumstances the pathogen reaches floral tissues causing blossom fall and/or infecting the embryonary cells still in development through the translocation of nutrients (Oku 1994a). In spinach seed crops, it was observed that spores of *S. botryosum* disseminated at the same time as the pollen and may reach the stigma of the flowers and infect the ovary, consequently allocating the fungus internally in the seed (Koike et al. 2001; du Toit & Derie 2003).

2.3.6 Phytotoxins produced by *Stemphylium* spp. and pathogenicity

Phytotoxic substances produced by the pathogen facilitate the infection process (Stackman & Harrar 1975) by causing degradation of epidermal cells (Oku 1994a). Mehta & Brogin (2000) demonstrated that *S. solani* produces a toxic metabolite responsible for foliar lesions in cotton plants as well as other hosts such as beans (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.) and potato (*Solanum tuberosum* L.). Phytotoxins produced by *S. vesicarium* caused brown lesions on young leaves of bean plants (Laidou & Thanassoulopoulos 2000) and pears (*Pyrus communis* L.) (Singh et al. 2002). Five major compounds (stemphylin, stemphyloxin II, stemphyperylenol, stemphol and a stemphol related compound) have been recognized in the main crop pathogenic *Stemphylium* species: *S. botryosum*, *S. herbarum*, *S. alfalfae*, *S. sarciniforme* (Barash et al. 1975; Andersen et al. 1995; Laidou & Thanassoulopoulos 2000). Such substances may be involved in plasma membrane disorders and are responsible for pathogenesis on plants (Singh et al. 2002).

2.3.7 *Stemphylium* spp. in New Zealand

In New Zealand, diseases associated with *Stemphylium* spp. have been catalogued since the 1970s. Between 1972 and 1979 *Stemphylium* spp. were isolated from several crops and in many parts of New Zealand (Tables 2.4 and 2.5). In Auckland, Landcare Research reported *Stemphylium* spp. from stems of asparagus, tomato, and pepper (Anonymous 2001). At the same time *Stemphylium* was associated with fern spots in asparagus in Hawke's Bay and Wellington and leaf spot of leeks (*Allium porrum* L.) in Hawke's Bay. In the Bay of Plenty *Stemphylium* was also isolated from kawakawa *(Macropiper excelsum)* (Anonymous 2001).

During the 1980s in Auckland, *Stemphylium* spp. were reported on leaves of onion and shallot (*Allium cepa*), tomato, lettuce and in black passionfruit (*Passiflora edulis* Sims) (Anonymous 2001). In the same period, in Hawke's Bay, Wellington and Waikato, the fungus was isolated from dead stems and leaves of asparagus (Anonymous, 2001). In Canterbury, the first report of *Stemphylium* spp. was in 1986 in lucerne crops. In 1991, the fungus was also isolated from lower leaf lesions in Hebe "Waireka" (*Hebe* sp. cv. Waireka) (Anonymous 2001). Specific occurrence of different *Stemphylium* spp. in New Zealand is summarized in Tables 2.4 and 2.5.

Table 2.4 Areas of occurrence and crops affected by *Stemphylium botryosum* in New Zealand.

Area	Crops affected
Auckland	tomato carrot tree lupin (*Lupinus arboreous*) pumpkin (*Cucurbita maxima*) asparagus
Taupo	lucerne (*Medicago sativa*)
Wanganui	white clover (*Trifolium repens*)

Source: (Anonymous, 2001).

Table 2.5 Areas of occurrence and crops affected by *Stemphylium vesicarium* in New Zealand.

Area	Crops affected
Auckland	tomato amaryllis (*Amaryllis* sp.) tree lupin pumpkin asparagus
Nelson Hawke's Bay Bay of Plenty Canterbury Wanganui Marlborough	asparagus

Source: (Anonymous, 2001)

2.3.7.1 Disease assessment

A major aspect of disease inspection is to assess the incidence and severity of a disease. Incidence can be assessed consistently whereas disease severity depends on descriptive keys or assessment keys. A descriptive key describes plants with different levels of disease and assigns a percentage infection. Assessment keys illustrate diseases at different levels by standard area diagrams which typify the development of a disease by category, number, index grade or percentage (Fox

1993a; Berg & Leath 1996). More recently, image analysis has been used to measure disease severity in an entire crop as well as in individual leaves. This methodology is more precise than subjective observations and nowadays computer software and digital imaging make such measurement simple (Vale et al. 2003).

2.3.8 *Stemphylium* spp. in pure culture (isolation, incubation, identification)

In the laboratory, fungi can be distinguished by their growth characteristics (colour, aspect and colony size) and the spores produced (shape, colour and size). The main characteristics of *Stemphylium* fungi under the microscope are pale brown to brown septate hyphae. Camara et al.(2002) and Mathur and Kongsdal (2002) reported that colonies of *Stemphylium botryosum* on potato dextrose agar (PDA) grow rapidly and mature in 5 days at 25 °C. The colonies have a velvety to cottony texture. On the surface, the colony colour is grey, brown, or brownish-black in colour, with the reverse of the colony being black.

Mehta (2001) working with *Stemphylium solani* reported that this species is slow growing, reaching 66 mm in diameter after 10 days growth on common culture media such as PDA and incubation conditions of 25°C, 12 hours fluorescent light. Additionally, some *S. solani* isolates do not sporulate under such conditions. The colonies had a velvety, cottony or immersed dark brown to black mycelia. Some isolates produce a yellow pigment in the medium that turns deep red with age.

Identification of *Stemphylium* species has relied on morphological characteristics such as variation in conidia, conidiophores (Figure 2.3) and ascospores (Neergaard 1945; Camara et al. 2002). Conidiophores are dark walled and may be simple or branched. They bear a number of vesicular swellings or nodes

(Mathur & Kongsdal 2002). However, such features may change according to the environmental conditions and the media used (Neergaard 1945).

2.3.8.1 Conidial morphology

The asexual spores (conidia) are solitary, light brown to black in colour, and rough- or smooth-walled. In general they are oblong or subspherical and rounded at the tips. These conidia have transverse and vertical septations (muriform conidia) and there is a typical constriction at the central septum (Figure 2.3). They are cicatrized (have thickened scars) at their base. Conidia are formed from the tips of nodose or irregularly swollen, pale green to brown, simple conidiophores, which grow singly or in small clumps (Raid & Kucharek 2003). Sporulation is not readily apparent to the naked eye. Conidia may be viewed microscopically on the surface of older lesions (Malloch 1981; Raid & Kucharek 2003).

Figure 2.3 Variability of conidiophores and conidia of *S. botryosum* (450 X) from (a) Onion; (b) Phlox; (c) Tomato (d) Zinnia. (Source: Ellis 1971).

The form of the conidia in the genus *Stemphylium* spp. is extremely variable (Figure 2.3). The growth medium may have a considerable influence on the morphology of the conidia (Camara et al. 2002). Separation of some common species of *Stemphylium* can be done using conidial characteristics using a key developed by (Ellis 1971) :

1. Spores rounded at apex

2. Spores smooth (on *Trifolium*).....*S. sarciniforme.*

2. Spores verrucose (on dead herbaceous stems).....3

3. Spores mostly constricted at median transverse septum.....*S. botryosum.*
3. Spores usually constricted at three major transverse septa.....*S. vesicarium.*

1. **Spores with pointed conical apex**

4. Spores constricted at median transverse septum, length-to-breadth ratio not more than 2:1.....*S. solani.*
4. Spores constricted at three major transverse septa, length-to-breadth ratio 3:1 or more .....*S. lycopercisi.*

Adapted from Ellis (1971).

Production of conidia therefore is desirable in studying *Stemphylium* spp. Neergaard (1945) mentioned that for most *Alternaria* species near ultra violet (NUV) rays of wave lengths between 2535 – 2800 Angstron units stimulated sporulation. However, for *A. porri* (Ellis) Cif sporulation was increased by intensive irradiation with visible light and not by NUV. For *Stemphylium* spp. visible light (Kulik 1995; Camara et al. 2002; du Toit & Derie 2002) and NUV light (Lamprecht & Knox-Davies 1984; Aveling & Snyman 1993) are also used to increase production of conidia.

Neergaard (1945) reported that this genus was a "very weak, facultative parasite markedly ubiquitous". Because of the great overlap in morphological characteristics described among *Stemphylium* spp. as well as variation within species, identification based on morphology can be difficult. For example, *S. botryosum* and *S. globuliferum* share characteristics such as oblong-muriform conidia rounded at the ends. Conidial length-width ratio ranges from 1 to 1.5 (27-42 x 24-30 μm), they are pale to dark brown in colour, usually with 3 horizontal and 1-3 vertical septa (muriform) and usually strongly constricted at the mid horizontal septum. The wall of the conidia is smooth to verrucose. *Stemphylium botryosum* and *S. globuliferum* both exhibit slow ascomatal development. *Stemphylium herbarum*, *S. vesicarium* and *S. alfalfae* have a conidial shape which is oval with a conidial length-width ratio of 2 (20-50 x 15-26 μm) and have fast maturing ascomata in culture (Ellis 1971; Stuville & Erwin 1990; Camara et al. 2002). In order to support morphological characteristics, other identification techniques have been developed. Mycotoxins and metabolites produced by different *Stemphylium* spp. can be used to differentiate species that are morphologically similar (Andersen et al. 1995). Advances in DNA sequencing have also been employed in an attempt to improve distinction of *Stemphylium* spp. (Camara et al. 2002; Moore & Frazer 2002a).

2.3.9 Phylogeny of *Stemphylium* spp.

Traditional systems of fungal classification emphasised morphology above all else. These are now being replaced by the use of molecular evidence of relationship and biological species concepts, although the old descriptions still have value (Moore & Frazer 2002b).

The detection of pathogens through nucleic acids (DNA and RNA) has some advantages over traditional methods. For example, all viable fungal propagules can be used (mycelia, spores, fruiting bodies) and the method is independent of the environment and host (Oliver 1993). Thanks to these techniques is it possible to detect unculturable pathogens and establish evolutionary relationships among species and strains (Oliver 1993; Moore & Frazer 2002b).

Phylogenetic distance relationships are typically shown as branching diagrams or trees. The total length of the branches separating any two sequences is drawn in scale proportional to the calculated evolutionary distance between them. The length of the lines in a tree is proportional to evolutionary distances calculated from the number of nucleotide base differences between two sequences. This is the essence of the distance method (Moore & Frazer 2002b). A simple explanation of the molecular approach is that the larger the number of generations separating related sequences from each other, the more different (or less related) the sequences are, because of accumulation of mutational changes (Emelyanov 2003).

A recent molecular phylogeny of *Stemphylium* has been developed by researchers at the Molecular Plant Pathology Laboratory, USDA/ARS (USA). Seventeen *Stemphylium* spp. were distributed into five major clusters (Table 2.6). Among them, the pathogens of *Trifolium* and *Medicago* are included in three clusters (Camara et al. 2002; Bradley et al. 2003).

Among the methodologies for distinguishing *Stemphylium* spp., Mehta (2001) reported that DNA sequencing using the ITS (internal transcribed spacers) region was able to separate *S. solani* isolates occurring on cotton and tomato foliage. The internal transcribed spacers are noncoding regions of DNA sequence

that separate genes coding for the 28S, 5.8S, and 18S ribosomal RNAs . These ribosomal RNA (rRNA) genes are highly conserved across taxa whereas the spacers between them may be species specific. The variation in the spacers has proven useful for distinguishing among a wide diversity of taxa which are difficult to identify (Shivji 1997; Kumar & Shukla 2005) .

Another phylogenetic approach to distinguish *Stemphylium* spp. was proposed by Camara et al. (2002) using the analysis of the *gpd* (glyceraldehyde-3-phosphate dehydrogenase) loci. The phylogenetic relationship using *gpd* loci is based on the fact that a major function of a gene is to encode the structure of a specific protein (Barondes 2000). Genes involved in metabolic pathways are a type of signature of a taxa or species (Emelyanov 2003) and *gpd* is one enzyme with catalytic activity, involved in the metabolism and glycolysis process (Mogri 2000). It is located in the cytoplasm and nucleus cells. The tree based on nucleotide sequences of genes encoding *gpd* has been proposed to re-organize yeasts classification (such as *Saccharomyces cerevisiae* Meien ex. Hansen and *Zygosaccharomyces rouxii* (Boutrox) Yarrow (Smith 1989). Camara et al. (2002) demonstrated that the ITS region did not generate a phylogenic tree supporting as many *Stemphylium* spp. groups as a tree derived from the analysis of the gene encoding *gpd*. The authors stated that the ITS region was not sufficiently informative to distinguish among morphologically described species. In contrast, the *gpd* gene DNA sequence was more reliable for establishing well supported relationships among species due to a greater resolving power.

Table 2.6 Five (A-E) major groups of *Stemphylium* spp. according to an evolutionary relationship based on the *gpd* (glyceraldehyde-3-phosphate dehydrogenase) gene sequencing (Camara et al. 2002).

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According to Camara et al. (2002) group E. includes *S. triglochinicola*, *S. loti*, and two clover pathogens, *S. trifolii* and *S. sarciniforme*. Such species have smooth spores and do not produce sexual reproductive structures in culture. Group C includes the lucerne pathogens and group D *S. solani*, pathogenic on tomato, eggplant (*Solanum melongena*) and pepper. A comparison of gene sequence data (DNA tests) combined with morphological and physiological characteristics of fungal colonies can provide distinction between species and isolates (Camara et al. 2002). In New Zealand, various species of *Stemphylium* have been identified (section 2.3.7). However, no studies have addressed a detailed description and recognition of seed borne *Stemphylium* spp. isolated from peas.

2.4 Seed borne plant diseases

Transmission of pathogens via any plant propagule may be of agricultural importance, because it provides an efficient way of transfer over time (from season to season) and space (from place to place). Seeds can be an important

source of initial inoculum for diseases which cause qualitative and quantitative losses to crops (Agrios 1988; Agarwal & Sinclair 1997; Hampton 2003).

Seed borne plant pathogens are microorganisms such as fungi, bacteria, viruses and nematodes (Agarwal & Sinclair 1997) . Seed borne pathogens are important in agriculture and horticulture because:

1. Infected seeds may not germinate or they have low vigour. The resulting decrease in seedling population can lead to fewer adult plants and a consequent reduction in crop yield (Kulik 1995; Wu 2001).

2. Infected seeds can be a source of inoculum which, under suitable environmental conditions, may introduce disease into the sown crop, adjacent healthy crops, or into geographic areas that are disease free , thus reducing yield and often quality (Kulik 1995; Khanzada et al. 2002).

Plant pathogenic seed borne fungi and bacteria are usually host specific and are found associated only with certain seeds. In addition, saprophytic microorganisms are often carried by seeds. These saprophytic microorganisms may be found on seeds of many different kinds of plants. Both pathogenic and saprophytic agents may be superficially attached to the outer seed surface or lodged in cracks in the seed coat or under it, but the pathogen may also be present within the cotyledons and other parts of a seed including the embryo (Kulik 1995; Maude 1996). Sowing infected seed introduces the pathogen randomly throughout the whole field, providing numerous foci of primary infection. This is usually more effective in starting a disease outbreak than wind-borne spores (Hampton 2003).

Many species of *Stemphylium* occur on a wide range of plants and are economically important pathogens of some agricultural crops (Simmons 1969; Stuville & Erwin 1990; Wu et al. 2001; Camara et al. 2002). *Stemphylium* spp. are known seed borne pathogens of, for example, lucerne (Lamprecht & Knox-Davies 1984) and onion (Aveling & Snyman 1993; Stivers 2004) and the causal agent of foliar diseases in several crops as already described in sections 2.3.1-2.3.3.4.

2.4.1 Seed infection mechanisms

Infected seeds are a result of the interaction among the host, pathogen and environment (Agrios 1988; Maude 1996; Brown 1997). The success of transmission varies with the pathogen strain and species, plant cultivar, growing conditions and age of the plant at the time of the infection (Brown 1997).

The maturing seed can be infected internally from the mother plant or by external transmission. The pathogen can be introduced through the flower, fruit stalk or seed stalk or even, directly from the seed surface (Grinstein et al. 1988; Hampton 2003; Stivers 2004). Infection of the ovule can also occur from infected or contaminated pollen (Brown 1997; Brown & Ogle 1997). Infection of onion seeds by *Stemphylium vesicarium* occurs via the seed stalk (Grinstein et al. 1988; Aveling & Snyman 1993; Stivers 2004). In spinach, du Toit & Derie (2003) suggested that protective measures should be taken prior to pollen shed to decrease the disease and chances of seed infection. Early infection of plants usually increases the level of seed borne infection as there is maximum opportunity for the ovule to become infected. Fungi may proliferate either intra, inter or extracellularly (Goodman et al. 1986b). However, transmission to seed does not occur if the cytoplasmic connections between the embryo and the infected maternal tissue are lost (Brown & Ogle 1997). At stage 3 of seed

development (section 2.1.5) the chances of infection are lower than in stages 1 and 2 (Agarwal & Sinclair 1997).

Some pathogens may penetrate directly through the seed surface (Oku 1994a). Legumes produce seeds in pods, which is a natural barrier to fungal penetration (Maude 1996) but seed can be infected via infected pods e. g. by *Ascochyta* spp. (Roger & Tivoli 1995). Additionally, during harvesting and processing seeds may become infected. The seed coat, which is an effective barrier to fungal infection, may suffer cracks that can be entry points for fungi (Coolbear 1995).

2.4.2 Seed health testing

Seed health testing is important in providing information for field performance, quarantine and seed certification requirements, and the need for treatment for specific pathogens (Hampton 2003). Routine health tests must provide reliable, economic, quick and accurate information (Saettler 1989; ISTA 2003).

2.4.2.1 Seed health testing methods

The choice of seed health test method depends on the purpose of the test (quarantine, seed certification, seed treatment). More than one method may be available for assessment of a seed borne pathogen (Hampton 2003). The International Seed Testing Association (ISTA) has internationally agreed tests for some important seed borne pathogens of various crops. Some of the common methods for seed borne fungi assessment cited by Malloch (1981), Mathur and Kongsdal (2002) and Hampton (2003), are :

1. Examination of ungerminated seed: inspection of dry seeds, washing seeds, embryo test.

2. Incubation tests: blotter test, freezing blotter test, agar plate test.

2.4.2.2 Incubation tests

Seed borne fungi are detected on a routine basis in seed testing laboratories by placing 200 – 400 surface sterilised seeds on nutrient agar or on a moist paper substrate (ISTA 2003). The seeds are incubated at a favourable temperature for fungal growth, often with 12 hours illumination from long wave ultraviolet or fluorescent lamps to stimulate fungal sporulation. After a determined period, usually 7 -10 days, the seeds are examined using a microscope to observe characteristic fruiting structures and colony growth forms (Kulik 1995). Procedures are described in the literature for detection of many economically important pathogens of some crops (Basset et al. 1978; Malloch 1981).

2.4.2.2.1 Agar plate test

This test consists of placing seeds on a suitable agar (commonly potato dextrose agar (PDA) or malt extract agar (MEA)) in Petri dishes. To reduce the development of saprophytes, seeds are usually surface sterilised by soaking for 2-3 minutes in 1% sodium hypochlorite (Hampton 2003). Through incubation tests it is possible to determine the type of inoculum, the amount of inoculum and the occurrence within a seed lot. The number of seeds with the pathogen is recorded and results are reported as a percentage infection (ISTA 2003).

A limitation of the incubation tests is the experience required for fungal identification. Additionally, for many pathogens, methods are not yet standardised (e.g. variations in media used, incubation conditions, etc.) (Hampton 2003; Ophel Keller 2003).

2.4.2.2.2 Freezing blotter test

In this test seeds are placed on moist filter paper at 20°C for two days to induce germination. Afterwards, the seeds are incubated for 24 hours at -20°C and then

transferred again for 20°C for a period of seven days. The freezing temperature prevents seed germination but the fungi can still sporulate (Hampton 2003).

2.4.3 Assessement of *Stemphylium* spp.

There is no standardised method for assessment of *Stemphylium* spp. on pea seeds. However, the fungus was observed in incubation conditions used for assessment of *Aschochyta pisi* on pea seeds (MEA or PDA at 20 ± 2°C, darkness for 7 days) (Wegrzycka 1990, 1991; K.D.R. Wadia and R.G. Bakker, personal communication, 10 February 2003).

The literature describes some methodologies for assessment of *Stemphylium* spp. For example, seed borne *Stemphylium vesicarium* in lucerne was assessed by Lamprecht & Knox-Davies (1984) who plated seeds on MEA media. Plates were incubated at 20 °C in 12 NUV (near ultra violet) light and 12 hours darkness for 10 days. *Stemphylium vesicarium* was identified through colony characteristics and the production of spores (conidia). Similarly, assessment of *S. vesicarium* on onion seeds was carried out by Aveling (1993) who used PDA and the incubation conditions described above.

ISTA (2003) describes a freezing blotter method for identification of *Alternaria radicina* Meier, Drechsler & Eddy (*Stemphylium radicinum*) in carrot seeds by placing 10 seeds evenly spaced in water soaked blotter papers in Petri dishes and incubating the seeds for 3 days at 20 ± 2°C in the dark. Then they are transferred to -20 ± 2°C for 24 hours and then incubated for 6 days at 20 ± 2°C in alternating 12 hours darkness and NUV. Martiniello and Porta-Puglia (1995) also assessed *S. botryosum* and *S. vesicarium* in berseem (*Trifolium alexandrinum* L.), lucerne and tall fescue (*Festuca arundinacea* Schreb.) seeds using the methodology described above.

2.4.4 Seed germination

Germination refers to a series of physiological events that result in a quiescent seed with low metabolic rate and low moisture content (5 – 15%) initiating the formation of a seedling from the embryo. For germination to occur seeds need to be hydrated under conditions of temperature and oxygen that encourage metabolic activity (Bewley & Black 1994; Copeland & McDonald 2001a) . The process starts with water uptake and physiologically, finishes with the elongation of the embryonic axis, usually the radicle (Bewley & Black 1994). However, in routine seed testing, germination is assessed as germinated normal or abnormal seedlings, which have been allowed sufficient time to develop their essential structures (ISTA 2003).

2.4.5 Germination testing methods

The objective of the germination test is to provide information for planting and comparison among different seed lots. Controlled laboratory germination testing methods have been developed as they are repeatable and less variable than testing in soil or in the field. In such tests the conditions of moisture, aeration and temperature are optimum and germination occurs quickly. Common media for germination tests are sand and germination paper (Hill 1999).

Germination tests are performed only on pure seed of a seed lot. For testing peas, 400 seeds must be used, which are set out in 4 replications of 100 hundred (ISTA 2003) . Seeds are placed on the substrate without touching each other and using the maximum space available. During the test period it is important to maintain moisture and aeration (Hill 1999).

According to the ISTA rules (ISTA 2003) germination testing of pea seeds can be performed either in sand or between paper at 20°C ± 2°C, and there is no need for procedures to break seed dormancy.

2.4.5.1 Between paper method (B.P.)

Seeds are placed on a sheet of damp germination paper and another sheet is placed on top, the bottom is turned up to prevent seeds from falling out and the paper rolled up. Paper sheets must be soaked and drained properly. Counting boards may be used for large seeds, such as peas, to arrange the seeds on the paper. These tests are labelled and the rolls stood upright in a basket which is placed in a plastic bag to prevent drying out. The basket is then transferred to the appropriate required temperature in a cabinet or room for the required number of days (ISTA 2003).

2.4.5.2 Sand

In this method seeds are placed in a square pattern with 30 mm spacing between seeds, either in or on the sand. The sand should have all particles passing through a sieve of 0.8 mm diameter holes but retained on a sieve having holes of 0.005 mm diameter. The sand must be washed and sterilised before use as a substratum in order to eliminate any bacteria, fungi, nematodes and foreign seeds. The pH range should be around 6.5 – 7.0 and de ionised water used to maintain moisture. For germination test of large seeds such as peas the sand should be moistened to 60% of water holding capacity (ISTA 2003).

2.4.6 Seedling evaluation

Germination in a laboratory test is the emergence and the development from the seed embryo of those essential structures which, for the kind of seed being tested, indicate the ability to develop into a normal plant under favourable conditions in soil (ISTA 2003) .

During the tests, seedlings are evaluated over the recommended test period and at each examination prior to the final evaluation only the normal seedlings are

removed. At completion of the test all remaining seedlings and seeds are examined and recorded in the categories: normal seedlings, abnormal seedlings, ungerminated seeds which are hard seed, fresh ungerminated seeds and dead seeds. Pea seeds are evaluated on the 5^{th} and 8^{th} days (ISTA 2003). Each replicate is assessed and recorded over the test period. At the final count all normal seedlings are summed for each replicate and seedlings and seeds of other categories are recorded. The results from the replicates are totalled and averaged to a percentage. It is necessary to understand the type of seed being tested, its seedling development and structures in order to classify them into normal or abnormal seedlings (Hill 1999).

Normal seedlings should be removed from the substrate at interim counts to avoid entanglement of their roots or collapse of the seedlings. Doubtful or damaged seedlings should be kept until the final count in order to reduce the possibility of incorrect evaluation. However, mouldy seedlings or decayed seedlings are removed to prevent spread of decay to the other seeds (Hill 1999).

2.4.6.1 Normal seedlings

Normal seedlings are those which can continue development into normal plants when grown under favourable conditions. Each species tested may have certain characteristics. A general rule for normal seedlings is to have their essential structures well developed, complete, in proportion and healthy. Normal pea seedlings may have a long and slender primary root, and secondary roots may be evident. A short or hardly distinguishable hypocotyl with a well developed epicotyl. Finally, cotyledons are round or oval and tend to be fleshy and remain within the seed coat (Schmitt 2000; ISTA 2003).

2.4.6.2 Development of pea seedlings during the germination test

The embryo of mature pea seeds has two large fleshy cotyledons containing the food reserves. At the beginning of germination the radicle emerges through the testa and elongates quickly, and secondary roots soon develop (Figure 2.4). The hypocotyl is not discernible but the epicotyl elongates considerably (Hill 1999).

Pea seedlings have a shoot system consisting of the elongated epicotyl and the terminal bud with the developing primary leaves. The cotyledons usually remain within the seed coat and the hypocotyl is not apparent. The root system consists of the primary root – usually with root hairs, and secondary roots, which may compensate for a defective or insufficient primary root (Schmitt 2000; ISTA 200

Figure 2.4 Stages of pea seedling development (Schmitt 2000).

2.4.6.3 Abnormal seedlings

ISTA (2003) defines abnormal seedlings as those that do not have the ability to develop into a normal plant when grown in soil under favourable conditions of temperature, moisture and light because of irreparable defects in essential seedling structures. There are three main categories of abnormal seedlings:

1. Damaged seedlings: in these seedlings the essential structures are missing or badly damaged and balanced development does not occur. Damage to the seed embryo may be as a result of mechanical handling, heat, drought or insect action. In this case is possible to observe cracks and splits in hypocotyls, epicotyls or cotyledons. Eventually, cotyledons or shoots are completely separated from other parts of the seedling and primary roots are missing or stunted (Schmitt 2000).

2. Deformed (or unbalanced) seedlings: those seedlings are a result of physiological or biochemical disorders. Usually they occur due to unfavourable growing conditions of the parent plant, poor ripening conditions, premature harvesting, effect of pesticides, poor cleaning procedures, inappropriate storage conditions, or natural ageing of the seed. A deformed seedling has retarded or spindly primary roots, short and thick, looping, twisted hypocotyls, epicotyls or mesocotyls. Inverted direction of growth and curled, discoloured cotyledons are also characteristic of deformed seedlings (Hill 1999).

3. Decayed seedlings: those seedlings with any of the essential structures so diseased as a result of primary infection that normal development is restricted. This may be caused by fungal or bacterial attack, often as a consequence of external damage or internal weakness (Hill 1999).

2.4.6.4 Ungerminated seeds

Ungerminated seeds may belong to three main categories: hard seeds, fresh ungerminated or dead seeds. Hardseedness is a form of dormancy common in legume species. The seed is unable to imbibe water due to an impermeable seed coat. Fresh ungerminated seeds imbibe water but development never takes place. Seeds that are not hard or fresh ungerminated are dead seeds and are usually soft,

discoloured, frequently mouldy and show no sings of germination (Hill 1999; A. Goldsack, personal communication, 24 March 2003).

2.4.7 Seedling emergence

Rapid and uniform crop establishment is desirable for pea crops in order to achieve high productivity. The roots should develop quickly, providing stability, facilitating water and nutrient uptake, and improving the chances of inoculation by *Rhizobium* bacteria. Fast shoot development allows the seedling to begin photosynthesis and attain more rapid independence from stored seed reserves for growth (Snoad 1985).

Both environmental factors and seed lot characteristics (i.e age, physical damage, vigour) may reduce emergence of seeds in the field. Fungi, if present on or in seeds, or in the soil, and under favourable environmental conditions may become active during germination and emergence of seedlings (Hampton 2003). For example, a major cause of pre emergence mortality in peas appears to be related to infection by *Pythium* spp., which infect and kills seeds before germination or in the early pre emergence phase. Infection can not only prevent emergence but may also reduce the growth rate of those seedlings that emerge, especially when environmental conditions are adverse (Mathews 1977).

2.4.8 Effects of *Stemphylium* spp. on seed colour

Seed borne fungi are sometimes recognizable to the naked eye, and in some cases it is possible to see a relationship between the presence of mould and alterations in the physical appearance of the seed: wrinkled, cracked, greenish or mouldy seeds (Hoffman et al. 1998). In lucerne, the visual effect of *Stemphylium vesicarium* attack is seen as changes in seed colour, where infected seeds are often darker compared with healthy ones.

For peas, seed colour varies according to the cultivar. Infected seed may or may not be discoloured, so the only means of ascertaining if seed is infected is to have a specific seed health test carried out (Gane 1972) (sections 2.4.2-2.4.3).

2.4.9 Effects of *Stemphylium* spp. on seed germination and seedling emergence

Germination can be affected by the presence of pathogens (Copeland & McDonald 2001b). Many pathogens become active when seeds are sown, and the activity of some may result in seed decay and damping off, resulting in poor plant stands in the field (Agarwal & Sinclair 1997).

There is sometimes a negative correlation between the germination percentage and the percentage of seeds infected by pathogens. Hoffman et al. (1998) reported that when 0.3% of seeds carried *Sclerotinia* spp., germination of soybean seeds (cv. A3304) was reduced from 99% to 52%. Linseed (*Linum usitatissimum* L.) infected with a range of fungi (mainly *Botrytis cinerea* Pers. (5- 6.5% infection) and *Alternaria linicola* Groves and Skolko(>20% infection)) had 44 – 73% germination (Cappelli & Ciriciofolo 1991).

Nascimento and West (1998) observed that during priming contamination by *Stemphylium*, *Alternaria* and *Cladosporium* (Link) in muskmelon (*Cucumis melo* L.) seeds reduced the germination from 100% to 85% with 7% of the seedlings being abnormal at 25°C. Wu et al. (2001) reported that the rate of emergence of pot marigold seeds was negatively correlated ($R^2 = 0.61$) with the amount of seed borne *S. vesicarium*. Lucerne seeds containing between 5 and 32.5% *Stemphylium vesicarium* germinated poorly in the laboratory (malt extract agar for 10 days, 20°C, alternating 12 hours dark and light) and under glasshouse conditions: 88% of non-infected seeds germinated while only 70% of infected

seeds germinated. Dark, olive-green lesions on contaminated seedlings appeared mainly in the transition zone between the root and hypocotyls (Lamprecht & Knox-Davies 1984). An unusual colour is a result of oxidation of phenolic compounds by phenol oxidases, which produces coloured end products. Those substances are melanins which confer the black or grey discoloration in damaged cells whereas anthocyanidins lead to the loss of pigmentation in damaged tissue (Burton 1982).

2.5 Seed treatment

The aim of seed treatment is to promote good seedling establishment, to minimize yield loss, to maintain and improve quality and avoid the dispersion of harmful organisms. A desirable seed treatment must be practical, efficient under varied conditions, safe to operators and for the environment, and economically viable (Hewett & Griffiths 1978; Agarwal & Sinclair 1997). Additionally, seed treatment should not be phytotoxic or negatively affect nodulation (Agarwal & Sinclair 1997; Desai 2004).

Seeds for within country use and for export are treated for control of seed borne inoculum. For quarantine, a 100% eradication of seed borne inoculum is desirable but may be not achievable. For eradication of inoculum highly selective fungicides, such as the systemics, are useful for some pathogens. Healthy, good quality seeds do not need treatment if there are no pathological or germination problems. However, in some countries it is common to apply a protectant treatment as conditions in the field may not be ideal for good seedling establishing (Agarwal & Sinclair 1997).

Many organisms associated with seeds may remain viable in seed lots for many years. The way in which seed borne organisms are carried influences both

longevity and the effectiveness of a particular treatment. Seed borne fungi may be carried on seeds, present in embryos or only accompanying seeds (Hewett & Griffiths 1978). Seed treatment may be divided into two main categories: seed disinfection and seed disinfestation (Agarwal & Sinclair 1997).

2.5.1 Seed disinfection

Seed disinfection refers to the elimination of inoculum established within the seed or seed coat tissues. The internal pathogens are controlled by thermotherapy (hot water for example) or by systemic fungicides. These products are absorbed, penetrate or diffuse into the seed or seedling. Seed disinfection chemicals are specific and applied after an assessment of the seed borne inoculum.

2.5.2 Seed disinfestation

Seed disinfestation involves the control of pathogens externally or passively present on the seed surface. Seed protection is made via fungicide treatment which protects the seed and seedling from seed and soil borne microflora. The method is suitable against facultative parasites which under ideal environmental conditions can cause seed rot and seedling blight. Seed protectant chemicals differ from crop to crop and from region to region.

Chemical treatment is commonly used for both seed disinfection and disinfestation (Halmer 2000). However, for legume seeds, chemical treatment may represent a market limitation as once seeds are treated they can not be used as feed (Agarwal & Sinclair 1997). Thermotherapy is another alternative for control of seed borne pathogens and may give more flexibility for the seed trade (Grondeau et al. 1992; Agarwal & Sinclair 1997). Recently, this method has also

been explored as an option for organic seed production (Banks 1998; Anonymous 2004a; Lennartsson & Roberts 2005).

2.5.3 Chemical seed treatment

Chemical seed treatment is the cheapest and most effective means of controlling many fungal seed borne pathogens. Fungicidal seed treatment may kill or inhibit seed borne fungi and may form a protective zone around seeds that may reduce decay and seedling blight caused by for example by soilborne pathogens (Agarwal & Sinclair 1997; Desai 2004).

Many common fungal pathogens (i.e. *Ascochyta*, *Alternaria*, *Botrytis*, *Fusarium*) colonize the seed coat or floral tissues with mycelium. Mycelia may penetrate to the inner structures such as cotyledons and the embryo. Such internally sited mycelia will be affected only by systemic fungicides (Hewett & Griffiths 1978). Seed health testing have been developed to indicate the fungal pathogens present on/in seeds (section 2.4.2) and the need for treating the seeds (Agarwal & Sinclair 1997; Wu 2001).

Even though chemicals may be beneficial in controlling seed borne pathogens they may also have detrimental effects on seed germination (Halmer 2000). Reduction in seed germination after seed treatment is expected if applied to physically damaged seeds or may occur after applying at higher than the recommended rates (J.G. Hampton, personal communication, 5 December 2004). This is likely to occur on large legume seeds. The larger the seed, the greater the potential for seed coat breakage during threshing and post-harvest (Vanderberg 1995) and consequently, the greater chances of phytotoxicity effects after seed dressing.

Some of the symptoms of phytotoxicity are: a short and thick or otherwise deformed seedling axis; curled discoloured and necrotic cotyledons or primary leaves; inverted growth, leaves yellow or white (due to chlorophyll deficiency) and spindly or glassy seedlings (Halmer 2000; Schmitt 2000).

2.5.4 Chemical pea seed treatment

Fungicide seed treatment has been used for the control of seed borne fungi such as leaf and pod spot (caused by *Ascochyta* spp), downy mildew and seed rot (caused by several fungi) (Jermyn 1986; Sheridan 2000; Kraft & Pfleger 2001). If there is more than one seed borne disease of concern, a single fungicide may not be effective against all pathogens (Mebalds et al. 1996). Virtually all pea seed lots sold in New Zealand are fungicide treated (J.G. Hampton, personal communication, 24 March 2005). Below are described the commercial products registered for use as pea seed treatments in New Zealand.

Aliette super: a systemic fungicide recommended against Ascochyta and Pythium diseases, damping off and downy mildew. The active ingredients are fosetyl- aluminium, thiram and thiabendazole (Anonymous 2002b; Anonymous 2003a) .

Apron 70 SD: contains captan and a systemic, metalaxyl. In addition to giving the same protection to diseases as Aliette super, it also protects against downy mildew. Metalaxyl gives protection against soil borne infection and being a systemic chemical it can protect young plants against damaging early infection (6 weeks after sowing) (Harvey 1986). Because of the high cost of this product it is usually recommended for use when planting peas in areas known to have high level of downy mildew (Harvey 1986).

Apron XL: this systemic fungicide contains only metalaxyl as the active ingredient. It is recommended for control of downy mildew, Pythium diseases and damping off (Anonymous 2002b; Anonymous 2003a).

Dithane M45: a contact fungicide for the control of a wide range of seed borne diseases and soilborne pathogens causing damping off. It is recommended for control of *Pythium* spp., *Fusarium* spp. and possibly *Ascochyta* spp. and *Septoria* spp. in pea crops as a seed treatment (Anonymous 2002b).

Orthocide 65 sp (Captan): a contact fungicide with action against Pythium, Fusarium and Ascochyta root rots (Anonymous 2002b).

Wakil XL: used for the treatment of pea seeds against damping off, downy mildew and Ascochyta diseases. Wakil XL contains metalaxyl, fludioxonil, and cymoxanil as active ingredients. It is a systemic fungicide absorbed through roots of germinating seeds (Anonymous 2002b).

2.5.5 Thermotherapy

Thermotherapy represents a relatively simple and preventative way of controlling seed borne microorganisms. It can be applied to small quantities of basic and prebasic seeds, which might be then multiplied. However, it is at present not practical for large seed lots (Grondeau et al. 1992). Thermotherapy use has been neglected due to capital cost, and lack of immediate urgency to develop new technology while there are other treatments available. However, the increase in organic production and the fungicide resistance that some pathogens have developed may increase studies on thermotherapy, especially for those seeds that can be used for feeding (Banks 1998; Forsberg 2004) such as peas.

There are two methods in which heat can be used to control seed borne pathogens: hot air and hot water.

Hot water: in water soak methods seeds are soaked in hot water for a period of time and subsequently dried. Effective modifications differ in temperature and duration of the treatment. The higher the temperature the shorter the time required. Crop varieties differ considerably in their sensitivity to injury from soaking for long periods (Desai 2004).

Hot air: seeds are heated in a revolving cage installed in a dry chamber or in ovens for a certain period of time according to the target pathogen and crop (Grondeau et al. 1992; Agarwal & Sinclair 1997).

Hot air can be less effective than hot water, but has the advantage that it causes less seed damage and is easier to set up than water soaking techniques (Agarwal & Sinclair 1997; Forsberg 2004). A hot water soak (50°C for 30 minutes) successfully eliminated bacterial infection from pea seeds (Boettinger & Bowers 1975; Grondeau et al. 1992). However, the treatment is restricted for those seeds that can withstand hot water, otherwise there is a reduction in germination (Floyd 1990; Grondeau et al. 1992; Mebalds et al. 1996).

2.5.5.1 Effects of hot water treatment on seed germination

Using a hot water soak for large legumes, such as peas and soybeans may create germination problems because the seed coat swells and sloughs off after soaking (Agarwal & Sinclair 1997). This may lead to reductions in germination after the treatment (Aveling & Snyman 1993).

The effects of the heat on seed germination depend on the temperature and time of exposure and the species/cultivar treated. For control of major fungal and bacterial seed borne diseases of eggplant and tomato, Nesmith (2004) reported 49.5°C for 25 minutes as the optimum for controlling the pathogens without causing serious damage to seed quality. Floyd (1990) reported the following temperatures and time for treatment of vegetable seeds against bacterial and fungal diseases (Table 2.7) that have no negative effect on germination.

Table 2.7 Hot water treatment temperatures and times recommended for vegetable seeds for control of bacterial and fungal diseases.

Vegetable	Temperature (°C)	Time (minutes)
Cabbage (*Brassica oleracea* var. capitata)	52	30
Broccoli (*Brassica oleracea* var. italica)	50	20
Brussels sprouts (*Brassica oleracea* var. *gemmifera*)	50	20
Cauliflower (*Brassica oleracea* var. *botrytis*)	52	25
Tomato	56	30
Celery (*Apium graveolens*)	50	30
Carrot	50	20
Pumpkin	55	15

Grondeau *et al.* (1992) observed that for peas of cultivar Solara, with a pre soaking germination of 93%, the treatments of 55°C for 30 minutes and 60°C for 15 minutes, reduced germination to 42% and 37%, respectively. For another pea seed lot (cultivar Belinda) 15 minutes soaking at 60°C corresponded to about a 20% germination decrease compared with the control (non treated seeds). Nevertheless, Bae et al. (2002) found that for soybean high temperature sterilization (40°C for 30 minutes) was effective for the control of bacterial pathogens with minimal effects on seed germination. The percentage of normal germinated seedlings was still high (96%) after the hot water soak treatment. Begum et al. (2004) also observed that a water soak at 52°C for 13 minutes was

not damaging to pea seed germination. However, there was only a reduction of 7% on fungal seed borne mycoflora using this treatment compared with untreated seeds. The period of 13 minutes in which seeds were exposed at 52°C perhaps was too short to damage the seeds or the pathogens.

The effect of temperature may be related to changes in plasma membrane protein composition (Campbell 1993a; Cookson 2001). At high temperatures seed cells experience disruption of membranes due to changes in lipid and protein bodies, loss of ribosomes and impaired respiratory capacity (Coolbear 1995). The results of those changes are a low rate of germination, low percentage of normal seedlings and/or a high percentage of deformed seedlings (Schmitt 2000; Copeland & McDonald 2001a).

2.5.6 Control of *Stemphylium* spp.

2.5.6.1 Chemical Control

Fungicides have been applied to the crop to control *Stemphylium* spp. which cause foliar diseases (section 2.3.1). Products such as chlorothalonil and copper hydroxide have been used in asparagus fields to manage Stemphylium leaf spot (Elmer 2001; Anonymous 2002b). Control of *S. vesicarium* was successfully achieved with tebuconazole and a mix of procymidone and chlorothalonil, whereas fosetyl-Al plus copper hydroxide was less effective in controlling Stemphylium leaf spot in garlic plants (Ureba et al. 1998) . In pears, thiram was found to be much more effective than metiram in preventing *S. vesicarium* infection in pear leaves and fruits (Marchi et al. 1995).

In vitro, experiments showed that mycelial growth of *S. vesicarium* was significantly inhibited by fungicides and 1% sodium hypochlorite. Tebuconazole, a carbendazim/flusilazole mixture and procymidone were the

most effective in inhibiting the fungus on PDA culture media .Thiram was not effective (Aveling & Snyman 1993).

Few studies have addressed the control of *Stemphylium* spp. through seed treatment, especially for pea seeds. Aveling & Snyman (1993) reported that tebuconazole and the carbendazim/flusilazole mixture at recommended dosages effectively reduced the percentage of *S. vesicarium* in onion seeds. However, none of the fungicide seed treatments used in their work completely eradicated the pathogen.

2.5.6.2 Control of *Stemphylium* spp. using hot water soak

Hot water treatment has been successfully used to reduce seed borne *Stemphylium* spp. For lucerne seeds a hot water soak (50 – 60°C for 30 minutes) significantly reduced seed infection by *Stemphylium* spp. (Lamprecht & Knox-Davies 1984) . Aveling & Snyman (1993) observed that a hot water soak (50°C for 20 minutes) was the most effective treatment in reducing *S. vesicarium* in onion seeds.While it gave better control than the fungicides, it had negative effects on germination and emergence compared with the untreated seeds and fungicide treated.

The efficacy of hot water methods in controlling seed borne pathogens is related to the high temperature effects on growth and reproduction of fungi. Lilly and Barnett (1951) reported that mycelial growth of *Neurospora crassa* stops at temperatures of 44°C. Other pathogens such as *Bipolaris sorokiniana* (Sacc.) Shoemaker may tolerate temperatures as high as 70°C for 48 hours (Couture & Sutton 1980). *Stemphylium* spp. may develop under a broad temperature range (from 18 to 42°C) (Neergaard 1945; Bashi et al. 1973; Ligero et al. 1998). Development of warm temperature strains of *Stemphylium* spp. occurs even at

temperatures around 40°C (Bashi et al. 1973; Stuville & Erwin 1990; Mehta & Brogin 2000). Therefore, a hot water soak treatment for controlling *Stemphylium* spp. should use temperatures greater than 40°C.

Fungal growth and reproduction is mediated by enzymes that are heat sensitive (Campbell 1993a; 1993b). Fungi digest food externally by secreting hydrolytic enzymes which decompose complex molecules to simple compounds that the fungus can absorb and use (Campbell 1993b). Hydrolases, esterases, carbohydrases, pectinase and oxidases are examples of essential enzymes. These enzyme systems are sensitive to heat and are gradually inactivated (Campbell 1993a; Ma et al. 2003).This decrease in activity may be seen in a lowered rate of fungal growth (Lilly & Barnett 1951). Activity of catalase and superoxide dismutase for instance increases when temperatures increase from 20 to 26°C ; however, at 30°C these enzymes are inactive (Xu & Huang 2004). The increase in temperature causes thermal agitation of enzyme molecules, disrupts hydrogen bonds and other weak interactions that stabilize the active conformation, and the protein molecule denatures (Campbell 1993a). The temperature of inactivation is not fixed unless the length of exposure is also considered (Lilly & Barnett 1951; Couture & Sutton 1980).

3 Seed borne *Stemphylium* spp. in pea

3.1 Introduction

In 2002, *Stemphylium* spp. were detected in pea seed lots during routine laboratory health tests for the Ascochyta complex with *Stemphylium* infection recorded as being as high as 70% for some marrowfat seed lots (K.D.R. Wadia and R.G. Bakker, personal communication, 10 February 2003).

Seed borne *Stemphylium* spp. have been quantified for various plant species but there is limited information about the status of *Stemphylium* fungi on or in pea seeds. Wu et al. (2001) reported a range of 32 to 67% of marigold seeds infected by *S. vesicarium*. Aveling & Snyman (1993) found 32% of onion seeds infected by *S. vesicarium*, whereas Koycu and Ozer (1997) reported only 0.2% infection caused by *S. botryosum*. However, no reports about the possible effects of *Stemphylium* spp. on pea seed germination or emergence have been found.

Some *Stemphylium* spp. are seed borne pathogens of forage legumes such as clover (*Trifolium* spp.), lucerne and some vegetable and flowers species (Stuville & Erwin 1990; Srivastava et al. 1995; Stravato et al. 1995; Berg & Leath 1996; Wu et al. 2001; du Toit & Derie 2003; Stivers 2004). Fungal infection of seeds has been negatively correlated with germination of several crops (Cappelli & Ciriciofolo 1991; Hoffman et al. 1998; Nascimento & West 1998). *Stemphylium* spp. have been negatively correlated with germination of lucerne (Lamprecht & Knox-Davies 1984), carrots (Coles & Wicks 2001) and onion (Grinstein et al. 1988).

Fungal infection may also reduce seedling emergence (Wu et al. 2001). The resulting decrease in seedling population can lead to fewer adult plants and a consequent reduction in crop yield.

A laboratory and a glasshouse experiment were designed in order:

1. To quantify the occurrence of *Stemphylium* spp. from New Zealand grown pea seed lots.

2. To determine if the presence of *Stemphylium* spp. was related to germination abnormalities or seed death *in vitro* (agar plate test).

3. To evaluate if *Stemphylium* spp. infection was associated with poor germination of pea seeds in laboratory conditions (BP tests).

4. To evaluate if seed borne *Stemphylium* spp. affected establishment and growth of pea seedlings in the glasshouse.

3.2 Material and methods

3.2.1 Seed borne *Stemphylium* spp. assessment

Eighteen commercial pea seed lots harvested between 2002 and 2004 in Canterbury provided by the New Zealand Seed Technology Institute Plant Diagnostic Laboratory (BioLinc) were assessed. The criterion used to select the seed lots was the suspected occurrence of *Stemphylium* spp. noted during commercial tests for fungi of the Ascochyta complex. The seed lots tested included 10 of cultivar Midichi and one of each of a further eight cultivars (Table 3.1).

Table 3.1 Cultivar and harvest year of pea seed lots assessed for the presence of *Stemphylium* spp.

Lot number	Cultivar	Harvest Year
1	Midichi	
2	Midichi	2002
3	Midichi	
4	Midichi	
5	Midichi	
6	Early Frosty	
7	Midichi	2003
8	Midichi	
9	Midichi	
10	Midichi	
11	Greenshaft	
12	Early onward	
13	Puget	
14	Oasis	2004
15	Rondo 95	
16	Meteor	
17	Bolero	
18	Midichi	

The presence of *Stemphylium* spp. was determined by plating 200 surface disinfected untreated seeds (4 replicates of 50 seeds each) per seed lot on MEA (Merck, KGaA/Germany) amended with 0.1% chloramphenicol (Biochemical BDH, England; 50 mg/ml). Seeds were soaked for 10 minutes in 1% NaOCl and rinsed in sterile distilled water before plating (10 seeds/Petri dish). Plates were incubated at 20°C for 14 - 21 days under 12 hours dark and 12 hours NUV light (Lamprecht & Knox-Davies 1984; Bradley et al. 2003).

Seed borne *Stemphylium* spp. were recorded by examining the seeds and colonies (Appendix 1) using a stereomicroscope (approximately x 30) and microscope (x 400). Characteristic conidia, fruiting structures (pseudothecia) and

colony growth forms were the main criteria used for assessment as described in sections 2.3.8 (Stuville & Erwin 1990; Kulik 1995; Mathur & Kongsdal 2002).

In a preliminary study using seed lots 8, 9 and 10, the seeds incubated were examined at the 10th, 14th and 21st day of incubation for the presence of *Stemphylium* spp. This was done to determine which incubation period was most appropriate for assessing the fungus on pea seeds. However, as days of incubation did not significantly affect the results (Table 3.2) 14 days was chosen as the incubation time for this experiment.

Table 3.2 Percentage of seeds infected with *Stemphylium* spp. after 10, 14 and 21 days of incubation on MEA (20°C under 12 hours dark and 12 hours NUV light).

Seed lot	Incubation days	% *Stemphylium* spp.
8	10	4[a] (11.60)[b]
	14	6 (13.10)
	21	5 (12.60)
LSD[c]		6.29[c]
9	10	7 (14.80)
	14	8 (15.80)
	21	8 (15.30)
LSD		8.30
10	10	9 (17.10)
	14	11 (19.20)
	21	12 (18.00)
LSD		12.33

Means from 4 replicates per treatment containing 50 seeds.
[a] Non-transformed means rounded to whole numbers. [b]Values in parentheses are means after angular transformation of percentage data for each replicate.
[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from analsysis of variance (ANOVA) analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

3.2.1.1 Status of the seeds with *Stemphylium* spp. growth

The agar substrate and incubation conditions allowed pea seeds to germinate. For those seeds on which *Stemphylium* spp. were detected the germination status of the seed (i. e. normal seedling, abnormal seedling, dead seed) (ISTA 2003) was recorded as described in section 2.4.6.

3.2.2 Laboratory seed germination

Fourteen of the eighteen commercial pea seed lots provided by the New Zealand Seed Technology Institute Plant Diagnostic Laboratory (BioLinc) were tested (Table 3.1). Seed lots 4, 5 8 and 10 had insufficient seeds for germination testing.

Standard laboratory germination tests were conducted using the between paper method (BP) (section 2.4.5.1) at 20°C with 4 replicates of 25 seeds each. The first count was done on the fifth day of the test and the final count after 8 days. Seedlings were evaluated and classified as normal or abnormal (ISTA 2003) (section 2.4.6). Ungerminated seeds were also recorded.

3.2.3 Seedling emergence and growth in glasshouse environment

Two commercial pea seed lots of cultivars Midichi (seed lot 18) and Rondo 95 (seed lot 15) provided by the New Zealand Seed Technology Institute Plant Diagnostic Laboratory (BioLinc) were tested for emergence. They were harvested in 2004 in Canterbury.

For this trial the treatments were seeds known to be infected with *Stemphylium* spp. and non infected seeds. Infected and non infected seeds were selected from plates which had been incubated for 14 days and used for *Stemphylium* spp.

assessment (section 3.2.1 and Appendix 1). Seeds with *Stemphylium* spp. and seeds with no fungal growth were carefully removed from the plates and sown 25 mm deep in plastic pots (150 x150 x190 mm) containing vermiculite. Seeds of cv. Rondo were sown on 10 June 2004 and seeds of cv. Midichi were sown on 18 August 2004.

The pots were placed in a Lincoln University Nursery Glasshouse. The trial was set out as a completely randomised design with 3 replicates of 6 seeds, where each pot represented a replicate (18 seeds per treatment). The pots were watered daily using a hose. Seedling population was determined 30 days after sowing. The number of expanded nodes in the main stem was counted. The seedlings were dug out and the length of the shoot and root measured. Seedling dry weight was determined after washing all the seedlings and drying them at 60°C for 48 hours. Temperatures in the glasshouse were monitored (Siemens electronic room sensor) and are shown in figure 3.1.

3.2.4 Data analysis

Results from the seed health test (*Stemphylium* spp. infection and seed and/or seedling status, such as dead, abnormal, normal) and the seed germination test were reported as a percentage. Percentage of seeds infected with *Stemphylium* spp., normal and abnormal seedlings, death and ungerminated seeds were angularly transformed prior to ANOVA analysis (Clarke & Kempson 1997). Means were calculated and then separated using the least significant difference at $P< 0.05$ (John 1998).

Figure 3.1 Maximum (—) , mean (—) and minimum (—) temperature (°C) during the experiment for the pea cultivars (a) Rondo 95; (b) Midichi.

Seedling population data were calculated by dividing the number of seedlings by the number of seeds sown. Data were expressed as a percentage. Values were angularly transformed prior to analysis of variance (Clarke & Kempson 1997).

Means of number of nodes and shoot and root length (mm) were separated using the least significant difference at $P< 0.05$ (John 1998). Mean values of seedling dry weight (g) are reported.

3.3 Results

3.3.1 *Stemphylium* spp. assessment

The average *Stemphylium* spp. incidence in the pea seed samples assessed was 7.3%, ranging from 0 to a maximum of 46% (Table 3.3 and Appendix 1). The majority (80%) of the seed lots had less than 10% *Stemphylium* spp. The mean percentage of seeds infected by *Stemphylium* spp. was 2.5, 5.0 and 14.5% for seeds harvested in 2002, 2003 and 2004 seasons, respectively.

3.3.2 Status of the seeds with *Stemphylium* spp. growth

Across all seed lots, of the seeds from which *Stemphylium* spp. were recorded, 45% had produced a normal seedling, 43% an abnormal seedling and 12% were dead.

3.3.3 Seed germination

Germination of the seed lots varied from 41% to 100% (Table 3.4). For Midichi seeds harvested in 2002 and 2003 the mean germination percentages were 61% and 79%, respectively. For seeds harvested in 2004 (cvs Greenshaft, Meteor, Oasis, Rondo 95, Puget, Early Onward and Bolero) the mean germination percentage was 94%.

Table 3.3 *Stemphylium* spp. infection (%) of pea seed lots harvested in Canterbury in 2002 (1-4), 2003 (5-10), 2004 (11-18). Seeds were incubated on MEA (20°C, in 12 hours dark and 12 hours NUV light) for 14 days.

Seed lot number	Cultivar	% of *Stemphylium* spp.
1	Midichi	2[a] (5.60)[b]
2	Midichi	2 (6.10)
3	Midichi	3 (9.80)
4	Midichi	2 (7.60)
LSD[c]		9.34
5	Midichi	0 (0.00)
6	Early Frosty	1 (2.63)
7	Midichi	3 (10.49)
8	Midichi	5 (13.07)
9	Midichi	8 (15.82)
10	Midichi	12 (19.22)
LSD		6.27
11	Greenshaft	2 (7.02)
12	Early onward	4 (10.76)
13	Puget	5 (12.93)
14	Oasis	5 (13.10)
15	Rondo 95	6 (13.69)
16	Meteor	18 (24.82)
17	Bolero	30 (33.17)
18	Midichi	46 (43.90)
LSD		4.96

Means from 4 replicates containing 50 seeds.

[a]Non-transformed means rounded to whole numbers.

[b]Values in parentheses are means after anguar transformation of percentage data for each replicate.

[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from ANOVA analysis and t = critical value (P = 0.05) of Student's t distribution for v degrees of freedom (df).

Table 3.4 Percentage of normal seedlings, abnormal seedlings and ungerminated seeds of pea seeds lots.

Seed lot	%Normal seedlings	%Abnormal seedlings	%Dead	%Fresh ungerminated
2002				
1	79[a] (63.50)[b] c	16 (23.00) a	2 (5.80) a	3 (7.00) a
2	40 (39.50) a	38 (38.30) b	9 (17.00) b	12 (19.90) b
3	64 (53.00) b	31 (33.90) b	5 (12.80) ab	0 (0.00) a
LSD[c]	8.62	8.26	7.99	9.59
2003				
6	91 (72.60) b	8 (16.20) a	2 (2.90) a	0 (0.00) a
7	80 (63.90) a	18 (23.90) a	2 (4.10) a	0 (0.00) a
9	79 (64.10) a	21 (26.10) a	0 (0.00.) a	0 (0.00) a
LSD	7.45	10.38	9.27	0.00
2004				
11	100 (90.00) d	0 (0.00) a	0 (0.00) a	0 (0.00) a
12	98 (84.23) cd	1 (2.88) a	1 (2.88) ab	0 (0.00) a
13	100 (90.00) d	0 (0.00) a	0 (0.00) a	0 (0.00) a
14	100 (90.00) d	0 (0.00) a	0 (0.00) a	0 (0.00) a
15	96 (80.18) bc	4 (9.82) b	0 (0.00) a	0 (0.00) a
16	95 (79.16) bc	4 (9.88) b	1 (2.88) ab	0 (0.00) a
17	91 (73.13) b	6 (13.90) b	3 (6.91) b	0 (0.00) a
18	71 (57.64) a	28 (31.66) c	1 (2.88) ab	0 (0.00) a
LSD	7.11	6.33	6.67	0.00

Means from 4 replicates of 25 seeds per lot. Seed lots with the same letter are not significantly different ($P<0.05$).

[a]Non-transformed means rounded to whole numbers.

[b]Values in parentheses are means after angular transformation of percentage data for each replicate.

[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from ANOVA analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

3.3.4 Seedling population

Seedling population 30 days after sowing was higher for those seeds not infected by *Stemphylium* spp. (5.7 seedlings pot $^{-1}$ for both cultivars) than for *Stemphyium* spp. infected seeds (Figure 3.2 and Appendix 3). For cv. Rondo 95 the

Stemphylium spp. infection significantly reduced ($P<0.01$) seedling population by 60% (2.3 seedlings pot $^{-1}$). For cv. Midichi there were 20% fewer seedlings per pot (4.6 seedlings pot $^{-1}$) but this was not significant ($P<0.05$).

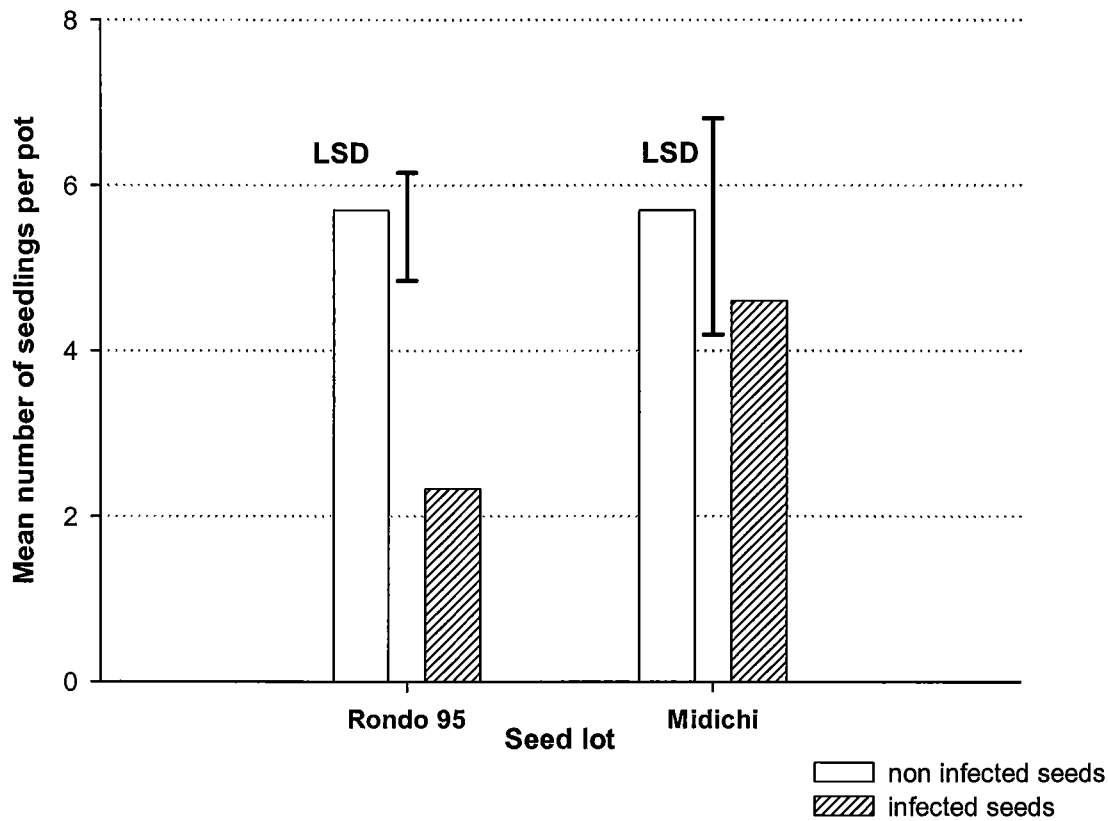


Figure 3.2 Mean number of seedlings established 30 days after sowing. Means of 3 replicates per treatment, with bars representing LSD at $P< 0.05$ derived from ANOVA analysis.

3.3.5 Number of nodes

The number of nodes on the seedlings was not affected by *Stemphylium* spp. seed infection. For cv. Rondo, seedlings from both non infected and infected seeds had an average of 3.83 nodes seedling^{-1}. For seed lot cv. Midichi, the number of nodes for seedlings from non infected seeds and infected seedlings was also not significantly ($P< 0.05$) different, 4.81 and 3.87 nodes seedling^{-1}, respectively.

3.3.6 Seedling shoot and root length

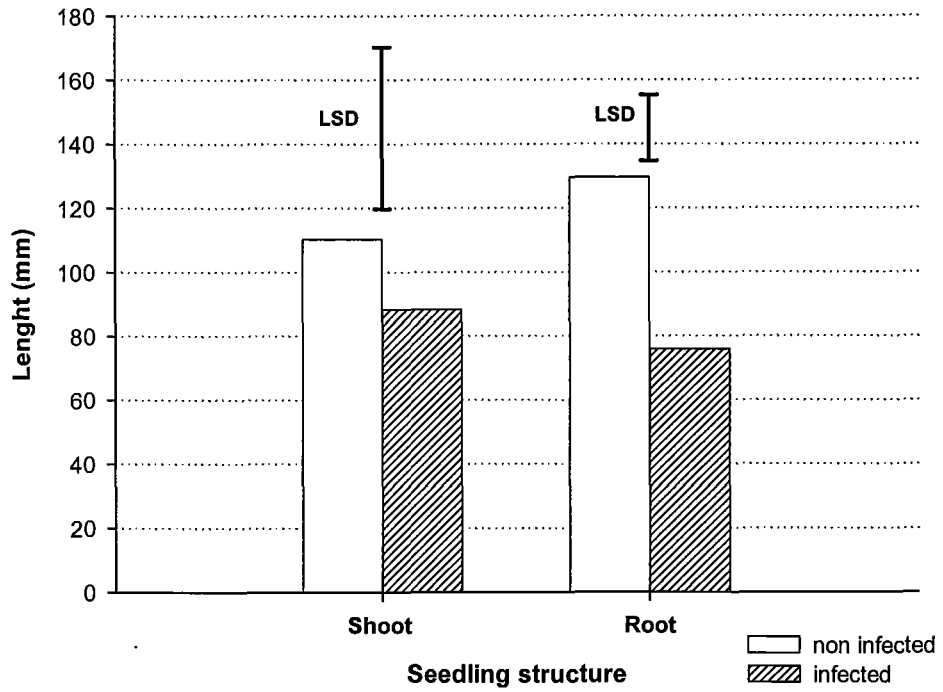
The primary root length of seedlings of cv. Rondo was significantly reduced ($P<0.01$) by *Stemphylium* spp. infection, from 130 mm to 76 mm (Figure 3.3). *Stemphylium* spp. infection reduced seedling shoot length by 20%, but this was not significantly lower than for non infected seedlings ($P<0.05$). For cv. Midichi seedlings *Stemphylium* spp. infection did not significantly ($P<0.05$) affect either shoot or root length.

3.3.7 Seedling dry weight

For both cultivars the seedlings from infected seeds were lighter than seedlings from healthy seeds.

Only means are reported because of the high number of seeds that were dead in the infected treatment, mainly for cultivar Rondo 95. The average seedling weight from non infected seeds was 0.18g seedling^{-1} for cultivar Rondo 95 and 0.34g seedling^{-1} for cultivar Midichi. Seedling weight from infected seeds of cultivar Rondo and Midichi was 0.09g and 0.25g seedling^{-1}, respectively.

(a) cv. Rondo 95



(b) cv. Midichi

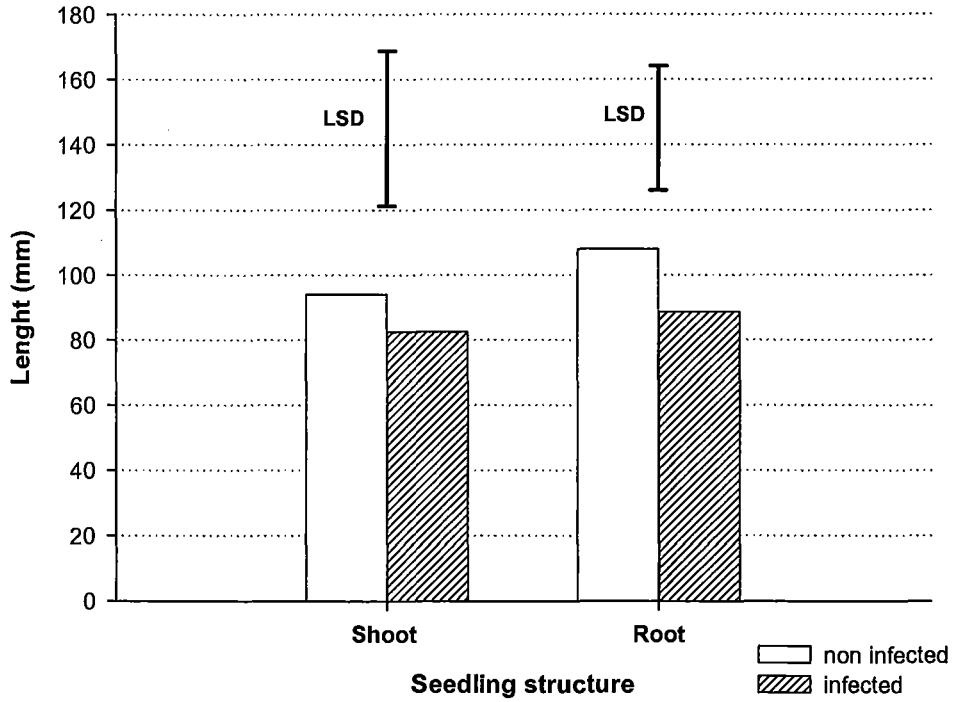


Figure 3.3 Shoot and root length (mm) of seedlings established 30 days after sowing (a) cv. Rondo 95 (b) cv. Midichi. Means of 3 replicates per treatment, with bars representing LSD at $P< 0.05$ derived from ANOVA analysis.

3.4 Discussion

3.4.1Seed health

The results obtained in this trial showed that *Stemphylium* spp. can be seed borne in peas occurring with at least another 8 fungal genera (Appendix 2). The surface disinfection (10 minutes in 1% NaOCl) did not eliminate *Stemphylium* spp. and this indicates that the pea seeds used here were infected and not only infested by *Stemphylium* spp. The incidence of *Stemphylium* spp. in the eighteen samples assessed varied from 0 to 46%, with the highest incidence found in seeds from the 2004 harvest. These seeds, when planted, may be a potential source of inoculum in the field (Stuville & Erwin 1990; Aveling & Snyman 1993).

In vitro, the fungus was not particularly associated with dead seeds. *Stemphylium* spp. apparently had no negative effect on seedlings, as overall 45% of the seeds in which *Stemphylium* spp. grew were normal. In contrast to Lamprecht & Knox-Davies (1984) and Coles and Wicks (2001), who found a relationship between infection caused by *Stemphylium* spp. and an increase in abnormal seedlings in lucerne and carrot, respectively (section 2.4.9), in this study just 43% of the seeds with *Stemphylium* spp. produced abnormal seedlings (usually a dark brown colouration in the hypocotyl region and twisted primary roots). This may indicate that some *Stemphylium* species or strains occurring on those seed lots may cause harm to the seedlings while others do not.

3.4.2 Seed germination

Germination is complex and depends on many factors, one of which may be seed health. In this experiment laboratory germination was not affected by *Stemphylium* spp. Seed lot 17 for example had high germination (91%) and high *Stemphylium* spp. infection (30%). In contrast, seed lot 2 had poor germination

(40%) but a very low *Stemphylium* spp. incidence (2.5%). The fact that no negative correlation between the laboratory germination results and the *Stemphylium* spp. infection was found (R^2= 0.0036), demonstrated that the fungi do not appear to interfere in the seed germination process itself.

3.4.3 Seedling emergence

Emergence of seeds infected by *Stemphylium* spp. was constrained compared with non infected seeds. The effect was more evident in the seed lot of cultivar Rondo 95 than in the Midichi seed lot. Only 40% of infected seeds from the cultivar Rondo 95 seed lot emerged. The seeds that did not emerge were rotted, having dark cotyledons and a barely formed root system. The effect of *Stemphylium* spp. infection on seedling performance depends on several factors. Seedling performance varied according to the seed lot used in this trial which may indicate a degree of resistance to *Stemphylium* spp. infection. Aditionally, it could be due to different species or strains of *Stemphylium* occurring in those seed lots. The data presented here are insufficient to draw conclusions about the effect of the fungus on emergence in the field, where conditions are extremely variable.

No differences in the development (node number) of seedlings from infected or uninfected seeds were observed. However, seedlings which emerged from infected seeds of cv. Rondo 95 had significantly smaller roots and smaller, but not significantly so, shoots. Also the weight of the infected seedlings was lower for both pea seed lots. *Fusarium* infected wheat (*Triticum aestivum* L.) seeds also produced lighter seedlings compared with non infected seeds, with this reduction in seedling weight mainly because of a lower root weight (Gilbert & Tekauz 1995). In this experiment, it was also observed that the primary root was

the main affected seedling structure, with a mean reduction of 23% in root length but no significant reduction in shoot length. *Stemphylium* spp. may interfere with absorption of water and nutrients especially in the young primary root tissues, which might be related to the deformities observed *in vitro* and the uneven establishment in the pot trial.

The negative effects of *Stemphylium* spp. infection on seedling performance were significant only for the seed lot of cultivar Rondo 95. For the seed lot of cultivar Midichi, even though it had the highest percentage of infection, seedlings were not significantly affected.

3.5 Summary

- *Stemphylium* spp. can be seed borne in peas and carried internally.

- *Stemphylium* spp. infection of the 14 pea seed lots assessed was not related to seed lot germination.

- For one seed lot of cultivar Rondo 95 *Stemphylium* spp. infection had a negative effect on seedling performance (emergence, seedling root size and weight). *Stemphylium* spp. infection did not significantly affect seedlings of one seed lot of cultivar Midichi.

- There was no effect of *Stemphylium* spp. on development of the seedlings (node numbers) for either cultivar.

4 Characterization of *Stemphylium* spp. isolated from pea seeds

4.1 Introduction

Stemphylium species are important seed borne pathogens in several crops such as forage legumes (lucerne, clover, trefoil), solanum species (tomato, eggplant, pepper), spinach, carrot and brassicas, like broccoli and cabbage (Lamprecht & Knox-Davies 1984; Wu 2001). *Stemphylium* spp. have been detected in pea seeds in Europe (Wegrzycka 1991) and more recently have often been observed in pea seed lots harvested in Canterbury (K. D. R. Wadia and R.G. Bakker, personal communication, 10 February 2003) (Chapter 3). Despite the successful isolation of *Stemphylium* species from both the foliage and roots of several species of plants in New Zealand (Anonymous 2001), there are no previous local reports of the occurrence of this genus in pea plants or seeds.

Host crops are affected by specific *Stemphylium* species. Onion and garlic are mainly affected by *S. vesicarium* while clover and lucerne plants and seeds may be infected by several species that may occur singly or in groups (such as *S. sarciniforme*, *S. botryosum*, *S. alfalfae*, *S. globuliferum*, *S. trifolii* and *S. vesicarium* (Stuville & Erwin 1990; Christensen & Wysong 1997; Bradley et al. 2003; Anonymous 2003c). Currently, there has been no detailed characterization of the *Stemphylium* species infecting pea seeds in the Canterbury region. The main diagnostic methods used to identify *Stemphylium* on culture media involve the evaluation of morphological and developmental characteristics (e.g. size and shape of conidia; occurrence of pseudothecia)

(Ellis 1971; Malloch 1981; Camara et al. 2002; Raid & Kucharek 2003). However, many of these characteristics overlap among species making their classification difficult (Camara et al. 2002; Bradley et al. 2003). To overcome such limitations, DNA tests have been extensively employed in phylogenetic and taxonomic studies of fungi. Such techniques can be extremely precise in distinguishing species that are difficult to characterize by microscopic observation (Mehta 2001; Camara et al. 2002).

The objectives of the work reported in this chapter are:

1. To describe the morphological and physiological characteristics of *Stemphylium* spp. isolated from pea seeds obtained from the New Zealand Seed Technology Institute Plant Diagnostic Laboratory (BioLinc).

2. To identify *Stemphylium* spp. through DNA sequencing carried by the Molecular Plant Pathology Laboratory, USDA/ARS, Beltsville, MD (USA) and correlate the results with their characteristics observed in culture on artificial media.

3. To identify the most prolific isolates and the most suitable conditions for production of spores. This information will be used for subsequent pathogenicity studies (Chapter 5).

4.2 Material and methods

A laboratory experiment was set up to describe the morphological and physiological characteristics of *Stemphylium* spp. isolated from pea seeds. Nine single spore isolates had been previously obtained from marrowfat pea

seeds harvested in Canterbury in 2002 (K.D.R. Wadia, personal communication, 8 February 2003). They were conserved on artificial media at controlled temperature (3-5°C) and were provided for this study by the Plant Diagnostic Laboratory (BioLinc).

4.2.1 Subculture

To verify fungal viability for future studies subcultures of the original isolates were prepared. The isolates were transferred to 95mm Petri dishes containing 16 – 20 ml of PDA (potato dextrose agar). Incubation conditions were 20°C, with 12 hours under fluorescent light and 12 hours darkness (Airtech Incubator, New Zealand) for 14 days. Once it had been established that all isolates were viable, a disc of approximately 5 mm of each isolate was taken from the colony margin where the fungus was actively growing and transferred onto PDA for incubation at 20°C.

4.2.2Culture in different media

The experiment was conducted at the Plant Diagnostic Laboratory (BioLinc, Lincoln University) in a factorial completely randomized design with four replicates. The factors were five different autoclaved artificial media:

- Potato dextrose agar (PDA)
- Malt Extract agar (MEA)
- Oat Meal agar (OMA)
- Prune extract agar (PEA)
- Pea seed extract agar (PSA)

The media were prepared and poured into Petri dishes (20 ml per plate). Details of preparation of media are given in Appendix 4. The artificial media were inoculated with a small disc (5 mm) of mycelium from the border of 14 day old *Stemphylium* spp. cultures grown on PDA using a sterilized cork borer and needle. One disc was placed in the centre of each Petri dish. The plates were incubated under the light and temperature conditions used previously (20°C; 12 hours under fluorescent light). The plates were assessed every two days during the 14 days of incubation.

4.2.3 Measurements

The diameter (mm) of the colonies (mycelial growth) was measured every two days for a total of 14 days using a digital caliper (Digimatic - Mitutoyo) (R.G Bakker, personal communication, 1 August 2003). Diameter values were used to calculate the area of the colonies ($A = \pi * r^2$) (Prosser 1994a). Visual characteristics of the mycelium, such as colour, texture and shape were recorded after 7 and 14 incubation days (Hawksworth 1974). Determination of the colour of the colonies was done by visual assessment using the Kornerup and Wanscher (1968) system. Shape was recorded as an irregular (I) or circular (C) pattern of colony growth (Cochrane 1958a; Russell 1981). Texture of the aerial mycelium was determined by observing the top of the

colonies and using the follow terminology:

(Hawksworth et al. 1995; Anonymous 2002a)

The presence of reproductive structures and sporulation were recorded after 14 days incubation, by viewing the colonies using a stereoscope microscope. A scale ranging from 0 (absent) to 5 (abundant) was used to quantify the presence of pseudothecia and conidia. The location of pseudothecia and conidia was also recorded. To further enhance sporulation, isolates were then subjected to two different light and temperature regimes: two replicates of each treatment were placed under near ultraviolet light (Phillips S10 4-65 W) at 20°C (Martiniello & Porta-Puglia 1995) and the other two replicates were left on a laboratory bench under natural light conditions (Bradley et al. 2003) and variable temperature (17 – 25°C). After 7 days, plates were examined for conidial presence and location of conidial production. In the case of positive sporulation, conidial dimensions (length and width, μm) of 30 conidia of each replicate were taken using a compound microscope at 400 x magnification (Hawksworth 1974). A conidial suspension was prepared adding 10 ml sterile distilled water to the plates and by gently scraping the surface of the colonies with a sterile glass rod and filtering the resulting spore suspension through a folded cheese cloth (Mehta 2001; K. D. R. Wadia, personal communication, 12 April 2003). Spore concentration (spores ml^{-1}) was measured using a haemocytometer. Additionally, pieces of *Stemphylium* isolate 7 colonies containing mycelia and conidia were covered by a thin layer of gold with a sputter coater (Souza 1998) for 2 minutes and then observed through a scanning electron microscope (SEM) (N. Andrews, personal communication, 23 September 2004).

4.2.4 Molecular identification of *Stemphylium* isolates

A small portion of the margin of 14 day old pure *Stemphylium* spp. cultures containing mycelia and/or conidia was transferred to slopes (centrifuge

Eppendorf tubes) containing approximately 1.5 ml of malt extract agar (MEA). The pure cultures of the isolates were sent to the Molecular Plant Pathology Laboratory, USDA/ARS, Beltsville, MD (USA). Identification of isolates was performed using DNA sequencing of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*). The *Stemphylium* isolates used in this study were compared with isolates of other regions and collectors (Appendices 8 and 9).

4.2.5 Data analysis

Growth rates of the isolates were compared by fitting a linear regression (y = ax + b) from the 2nd to the 10th day of incubation, where "y" represents the colony area (mm^2) and "a" the growth rate (mm^2day^{-1}) (Prosser 1994a). Analyses of variance (ANOVA) were performed using the statistical package Genstat 6th edition (Lawes Agricultural Trust, 2002). Growth rates (mm^2day^{-1}) were separated using the least significant difference ($P< 0.05$) (Clarke & Kempson 1997). In the case of conidial dimensions, maximum and minimum values for length and width are reported.

4.3 Results

4.3.1 Area of colonies and growth rate

According to their growth rate, isolates were grouped into slow (< 250 mm^2day^{-1}), medium (250-450 mm^2day^{-1}) and fast (> 450 mm^2day^{-1}) growing groups. There was a highly significant interaction ($P < 0.001$) between isolate and media. Broadly, most isolates grew faster on OMA and PSA, with overall means of 523.2 and 561.5 mm^2 day^{-1}, respectively (Table 4.1). On PDA, the mean growth rate for all isolates was only 287.0 mm^2 day^{-1}, and this value

differed stastistically from the growth rates obtained on OMA and PSA. On MEA isolates 2, 5 and 6 were slow growing (96 mm^2day^{-1}) (Table 4.1 and Figure 4.1). Isolates 1 and 9 had an intermediate growth rate (364.3 mm^2day^{-1}) but were not statistically different from the faster ones (isolates 3, 4, 7 and 8) with an average of 508.2 mm^2day^{-1}.

Table 4.1 Growth rate (mm^2 day^{-1}) of *Stemphylium* spp. isolates on five different artificial media.

	Isolate growth rate (mm^2 day^{-1})									
Media	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Mean**
MEA	321.4	49.0	450.6	512.8	74.9	164.2	515.8	554.4	407.3	338.9
OMA	382.7	608.2	465.2	572.7	497.8	566.0	630.7	543.7	442.0	523.2
PDA	235.2	67.8	93.1	481.5	188.4	253.9	316.4	714.2	232.7	287.0
PSA	316.9	658.3	443.4	598.0	504.3	678.1	579.5	724.8	550.0	561.5
PEA	317.1	150.9	409.2	493.0	117.7	176.9	506.5	555.4	372.4	344.3
Mean	314.6	306.8	372.3	531.6	276.6	367.8	509.7	618.5	400.8	411.0

* LSD (isolate x media) = 77.01; mean R^2= 0.9534
Mean of 4 replicates per treatment.

Results were similar on PEA, as isolates 2, 5 and 6 were again slow growing (148.5 mm^2day^{-1}) and were significantly different from isolates 1, 3 and 9 (317.1, 409.2 and 372.4 mm^2day^{-1}, respectively). Isolates 4, 7 and 8 had the fastest growth rates (518.3 mm^2day^{-1}).The growth of isolates 1 and 9 was relatively slow on OMA (412.3 mm^2day^{-1}). On this media, isolates 3, 5 and 8 had a medium growth rate (502.2 mm^2day^{-1}). Fastest growth was observed for isolates 2, 4, 6 and 7 (594.4 mm^2day^{-1}). However, only the growth rates of isolates 1 and 2 were significantly different ($P<0.05$) from other isolates. PDA

did not promote abundant growth of *Stemphylium* isolates and most colonies grew poorly on this medium. Compared with other isolates, 4 (481.5 mm^2day^{-1}) and 8 (714.2 mm^2day^{-1}) grew faster. On PSA isolates 1 and 3 grew slowly (380.1 mm^2day^{-1}) while isolates 4, 5, 7 and 9 showed medium growth (557.9 mm^2day^{-1}). Fastest growth was observed in colonies 2, 6 and 8 (687.0 mm^2day^{-1}).

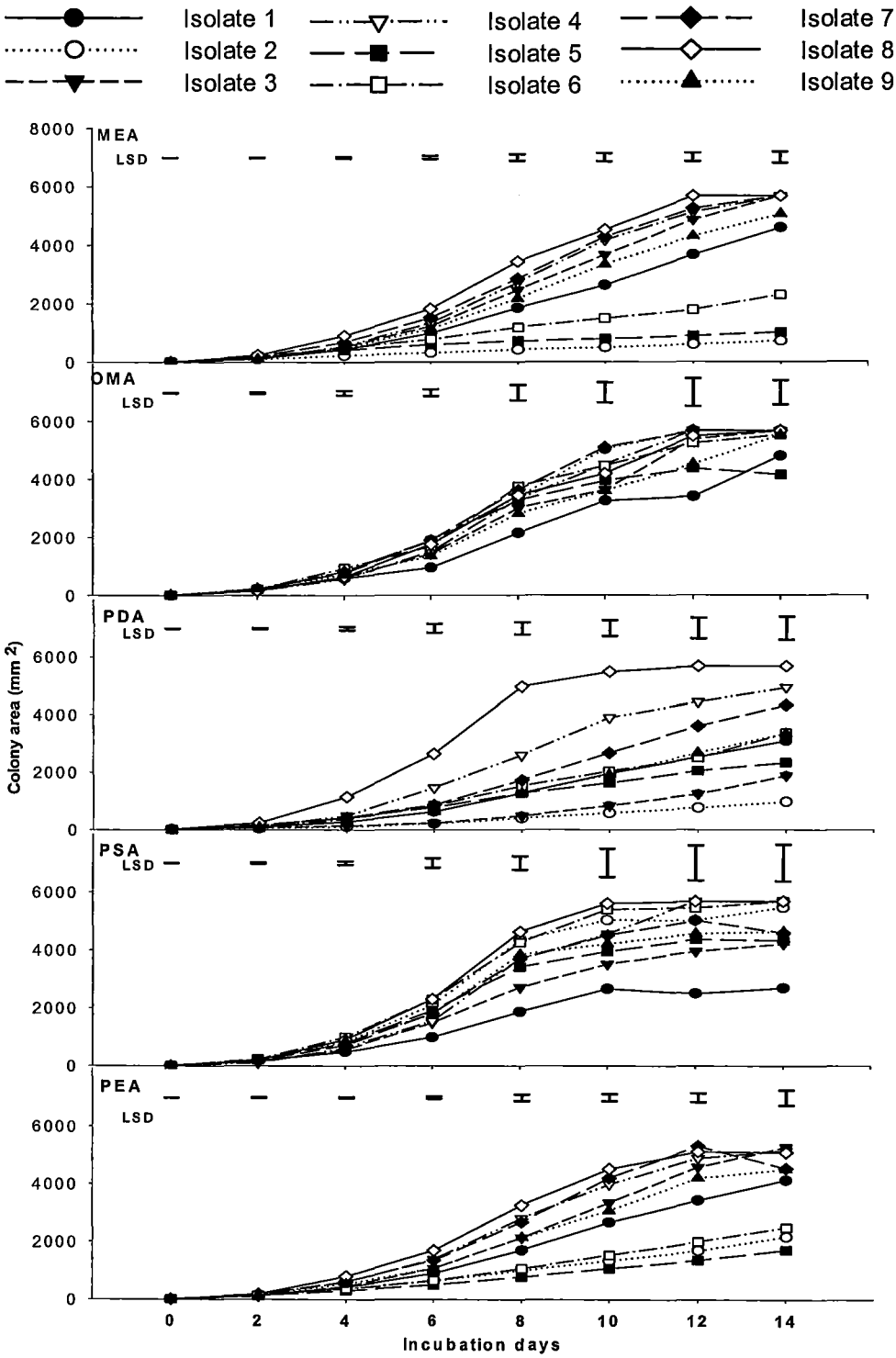


Figure 4.1 Colony area (mm^2) growth of nine *Stemphylium* spp. isolates on five different culture media over time (14 days incubation; 20°C; NUV light). Means of four replicates per treatment, with bars representing LSD at $P < 0.05$.

4.3.2 Visual characteristics

4.3.2.1 Colour, shape and texture

The colour of the surface of the colonies ranged from white to grey to brown, according to the medium on which they grew. In general, the colour of the bottom of the plates was yellow to brown. The description of the colour of the isolate colonies is given in Appendices 5 and 6. A more detailed view of each isolate is also presented in Figure 4.2. In terms of visual characteristics of the mycelia on MEA, isolates 2, 5 and 6 presented similar irregular shaped colonies with a cottony texture. The surface colony colour was orange grey to grey and the bottom was brown to orange-dark brown. Isolates 4 and 7 had a downy texture and circular shape with mycelial colour ranging from brownish orange to dark brown. Isolates 3 and 9 also had a circular shape and downy texture, but the mycelial colour was greyish orange. Isolates 1 and 8 had a circular shape and downy mycelia. The colour of the surface of these last colonies was light brown and white-orange grey. Isolates 4 and 7 were similar on OMA media, with a downy texture and circular shape. The mycelial colour was white-brown. Isolates 2 and 5 also had downy and circular mycelia with colour varying from light brown to grey. Isolates 3 and 9 again were similar, with white mycelia, a circular shape and downy texture. Isolates 1 and 6 also had downy mycelia, however, isolate 1 grew irregularly and the colour ranged from yellowish brown to white whereas colonies of isolate 6 had a brownish orange to white surface. Colonies of isolate 8 were brownish orange, circular and cottony in texture. On PDA isolates 4 and 7 again produced similar light brown to brownish orange, downy and circular colonies. Isolates 1, 5 and 6 were analogous, with an effuse texture, but isolate 5 had an irregular shape whereas isolates 1 and 6 a circular shape. However, the surface mycelia were consistently brownish orange.

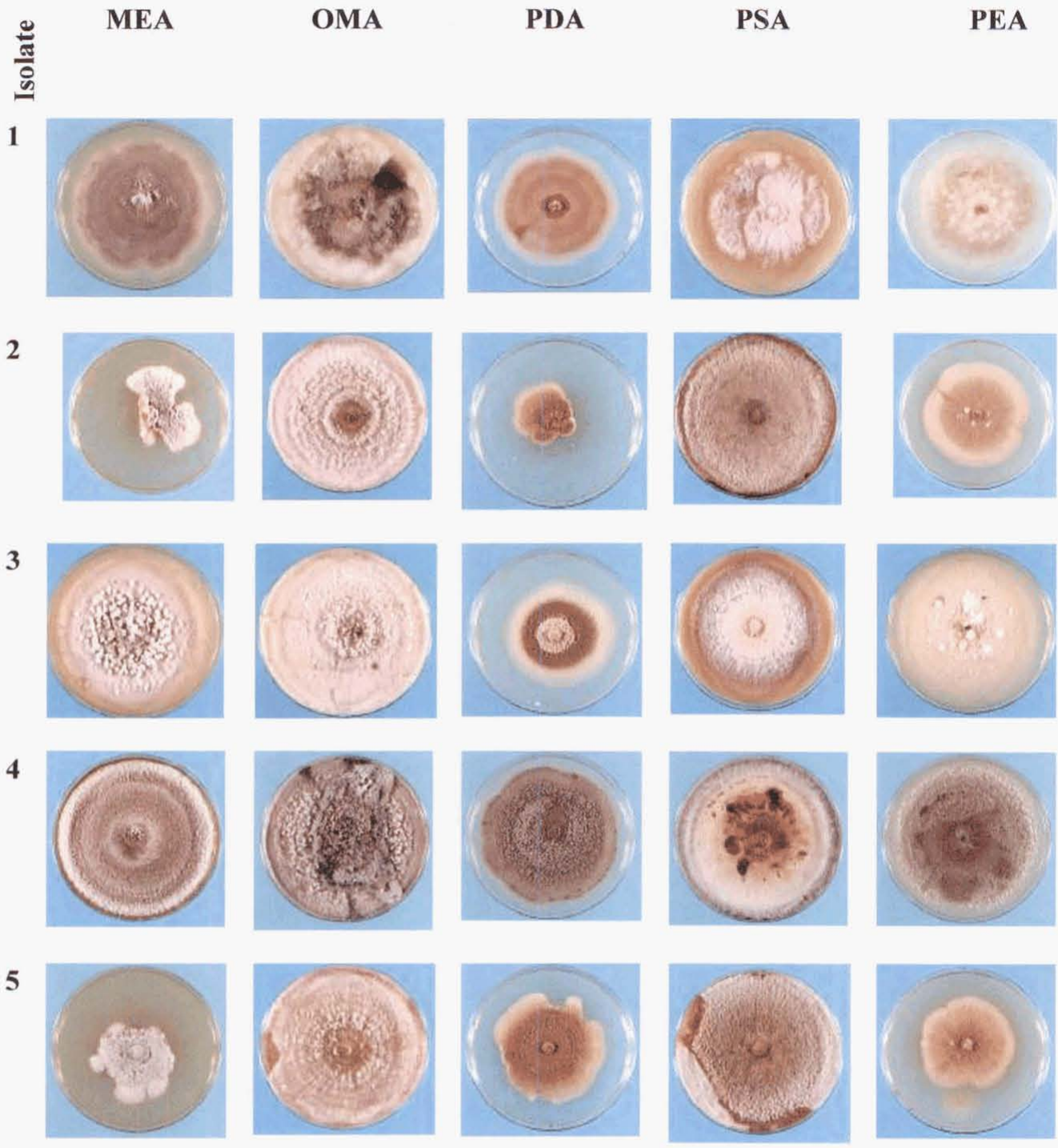


Figure 4.2 Surface view of the colonies of the isolates after 14 days incubation (20°C and fluorescent light) on different media.

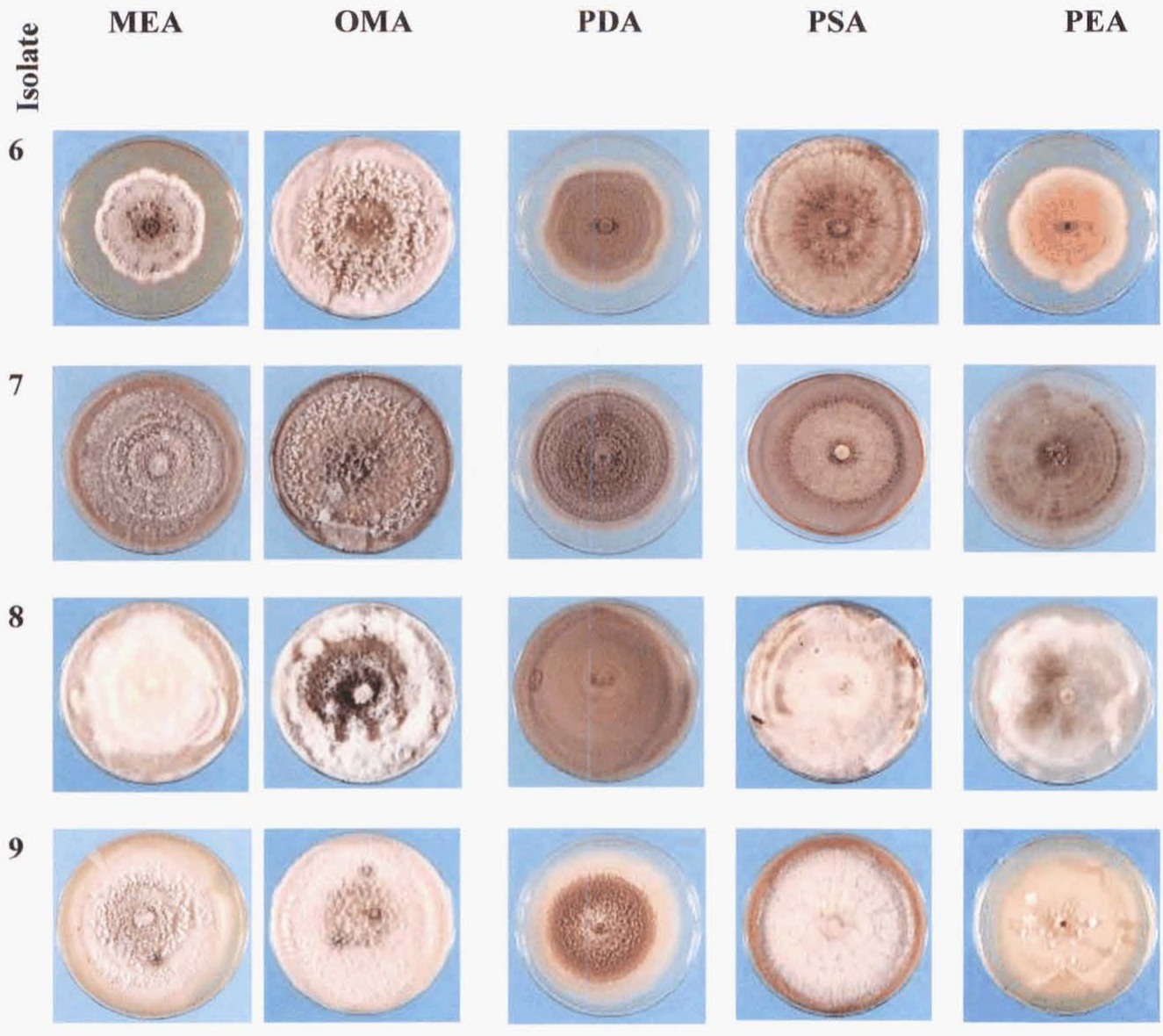


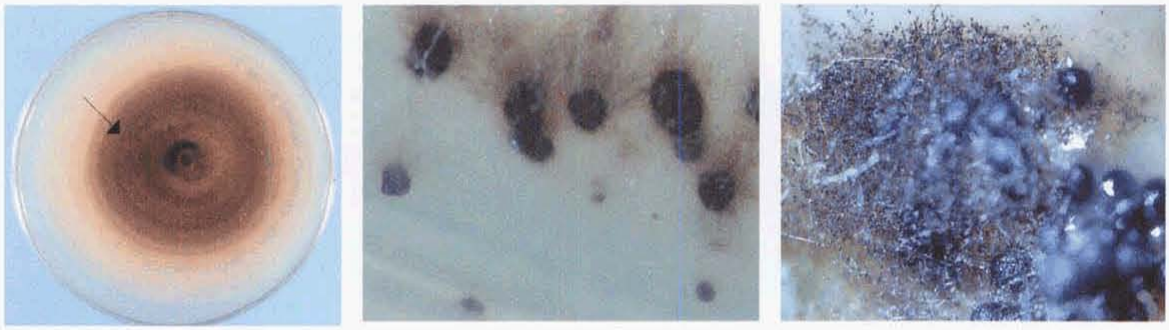
Figure 4.2 Surface view of the colonies of the isolates after 14 days incubation (20°C and fluorescent light) on different media continued.

On PSA all isolates were circular in shape, except isolate 1. All the isolates also produced a downy mycelia with the exception of isolate 7 which was effuse. Colonies of isolates 2 and 3 were very similar, with colour ranging from orange grey to white. Further, the colour of colonies of isolates 5 and 6 was similar (ranging from pale orange to white-orange grey). On PEA all isolates had a circular pattern of growth. Isolates 1, 4 and 7 had effuse mycelia while the aerial mycelia of isolates 2,3,5,6, 8 and 9 were downy. On plates of isolates 4 and 7, dark concentric rings were observed in the bottom of the plate. Similarities were observed among isolates 2, 3 and 9 which all had orange grey-orange white mycelial colour. Isolates 5 and 6 were also similar in colour (a brownish orange colour).

4.3.3 Production of reproductive structures

After 14 days incubation (20°C under fluorescent light) on MEA all isolates produced sexual fruiting bodies (pseudothecia) except isolate 4 (Figure 4.4). Isolates 1, 3, 6 and 7 produced abundant pseudothecia, which were distributed over the entire colony surface. Other isolates, in contrast, produced few fruiting bodies which were mostly concentrated either at the centre or at the edge of the colony. There was a substantial production of pseudothecia on OMA by all isolates, mostly dispersed evenly over the plate. The exception was isolate 8 with a score below 1. Only isolates 1, 3 and 9 (average scores 1, 4 and 2, respectively) produced pseudothecia on PDA. Fruiting bodies formed in concentric rings all over the plate in the case of isolates 3 and 9 (Figures 4.2 and 4.3). Pseudothecia were observed mainly in the centre. For isolate 1, fruiting bodies were concentrated in the edge of the colony. No pseudothecial production occurred in isolates 4, 7 and 8 on PSA. Isolates 1, 2, 3, 5 and 9 produced few pseudothecia (ranging from 0.5 – 2.5) on this media, while

isolate 6 produced abundant fruiting bodies all over the plate. On PEA, all the nine isolates produced pseudothecia, with scores ranging from 1.75 (isolate 4) to 5 (isolates 6 and 7).



(a) (b) (c)

Figure 4.3 Detail of pseudothecia produced by isolate 9 on PDA media.

(a) Concentric rings (arrow on obverse view) ; (b) fruiting bodies on the reverse of the plate (20-45x); (c) fruiting bodies and spores observed on the colony surface (under magnification 20 – 45x).

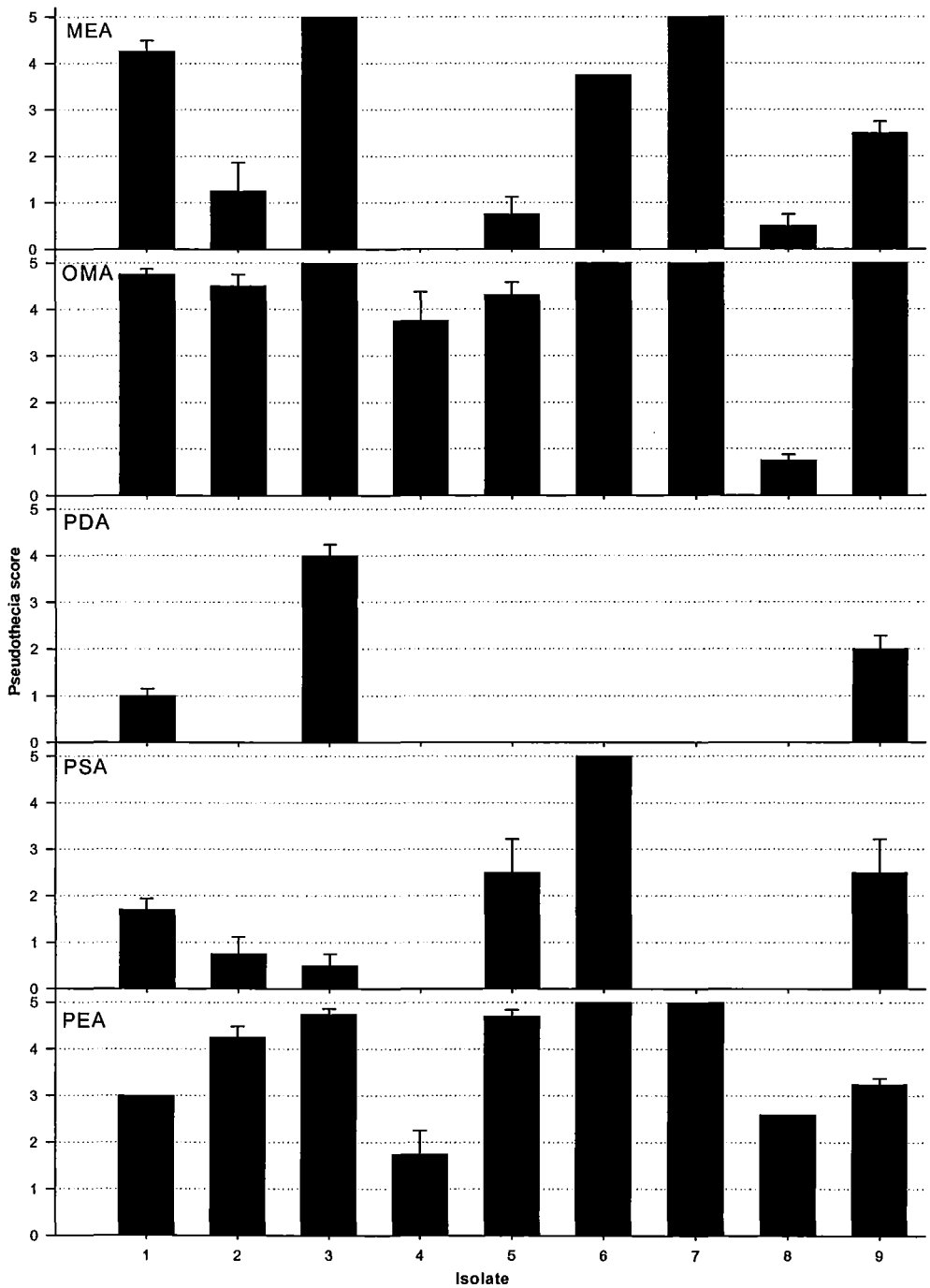


Figure 4.4 Score of presence of fruiting bodies (pseudothecia) of nine *Stemphylium* isolates on different artificial media (0 = absent; 5 = abundant). Mean of 4 replicates per treatment, with bars representing one standard error.

4.3.4 Conidial production

There was no production of conidia on PDA and PSA for any of the isolates. OMA was the media that promoted most conidial production although only 5 of the 9 isolates produced conidia (Figure 4.5). Conidia of isolates 2, 4, 6, and 7 were observed after 20 days incubation (7 days exposure to NUV/natural light). Conidia of isolate 5 were observed five days later. Among them, only isolate 7 presented a high score for sporulation.

On MEA only isolates 1, 2, 6 and 7 produced conidia. Isolate 7 was the most prolific, producing abundant conidia by the 14 th day of the initial incubation. The other three isolates produced conidia only after 7 days (isolates 1 and 6) or 11 (isolate 2) days exposure to NUV light or natural light (Appendix 7). On this medium isolate 6 produced conidia exclusively under natural light exposure, whereas isolates 1 and 2 only produced conidia under NUV light. Isolate 7 produced conidia in both light regimes with no difference ($P<0.05$) in spore production under NUV or natural light. This was assessed by measuring spore concentration with a haemocytometer (1.8×10^4 spores ml^{-1} under natural light and 2.5×10^4 spores ml^{-1} under NUV light).

On PEA only isolates 5 and 7 produced conidia by the 20th day, and these were concentrated mainly at the border of the colonies. On this medium there was no difference in conidial score after exposure under natural or NUV light. No conidia were produced on any medium by isolates 3, 8 and 9.

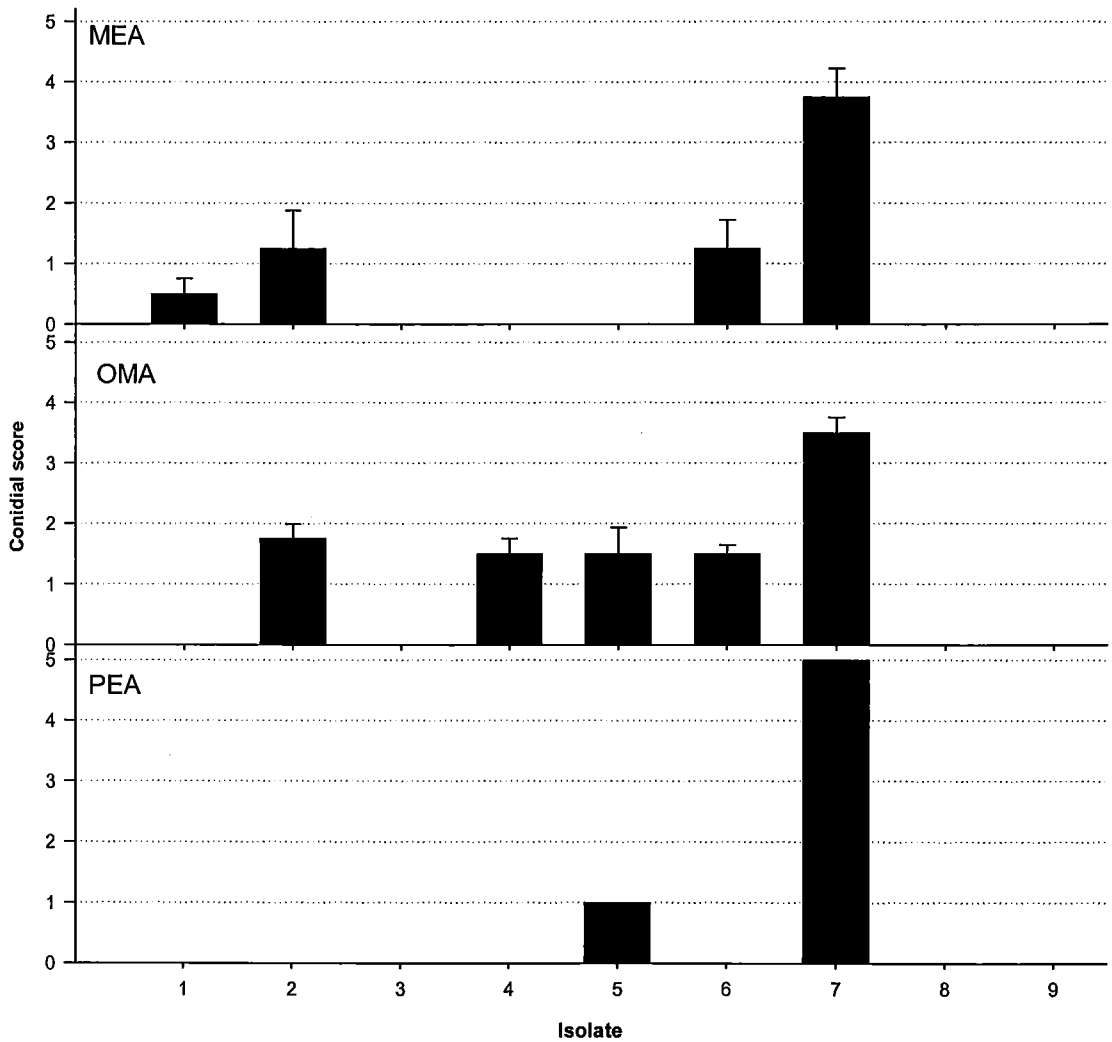
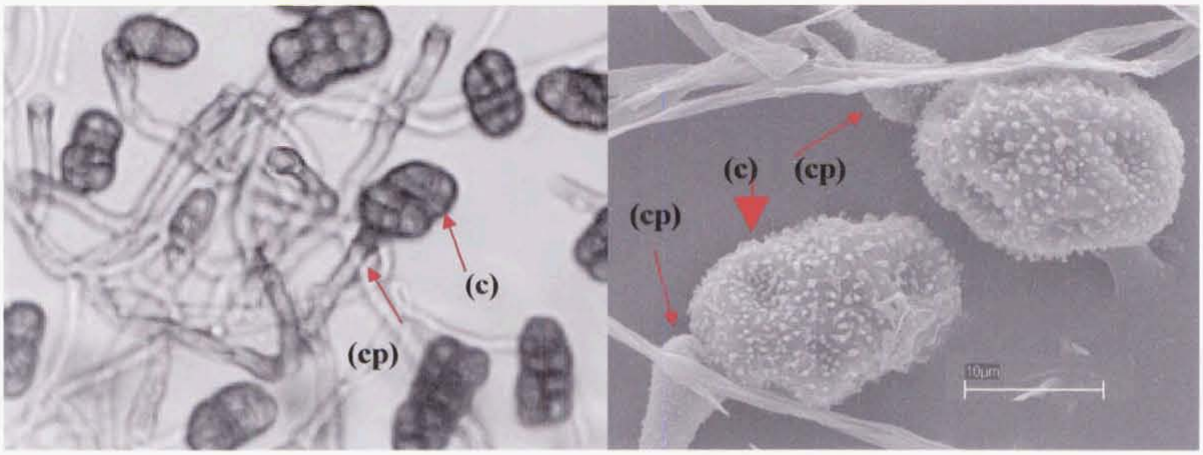


Figure 4.5 Score of conidia production of nine *Stemphylium* isolates on different media (0 = absent; 5 = abundant).

Mean of 4 replicates per treatment, with bars representing one standard error.

Conidia of isolates 1 and 7 (Figure 4.6) were predominantly verrucose whereas those of isolates 2 and 6 were smooth. For the other isolates (4, 5, 6) the texture of conidia ranged from smooth to verrucose.



(a) (b)

Figure 4.6 Conidia produced by isolate 7.

(a) Microscope view (400 x magnification) (b) detail of verrucose surface of conidia and conidiophores (scanning electronic microscope). Arrows indicate conidia(c) and conidiophores (cp).

The maximum and minimum values of conidial length and width (µm) are presented in Table 4.2.

Table 4.2 Minimum and maximum length (L) and width (W) (µm) of conidia of nine isolates on different artificial media.

Isolate	Media					
	MEA		OMA		PEA	
	L (min-max) (µm)	W (min-max) (µm)	L (min-max) (µm)	W (min-max) (µm)	L (min-max) (µm)	W (min-max) (µm)
1	15.0–30.0	10.0–18.0	–	–	–	–
2	20.0–30.0	10.0–23.0	13.8–25.0	10.0 – 19.0	–	–
3	–*	–	–	–	–	–
4	–	–	12.5–22.5	10.0 – 13.0	–	–
5	–	–	12.5–23.8	10.0 – 20.0	11.0 – 25.0	8.8 – 18.0
6	12.0– 26.0	10.0–21.0	12.5–27.5	11.0 – 21.0	–	–
7	14.0–29.5	10.0–29.0	15.0–24.0	12.0 – 19.0	12.5 – 28.3	9.0 – 18.0
8	–	–	–	–	–	–
9	–	–	–	–	–	–

*none produced. Means of 30 conidia per replicate.

4.3.5 Phylogeny analysis

DNA sequencing of the *gpd* gene placed isolates 1 and 9 in group C as they grouped together with *S. astragali* (Table 4.3 and Appendices 8 and 9). Isolate 3 also aligned close to *S. astragali*. Isolates 2, 5 and 6 were included in group E, forming a *Stemphylim* spp. cluster closely related to *S. loti* and *S. sarciforme*. Isolates 4 and 7 were also placed in group C, which in addition to *S. astragali,* also includes *S. herbarum, alfalfae, vesicarium, majusculum* and *gracilariae*. These two isolates were identified as *S. herbarum*. In the phylogenetic tree (Appendix 8) these two isolates are placed down in the branch, near *S. herbarum* EGS 38-091 and EGS 12-171 and distant from *S. alfalfae* and *S. vesicarium*. Isolate 8 could not be analysed due to difficulties in growing this isolate. The isolates 11 and 12, that were obtained from experiment 3 were also grouped in the *S. herbarum* branch. They were not included in the morphology study in this chapter. More details about these two isolates are given in section 5.3.4.

Table 4.3 Phylogenetic analysis of the *Stemphylium* isolates from marrowfat pea seeds.

Isolate	Group	*Stemphylium* species
1	C	*S. astragali*
2	E	*Stemphylium* spp. close to *S. loti / S. sarciniforme*
3	C	*Stemphylium* spp. close to *S. astragali*
4	C	*S. herbarum /vesicarum / alfalfae*
5	E	*Stemphylium* spp. close to *S. loti / S. sarciniforme*
6	E	*Stemphylium* spp. close to *S. loti / S. sarciniforme*
7	C	*S. herbarum /vesicarum/ alfalfae*
9	C	*S. astragali*

4.4 Discussion

This laboratory study of the *Stemphylium* fungi isolated from pea seeds revealed that more than one species can be seed borne. Culture growth on artificial media demonstrated visual differences among the isolates. However, the characteristics observed in culture media were not fully in agreement with the DNA sequencing identification of the isolates. Distinction between the isolates was even more complex because of major differences in morphological and physiological characteristics when the same isolate was grown on different media.

4.4.1 Colony growth

In this experiment the nine different *Stemphylium* isolates had different growth rates according to the culture media. PSA and OMA were the media that promoted faster growth of most isolates. Colonies grew 95% and 82% fastest on PSA and OMA, respectively than on PDA.

Isolates 4, 7 and 8 grew 62% faster on all media than isolates 1, 3, 5 and 9. These two groups were consistently fast or slow growers irrespective of media. Isolates 2 and 6 had variable and intermediate growth rates according to the media.The medium influenced the growth rate of the colony presumably because of the nutrients each provided. PSA and OMA may contain nutrients which promote mycelial growth of *Stemphylium* spp. compared with the other media (Appendix 4). This might explain the faster growth of all isolates on these two media. They are made from fresh plant parts and are rich in sterols and other growth factors (E. E. Jones, personal communication, 8 December 2004).

The nutrients available in the substratum are absorbed and support mycelium growth (Carlile 1994). Glucose diffuses rapidly through agar and its utilization by colonies growing on agar may result in nutritional limitation (Prosser 1994a). Additionally, there is a variation in pH as the fungus exploits nutrients (especially nitrogen) from the medium (Curren 1968). The pH of the medium influences the uptake of amino acids by determining the charge of the amino acid carrier protein. The optimum pH for the growth of fungi varies with strain, species and nutritional environment but in general, uptake of nutrients occurs most rapidly at pH 5- 6 (Garraway & Evans 1984). The pHs of the media used in this study were in this optimal range, from 5.5 ± 0.2 to 6 ± 0.2 (Atlas 1993). Alterations in pH and nutrient concentration with time were not recorded in this experiment but it is evident that the nutritional status of the media influenced the growth and morphological features of the isolates.

PDA is a standard media used for culture of *Stemphylium* species (Barash et al. 1978; Hawksworth et al. 1995; Cho et al. 2001) . Colonies of *S. botryosum* are reported to grow quickly on PDA (Mathur & Kongsdal 2002) while *S. solani* is slow growing on PDA (Mehta 2001). Aveling & Snyman (1993) reported a colony area development of 4400 mm^2 in six days from *S. vesicarium* on PDA. In the present experiment only isolate 8 grew fast on PDA, but achieved only 2634 mm^2 by the sixth day. Four out of nine of the isolates had intermediate growth on this media and four isolates a slow growth rate. Three main groups of *Stemphylium* were distinguishable based on colony growth on different media (Table 4.4).

Table 4.4 *Stemphylium* spp. isolates grouped according to growth rate on five different culture media.

Growth rate	Medium				
	MEA	**PEA**	**PDA**	**OMA**	**PSA**
Slow *1	2,5,6	2,5,6	1,2,3,5	-	1,3,5
Intermediate *2	1,3,9	1,3,9	4,6,7,9	1,3,9,5	4,7,9
Fast *3	4,7,8	4,7,8	8	2, 4, 6,7, 8	2,6,8

*1 slow = <250 mm^2day^{-1}
*2 intermediate = 250 - 450 mm^2day^{-1}
*3 = > 450 mm^2day^{-1}

This clearly shows media preference by some isolates over others and the often poor growth obtained on PDA, which is the most widely used culture medium for *Stemphylium* by other workers. However, this media also did not encouraged or promote the production of conidia by any of the isolates tested (section 4.3.4). For MEA and PEA there was a correlation between the growth and the species. Slow growth was observed for all related *S. loti/ sarciniforme* isolates (2, 5 and 6) whereas *S. astragali* related isolates (1, 3, and 9) had intermediate growth. The fastest growth was observed for *S. herbarum/ vesicarium/ alfalfae* isolates (4, 7) and isolate 8.

4.4.2 Colony characteristics

Visual colony characteristics are often used to distinguish fungi at genus or species level. Derived from colony features, the present isolates were grouped into five sets (Table 4.5). The five groups (i, ii, iii, iv and v) were formed according to similarities in colour, shape and texture of the colonies on the media (Figure 4.2 and Appendices 5 and 6).

The visual aspect of the majority of the isolates agrees with colony characteristics described by Ellis (1971) and Mathur and Kongsdal (2002) for *Stemphylium* spp. However, it is notable that media type did alter the appearance of the colonies with time. When growth conditions become unfavourable, secondary metabolites or staling products are often produced at the colony centre. The result is a change in the branch angle, hyphal diameter and mycelial extension rates (Prosser 1994b). This might be the reason for irregular shapes observed in isolates 2 and 5 on MEA and PDA and variability in colony growth rate among isolates as well as among media.

As colonies became older the production of spores and other structures starts. The mycelium can also change substantially in colour and possibly in texture. Generally the colony surface became darker with age and changed from a floccose to a downy texture. It is also possible to distinguish concentric dark rings formed due to the production of fruiting bodies. Again the nutritional status of the media is related to the presence and abundance of these structures. Often a media high in carbon promotes mycelial (vegetative) growth, whilst a media low in carbon (low carbon to nitrogen ratio) promotes reproduction (E. E. Jones, personal communication, 8 December 2004). As exhaustion of nutrients occurs, the production of reproductive structures for dispersion (asexual) or survival (sexual) is initiated (Carlile 1994).

Table 4.5 *Stemphylium* spp. isolates grouped according to the visual characteristics (colour, shape and texture of the colonies) on five different media.

Group	Isolates	*Stemphylium* species
i	2,5,6	all *Stemphylium* related to *loti/ sarciniforme*
ii	4,7	*S. herbarum/ vesicarium/ alfalfae*
iii	3,9	*S. astragali and Stemphylium* spp. close to *S. astragali*
iv	1	*S. astragali*
v	8	Not identified

i = includes colonies with irregular shape on MEA and PDA and circular shape on OMA, PSA and PEA.
Cottony texture on MEA; downy texture on OMA and PSA and effuse on PDA and PEA. The colour was nearly an orange grey surface colony, dark brown colony base on MEA. On OMA colonies were light brown to white on the top and brownish orange in the base. On PDA the surface of the colonies was brownish orange. On PSA the colonies surface were white to pale orange, while on PEA they were brownish orange to orange grey.

ii = the colonies had circular shape on all media. On MEA the colour of colony surface was orange- grey brownish and the mycelia were downy. On OMA it was observed a white and brown mycelia with downy texture. On PDA the mycelia had a brown surface and dark brown base, and a downy texture. On PSA colonies had white to light brown surface and downy and effuse texture. On PEA colonies had yellowish brown surface, a effuse texture with fruiting bodies forming visible rings in obverse view.

iii = includes colonies of circular shape on all media. The texture was cottony on OMA and downy on all the other four media. The colour of mycelia surface on MEA was greyish orange while on OMA and PSA was white.On PDA and PEA the colonies were orange in colour.

iv = in this group, colonies had circular shape on MEA, PDA and PEA and irregular on OMA and PSA. The texture was downy on MEA, OMA and PSA and effuse on PEA and PDA. The colour of colonies surface was greyish orange on PEA and PSA, and ranged from brownish orange to white on PDA, The colours of mycelia were light brown on MEA and yellowish brown on OMA.

v = isolates which colonies were circular in shape on all the five media. They had a downy texture on MEA, PDA, PSA and PEA and a cottony texture on OMA. The surface of colonies were white on MEA and PSA whereas on OMA was olive brown on OMA. On PDA and PEA colonies were brownish orange.

4.4.3 Production of reproductive structures

4.4.3.1 Pseudothecia

Media had a considerable effect on the production of fruiting bodies (Table 4.6). All the isolates produced pseudothecia on OMA and PEA. On MEA eight out of nine isolates produced pseudothecia (Figure 4.4). PDA and PSA did not favour the production of fruiting bodies. Among the isolates, 3 and 9 consistently produced abundant pseudothecia on all media.

OMA and PEA may have substances, such as sterols, which are required by the fungus for reproduction (Hendrix 1970). In contrast, PDA is poor in sterols (E. E. Jones, personal communication, 8 December 2004) and therefore may not have supported the production of reproductive structures by *Stemphylium* spp. isolates

Table 4.6 Nine *Stemphylium* spp. isolates grouped according to pseudothecia production on five different media.

Score	Media				
	MEA	OMA	PDA	PSA	PEA
0-1	**4,5,8**	**9**	**1,2,4,5,6,7,8**	**2,3,4,7,8**	**-**
1.1-3	**2, 9**	**-**	**9**	**1,5,9**	**1,4,8**
3.1-5	**1,3,6,7**	**1,2,3,4,5,6,7,8**	**3**	**6**	**2,3,5,6,7,9**

The production of fruiting bodies is an important feature of distinction among *Stemphylium* species (Stuville & Erwin 1990). Pseudothecia are the primary sexual reproductive structures of teleomorph *Pleospora* spp. Some species of *Stemphylium* (*Pleospora*) do not produce pseudothecia on culture media (e.g. *S. loti* and *S. sarciniforme* growing on V-8 juice agar, 25°C for 4 weeks)

(Stuville & Erwin 1990; Bradley et al. 2003),while others such as *S. herbarum*, *S. vesicarium* and *S. alfalfae* are well known for the production of fruiting bodies in culture (Stuville & Erwin 1990; Bradley et al. 2003) . Aveling (1992) observed immersed dark-brown globose pseudothecia produced by *S. vesicarium* on PDA. In contrast, S. *botryosum* and *S. globuliferum* show only slow ascomatal development on artificial media (Ellis 1971; Camara et al. 2002; Bradley et al. 2003). In this experiment (20°C for 14 days), isolates 2, 5 and 6 (identified as *S. loti* and *S. sarciniforme*) produced pseudotechia, especially on OMA and PEA, which enphasises that characterization of species only by morphological and physiological can be deceptive due to differences in the culturing conditions provided for fungal growth.

Despite pseudothecial production, no fruiting body maturation was observed for any of the isolates. Maturation of pseudothecia and the formation of ascospores usually requires exposure to low temperatures (10 – 15°C) (Suheri & Price 2001; Llorente & Montesinos 2004) or occurs with increased age of culture (from 2 – 12 months) (Cochrane 1958b; Stuville & Erwin 1990). This may explain the lack of mature pseudothecia in the current study.

4.4.3.2 Conidia

PEA, OMA, and MEA supported conidial production, with OMA and MEA being the media promoting most asexual sporulation. Conversely, PSA, despite fast colony growth, did not support conidial production. Conidial production was also not observed on PDA. *Alternaria alternata* grown in PDA under NUV produced less conidia than on V8 juice agar (Masangkay et al. 2000).Cho et al. (2001) also reported difficulties inducing sporulation of *S.*

solani on PDA. In this experiment isolate 7 produced abundant conidia while isolates 2, 4, 5 and 6 developed few conidia. Isolate 1 only produced conidia on MEA and isolates 3, 8 and 9 did not produce any (Table 4.7 and Figure 4.5).

The effect of media on conidial production can be explained by the fact that microorganisms tend to reproduce more effectively under certain stress conditions, for example, limited nutrients such as glucose (Cochrane 1958a; Carlile 1994). Nutrient concentration is depleted as the fungal colony grows, with an accumulation of inhibitory end products, production of secondary metabolites and changes in pH (Prosser 1994a). Curren (1968) reported that a drop of pH from 5.6 to 3.4 in 9 days of incubation negatively affected the uptake of nitrogen compounds by *S. radicinum* isolates. As a result the growth of *S. radicinum* was inhibited. The most evident changes resulting from growth limitation are the development of differentiated structures such as fruiting bodies and spore bearing aerial hyphae (Prosser 1994a). According to Griffin (1981) the changes from vegetative hyphae to conidiophores and to conidia may be dependent on genetics. Enzyme activity is needed for the transcription of RNA and synthesis of polysaccharides which form the spores themselves. The control of these metabolic processes is regulated by genes but may depend on substrate limitation and the presence or absence of inhibitors and activators. In this experiment, it is likely that both factors (genetic and nutritional) contributed to the production or lack of production of *Stemphylium* conidia. Sterols once more may have an important role in the production of conidia. According to Hendrix (1970) presence of sterols (particularly ergosterol) increased conidiation threefold on a natural media. Sterols also

replaced the ultraviolet light requirement for *S. solani* conidial production (Sproston & Setlow 1967).

Table 4.7 Nine *Stemphylium* spp. isolates grouped according to conidial production on five different media.

Score	**Media**				
	MEA	OMA	PDA	PEA	PSA
0-1	**1, 3, 4, 5, 8, 9**	**1, 3, 8, 9**	-	**1, 2, 3, 4, 6, 8, 9**	-
1.1-3	**2, 6**	**2, 4, 5, 6**	-	**5**	-
3.1-5	**7**	**7**	-	**7**	-

Recognition of many fungi relies on the examination of spores. The presence of asexual spores (conidia) and their shape and size were the main characteristics observed in this experiment. Conidia of the isolates that did sporulate readily were typical of the *Stemphylium* genus as described by Ellis (1971) and Stuville and Erwin (1990). A comparison between microscopic characteristics of the spores produced during the experiment and the description by Ellis (1971) and Mathur and Kongsdal (2002) narrowed them to five main species: *S. botryosum, S. sarciniforme, S. globuliferum, S. herbarum* and *S. vesicarium*. However, since the size of the conidia of the same isolate changed according to the media, this makes the comparison with literature extremely limited and often confusing and unreliable. This variability in spore size and medium was already indicated by Neergaard (1945) regarding *Alternaria* species.

Media had an effect on spore size of isolates 2, 5, 6 and 7 (Table 4.2). In isolates 3, 8 and 9 because of the lack of conidia production under the

conditions provided, it was only possible to confirm their identity as *Stemphylium* species by their growth and pseudothecia production on culture media. Identification of the isolates 3 and 9 was, however, possible through DNA sequencing (section 4.3.5).

It was also observed that after a period of 14 days of incubation (20°C, fluorescent light) only isolate 7 sporulated. The other isolates produced conidia only after exposure to natural light or NUV light. NUV light was used by Martiniello and Porta-Puglia (1995) and Lamprecht & Knox-Davies (1984) to induce production of *S. vesicarium* conidia under laboratory conditions. Six of the nine isolates used in the experiment sporulated after exposure to NUV light. For isolate 7 the visual conidial score was higher under natural light. However, the haemocytometer count of conidial concentration was slightly higher but not significant, under NUV. This can be explained by the fact that spores could be embeded in the mycelia and a subjective visual observation was not totally accurate.

4.4.3.3 Influence of the medium on mycelia growth, pseudothecia and conidia

MEA, OMA and PEA were the media which supported greatest pseudothecial and conidial production in this study. OMA was favourable for mycelia growth whilst in MEA and PEA growth was intermediary (section 4.3.1). As already described, PEA and OMA may contain promoters for growth and reproduction that were absent in PDA. PSA is also rich in growth promoters reflecting the greatest growth rate achieved by the isolates on this media. However, sexual and asexual reproduction were restricted. Probably in this

medium the nutrient content promoted vegetative growth instead of reproduction (E. Jones, personal communication, 8 December 2004).

4.4.4 DNA sequencing of the isolates

The molecular tests performed by the USDA laboratory demonstrated that more than one species had been isolated from pea seed samples. DNA sequencing of the *gpd* gene showed that at least five species were present. Among them, two are considered semi saprophytic (*S. vesicarium* and *S. herbarum*) and three (*S. loti, S. sarciniforme* and *S. astragali*) reported as being pathogenic (Ellis 1971; Ligero et al. 1998; Camara et al. 2002).

The DNA sequencing data and culture growth in artificial media were similar to a certain extent. However, isolates of the same species may present differences in morphology and physiology which can be confounding. For instance, although isolates 4 and 7 (according to the molecular tests) belong to the same *Stemphylium* group (*S. herbarum*) and had similar growth and morphological colony characteristics, in culture they differed considerably in their relative production of pseudothecia and conidia. The reason for that could be the intrinsic characteristics of each isolate. Other studies have already verified differences in the ability to produce spores among strains of the same species of *Alternaria* (Neergaard 1945) and *Stemphylium* (Curren 1968).

According to Camara et al. (2002) *Stemphylium* species can be grouped into five main groups (A-E) (Table 2.6).The observation of colony features and conidia of two of the isolates in this study (4 and 7) would classify them in *S. botryosum* (group B in the phylogenetic tree suggested by Camara et al.

(2002)) instead of group C (*S. alfalfae*, *S. vesicarium*, *S. herbarum*). However, these two species (*S. herbarum* and *S. botryosum*) are morphologically very similar and distinction based only on colony characteristics and asexual spores may be misleading. So far, DNA sequencing of the gene encoding *gpd* has been the most accurate approach to differentiate the *Stemphylium* spp. compared with other DNA sequences such as the ITS region (2002) (section 2.1.27). *Stemphylium herbarum* is one of several species attacking lucerne (Camara et al. 2002) and has also been reported as pathogenic on chicory (*Cichorium intybus*) (Stravato et al. 1995). Likewise, *S. botryosum* attacks lucerne (Stuville & Erwin 1990) , spinach (Koike et al. 2001; du Toit & Derie 2002) and lettuce (Barash et al. 1978).

DNA sequencing revealed that isolates 1, 3 and 9 were *S. astragali*. This species is pathogenic on Chinese milk vetch (*Astragalus sinicus*) crops (Anonymous 2003c). On artificial media these isolates had slow to intermediate growth rates and low sporulation (conidia) ability (sections 4.3.1 and 4.3.4). However, the isolates did differ in visual colony characteristics falling into 2 groups and in the production of sexual fruiting bodies (section 4.3.2 and 4.3.3). Only isolate 1 produced conidia and the conidial morphology could be confused with spore morphology of *S. vesicarium* (Ellis 1971) as this isolate had oblong ellipsoidal verrucose spores that were pale brown. In this group, conidia had several septa and the size ranged from 10-18 μm in width and 15-30 μm in length. Dimensions of *S. vesicarium* conidia vary from 15-26 μm (width) and 20-50μm (length) according to Ellis (1971).

Isolates 2, 5 and 6 were grouped together in a cluster near *S. loti* and *S. sarciniforme*. These isolates together had the same colony appearance and

characteristics although they varied slightly in terms of growth rate, pseudothecial and conidial production. Both species (*S. loti* and *S. sarciniforme)* are known to be causal agents of leaf spot in trefoil (*Lotus corniculatus*) and clover (*Trifolium* spp.), respectively (Bradley et al. 2003; Anonymous 2003c).

4.5 Summary

- According to culture media studies and subsequent DNA sequencing of the *gpd* gene, a group of *Stemphylium* species was identified as seed borne in pea seeds. All the species recovered are recorded as pathogenic to legume species. They belong to three main groups which include *S. herbarum* and S. *vesicarium*, which are semi saprophytic (Neergaard 1945; Basset et al. 1978) and pathogenic to lucerne (Stuville & Erwin 1990) as well as asparagus (Hausbeck 2003) and chicory (Stravato et al. 1995). *Stemphylium astragali* is pathogenic on Chinese milk vetch and *S. loti* and *S. sarciniforme* have been recorded as causal agents of leaf spot in birdsfoot trefoil and clovers (Anonymous 2003c).

- Observations on culture media were not entirely consistent with the results of molecular identification. This suggests that attempts to identify these species solely by growth characteristics on culture media may be misleading.

- OMA and MEA were the most suitable media for conidial production of the isolates. NUV light or natural light enhanced the production of conidia over fluorescent light. Isolate 7 (*S. herbarum*) was the most prolific in terms of conidial production.

5 Pathogenicity of *Stemphylium herbarum* on pea plants

5.1 Introduction

Stemphylium spp. are foliar pathogens of many crops (Lamprecht & Knox-Davies 1984; Martiniello & Porta-Puglia 1995; Stravato et al. 1995; Berg & Leath 1996; Christensen & Wysong 1997; Mehta & Brogin 2000; Suheri & Price 2000; Coles & Wicks 2001; Wu et al. 2001; du Toit & Derie 2002; Anonymous 2003c; Jones 2005). *Stemphylium* spp. infection occurs when there is free water available on the surface of leaves (Bradley et al. 2003). Characteristic symptoms range from oval slightly sunken brown lesions in forage legumes such as lucerne and clover (Stuville & Erwin 1990; Bradley et al. 2003) to purple spots on the stems of asparagus (Hausbeck 2003). The fungal presence on legume plants is related to foliage damage (Koike et al. 2001) and early senescence of leaves (Stuville & Erwin 1990; Berg & Leath 1996).

There is limited information about the pathogenicity of *Stemphylium* spp. on pea plants. *Stemphylium* spp. have been isolated from peas in North America (Camara et al. 2002), Europe (Wegrzycka 1990; Wegrzycka 1991) and New Zealand (K.D.R. Wadia and R.G. Bakker, personal communication, 10 February 2003). Studies have demonstrated that a strain of *S. sarciniforme* was unable to cause infection and symptoms in pea plants (Thanutong et al. 1982). However, no studies have been carried out to investigate the effects of *S. herbarum* on peas.

The aim of the work reported in this chapter was to determine the pathogenicity of *S. herbarum* in peas. The pathogenicity study consisted of artificial inoculation of

S. herbarum spores onto above ground tissues of seedling and adult pea plants, and involved two phases. Firstly conidia of *S. herbarum* from pure cultures were applied to healthy pea plants to determine whether infection occurred, as indicated by symptom development (lesions). Secondly, the fungus was re-isolated from lesioned leaves. Glasshouse and laboratory experiments were conducted in order to:

1. Identify if the *S. herbarum* isolated from experiment 2 was able to infect pea plants and produce symptoms, such as spots on leaves, stems and pods, and could be re-isolated from the plants – fulfilling Koch's Postulate.

2. Determine when pea plants are most susceptible to *S. herbarum*.

5.2 Material and methods

Pathogenicity testing of *Stemphylium herbarum* was performed in a glasshouse (Lincoln University Nursery) and in a controlled environment room (New Zealand Seed Technology Institute) at Lincoln University, Canterbury, New Zealand. The experiment was organized in three stages according to the development of the pea plants (sections 2.1.4.1 and 2.1.4.2), as a complete randomised block design with six replicates of six plants each. The factors were four types of inoculum (sterile water, sterile water plus Tween, conidial suspension and conidial suspension plus Tween) and the three plant development stages (seedling, flowering and pod stages) – a total of 72 observation units.

5.2.1 Inoculum preparation

Inoculum was prepared using a strain of *S. herbarum* (BioLinc 7) isolated from marrowfat pea seeds in Canterbury, New Zealand in 2002 (K. D.R. Wadia, personal communication, 8 February 2003). This isolate had demonstrated rapid and

abundant sporulation in a previous experiment (Chapter 4) and was identified as *S. herbarum* by the Systematic Botany and Mycology Laboratory, USDA-ARS, Beltsville (USA) (N. O'Neill, personal communication, 23 October 2004).

Pure colonies of this isolate were grown in sterile Petri dishes (95 mm) containing approximately 20 ml of artificial autoclaved malt extract agar media (Merck) incubated for 14 days under near ultra violet light and at 20 ± 3°C. The spore suspension was prepared by adding 10 ml sterile distilled water, gently scraping the surface of the colonies with a sterile glass rod, and filtering the suspension using a folded cheese cloth in order to remove pieces of mycelia and pseudothecia (Mehta 2001; K. D.R. Wadia, personal communication, 12 April 2003). Conidial concentration (spores ml^{-1}) was measured using a haemocytometer. The actual conidia concentration applied to the plants was 2×10^4 conidia ml^{-1} at the pod stage and 3×10^4 conidia ml^{-1} at the seedling and flowering stages. These conidia concentrations were the maximum achieved by growing the fungus on MEA as previously described.Two conidial suspensions were prepared, with 0.1% Tween 20 surfactant (Boehringer Mannheim, Germany) being added to one (Kabeere 1995; Koike et al. 2001; du Toit & Derie 2002).

5.2.2 Plant inoculation

Marrowfat pea seeds, cultivar Midichi, free from *Stemphylium* spp. (Table 3.3) were sown at 25 mm depth in plastic pots (150 x150 x190 mm) containing approximately 2,800 g of a 3-4 month potting mix (400 l bark; 100 ml pumice, osmocote 15N – $4.8P_2O_5$ -10.8 K_20, Ag lime 500 g). Seven to eight seeds were sown per pot, with the objective being to obtain six plants per pot. After seedling emergence any extra seedlings were removed. Each pot was considered a replicate,

and each inoculum treatment was applied to six replicates. This was done for three plant growth stages: seedlings, flowering plants and pod bearing plants.

The plants were grown on a glasshouse bench and watered daily using a common hose. Maximum and minimum temperatures were recorded (Siemens electronic room sensor). The mean temperature in which plants grew in the glasshouse prior to inoculation was 18.6°C, with a maximum of 30.4°C and minimum of 13.3°C (Figure 5.1).

Seedlings were inoculated 4 weeks after sowing when they had 3 to 5 nodes (vegetative stage 3:2; Trawally, 1984). Plants at the flowering stage were inoculated when full bloom was observed (Table 2.1) and had approximately 12 nodes. Pods were inoculated at reproductive stage 6 (Table 2.1) when plants had approximately fourteen to fifteen nodes (Gane et al. 1984; Trawally 1984). A previous study had shown that there was no difference ($P<0.05$) in *S. herbarum* infection in plants with wounded or non wounded leaf tissue (Appendix 10). Therefore, in this experiment only plants with intact foliage were used. Plants did not receive any fungicide spray either pre or post- treatment application. Sulphur (Microsul sulphur 15 ml/10 l) was used in order to minimise the occurrence of other fungal diseases such as powdery mildew. Only plants that were inoculated at the flowering and pod stage were sprayed with sulphur and only once at the 3- 5 node stage (approximately 4 weeks after sowing).

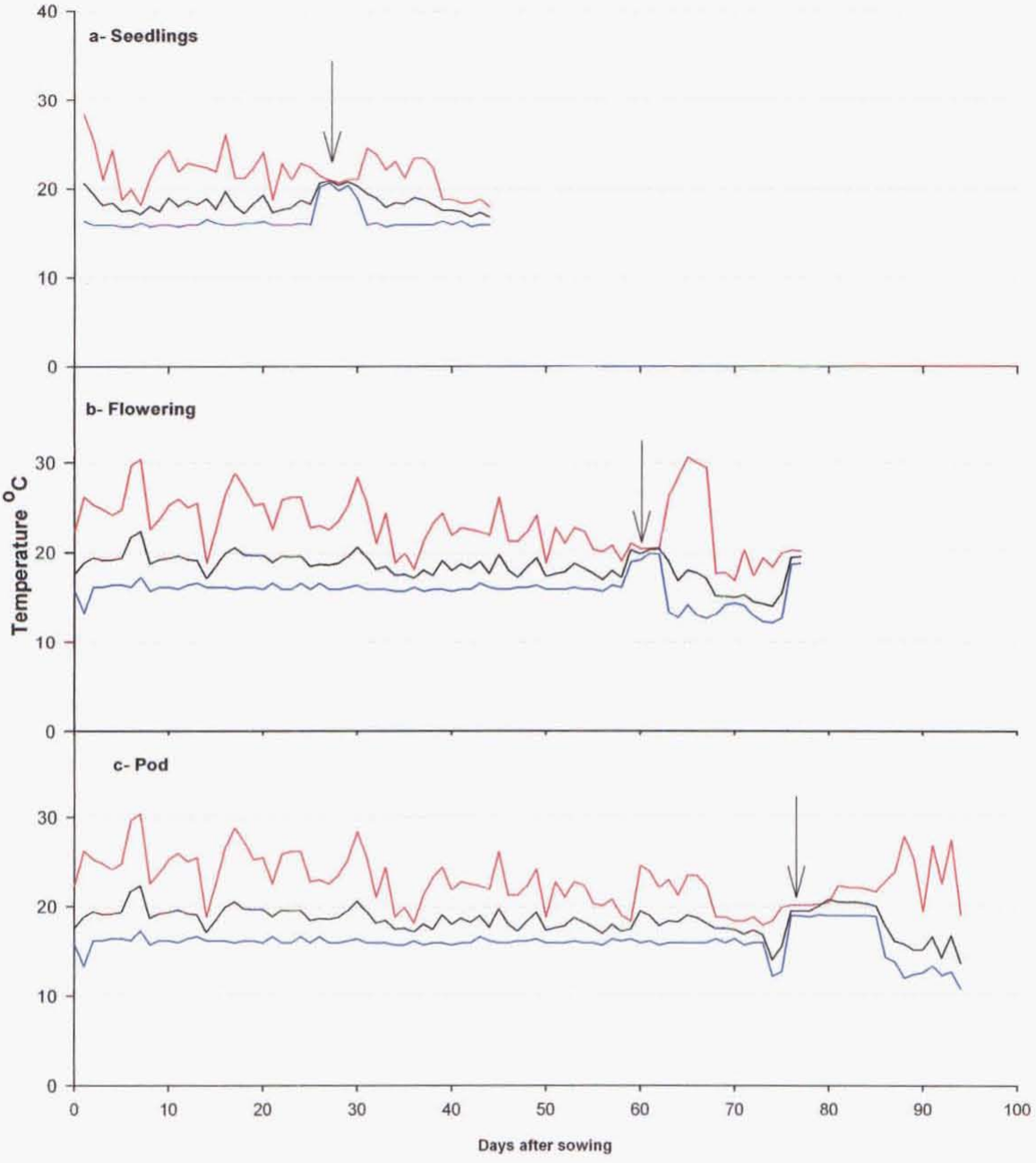


Figure 5.1 Maximum (—) , mean(—) and minimum(—) temperature (°C) during the experiment for the three plant stages: (a) seedling; (b) flowering; (c) pod.

Arrows indicate inoculation and subsequent incubation (4 days) period.

Above ground plant parts were inoculated using a low pressure spray bottle to run-off point (approximately 10 ml per plant) with the treatments outlined in Table 5.1. Control plants were sprayed with sterile water or sterile water plus 0.1% Tween 20. Only plants in the pod stage received the treatment only in the top part of the canopy (between nodes 7 and 16).

Table 5.1 Treatments used in the *S. herbarum* pathogenicity experiment.

Acronym	Treatment
W	Sterile distilled water
WT	Sterile distilled water plus Tween
S	*Stemphylium herbarum* conidial suspension
ST	*Stemphylium herbarum* conidial suspension plus Tween

5.2.3 Incubation

Clear plastic bags (450 x 750 x 30mu polyethylene) covers were placed over the pots after inoculation to maintain high humidity (Appendix 11). The plants were transferred from the glasshouse to a controlled temperature room (at the New Zealand Seed Technology Institute) where conditions were suitable for fungal infection (Gilchrist et al. 1982). Temperature inside the plastic bags ranged from 20 to 22°C (monitored by a Hobbo 4 channel logger – Onset Computer Corporation, Bourne, Maryland, USA) and the photoperiod was 12 hours (116 W white fluorescent light). These conditions were continued for 96 hours following inoculation. Afterwards, the plastic bags were removed and plants placed back in the glasshouse, where they stayed for a further 14 days. Control plants and plants inoculated with *S. herbarum* were kept apart to avoid cross contamination.

5.2.4 Symptom assessment and leaf senescence

After removal of plastic bags, symptom appearance on plants was recorded every two days for 14 days. At the end of this period, the percentage infection was assessed and subsequent re-isolation carried out.

Disease severity was assessed on leaves and stems using a 0 – 5 scale, based on the number of lesions and percentage of the leaf area diseased as outlined in Table 5.2.

Table 5.2 Disease severity score for *S. herbarum* disease assessment (Grinstein et al. 1988; Roger & Tivoli 1995).

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Lesions on stems were assessed once at 18 days after inoculation (14 days after they were uncovered). The area of diseased leaf tissue was measured by digitally photographing the leaves and analysing pictures using Quant Image software (Vale et al. 2003).

Eighteen days post inoculation, foliar tissues were collected from all the treatments. From each pea seedlings replicate, two leaves were randomly collected from the third and fourth plant node. Pieces of leaves without disease lesions and leaves with lesions were used for low temperature scanning electron microscopy (LTSEM) observation. The leaves were freeze dried in liquid nitrogen at -176 °C (Read 1991;

Souza 1998) and then fractured (N. Andrews, personal communication, 11 October 2004). The leaf tissues were observed through a scanning electron microscope (Leica 440) and an Oxford Link Isis X-ray ED analysis system (N. Andrews, personal communication, 14 November 2004).

At the flowering stage, two leaves per replicate from nodes ten and eleven and two flowers per replicate were chosen at random. From plants at the pod stage leaves from the thirteenth and fourteenth nodes and pods were harvested. These leaves were also plated on agar for re-isolation of *S. herbarum* (section 5.2.5).

Leaf senescence was also measured 18 days after each inoculation by counting the number of leaves of seedlings and flowering plants with more than 50 % of the leaf area senesced for each replicate (Hay & Walker 1989; Rizvi & Nutter 1993).

5.2.5 *Stemphylium herbarum* re-isolation

Leaf tissues were surface disinfected with a solution of 1% NaOCl for ten minutes and rinsed with sterile distilled water. Tissue pieces were placed on MEA (malt extract agar) plus 0.1% chloramphenicol (50 mg/ml) then incubated at 20 °C for 10 days under 12 hours dark and 12 hours near ultra violet light (Lamprecht & Knox-Davies 1984; Bradley et al. 2003). Leaves were then examinined using a stereomicroscope (approximately x 30) and microscope (x 400). Characteristic colony growth, conidia and fruiting structures (pseudothecia) were compared with the original inoculum *S. herbarum* (isolate 7; section 4.3.1- 4.3.4). The number of lesioned leaves in which *S. herbarum* grew was recorded.

5.2.6 Data analysis

Analyses of variance (ANOVA) were performed using the statistical package Genstat 6 th edition (Lawes Agricultural Trust). Diseased tissue area and disease severity data were rank-ordered prior to performing a non parametric one way ANOVA using the Kruskal-Wallis test. When there was a significant difference found, to compare treatments Mann Whitney U (Wilcoxon Rank Sum) tests (Lowry 1999) at a 5% level of significance were done on pairs of treatments. Senesced leaves data were separated using the least significant difference ($P< 0.05$) (John 1998).

5.3 Results

5.3.1 Symptoms on seedlings

The pea seedlings inoculated with water (W) and water plus Tween (WT) did not produce any symptom on leaves (Figure 5.2 and 5.3). However, seedlings inoculated with *S. herbarum* suspensions - conidial suspension (S) and conidial suspension plus Tween (ST) showed symptoms. The mean disease scores for these last two treatments (S and ST) were significantly ($P<0.01$) higher than those of the untreated controls (Figure 5.2). Immediately after the incubation period seedlings inoculated with ST had a mean disease score of 2.5, whereas seedlings treated with S had a score of 1.5, but these values were not significantly different ($P<0.05$) at this assessment time or any other assessment time. From twelve days after inoculation there was no substantial change in the foliar symptoms (Figures 5.2 and 5.5).

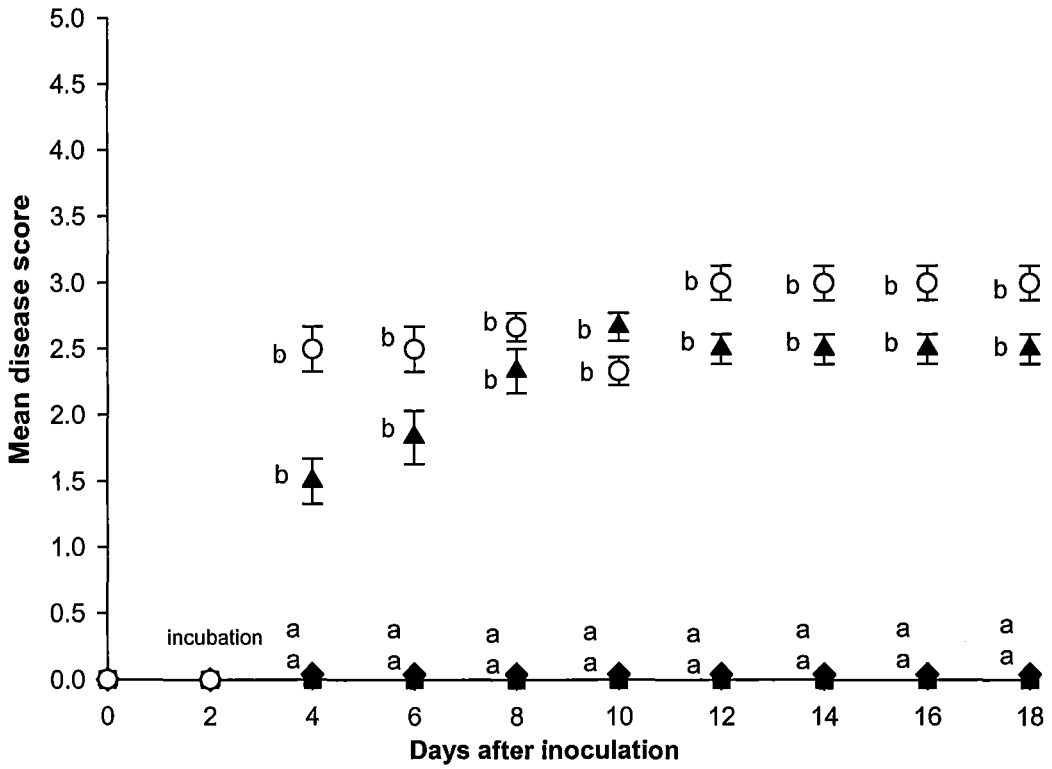


Figure 5.2 Mean disease score on leaves of pea seedlings from 0 to 18 days after inoculation . Treatments were: ♦ Sterile distilled water; ■ Sterile distilled water plus Tween; ▲ *S. herbarum* conidia suspension; ○ *S. herbarum* conidial suspension plus Tween.

Bars represent one standard error for 6 replicates. At each assessment time treatments with the same letter are not significantly different ($P<0.05$) according to Mann-Whitney U test.

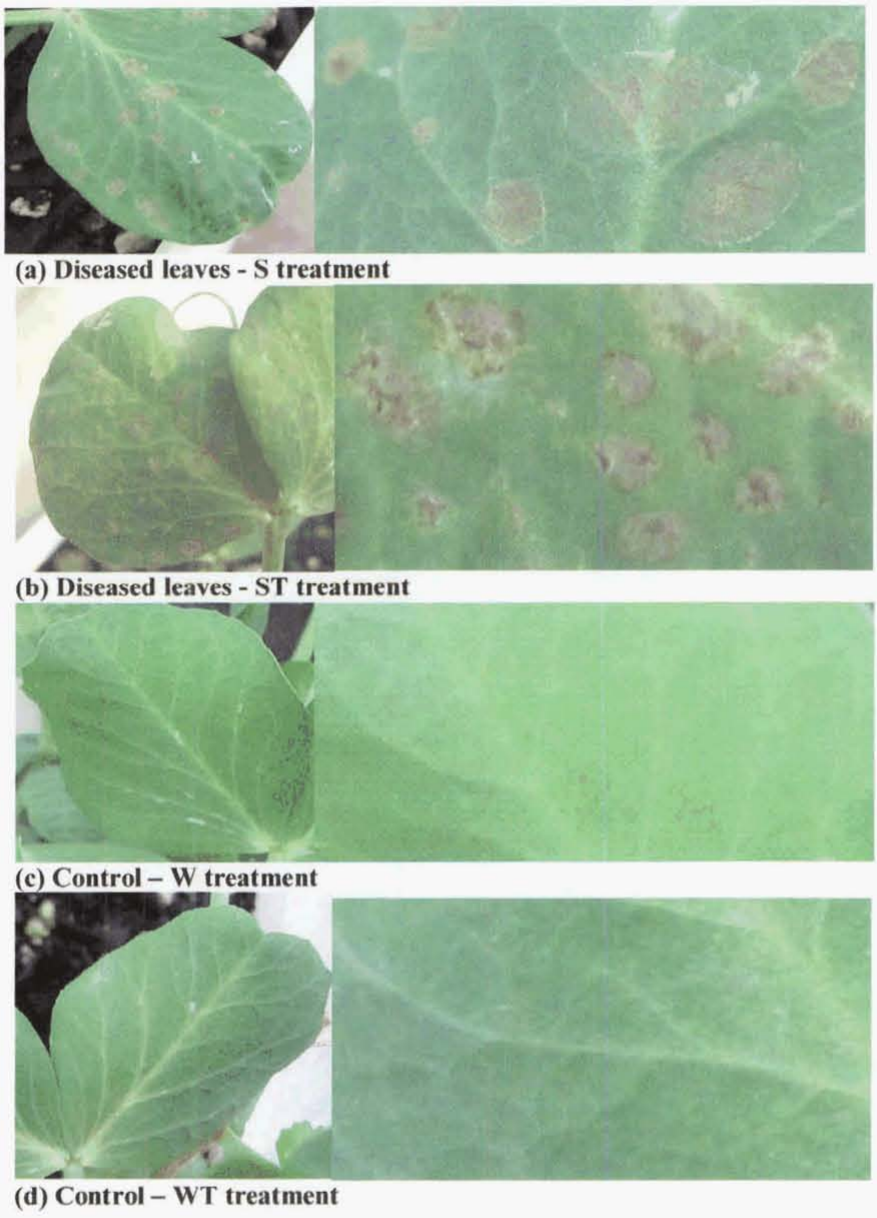


Figure 5.3 Symptoms observed on pea seedling leaves 11 days after inoculation. (a) leaves inoculated with *S. herbarum* conidial suspension (S); (b) leaves inoculated with *S. herbarum* conidial suspension plus Tween (ST); (c) leaves inoculated with sterile distilled (W); (d) leaves inoculated with sterile distilled water plus Tween (WT).

5.3.1.1 Area of diseased tissue on seedlings

After 18 days the leaves of seedlings inoculated with S and ST had an area of diseased leaf tissue of 16.5 mm^2 and 41.6 mm^2 (Figure 5.4), which represented 3.0

and 8.4% of the total leaf area affected, respectively. Plants inoculated with W and WT had 0% diseased tissue (Figures 5.3 and 5.4), which was highly significantly different from the S and ST treatments (P <0.001). Addition of Tween 20 to *S. herbarum* conidial suspension did not significantly (P<0.05) increase the area of diseased leaves (Figure 5.5).

Scanning electron microscope examination of leaf tissue showed that fungal penetration into plants inoculated with S and ST had occurred through the epidermis and stomata (Figures 5.6 ,5.7, 5.8 and 5.9).

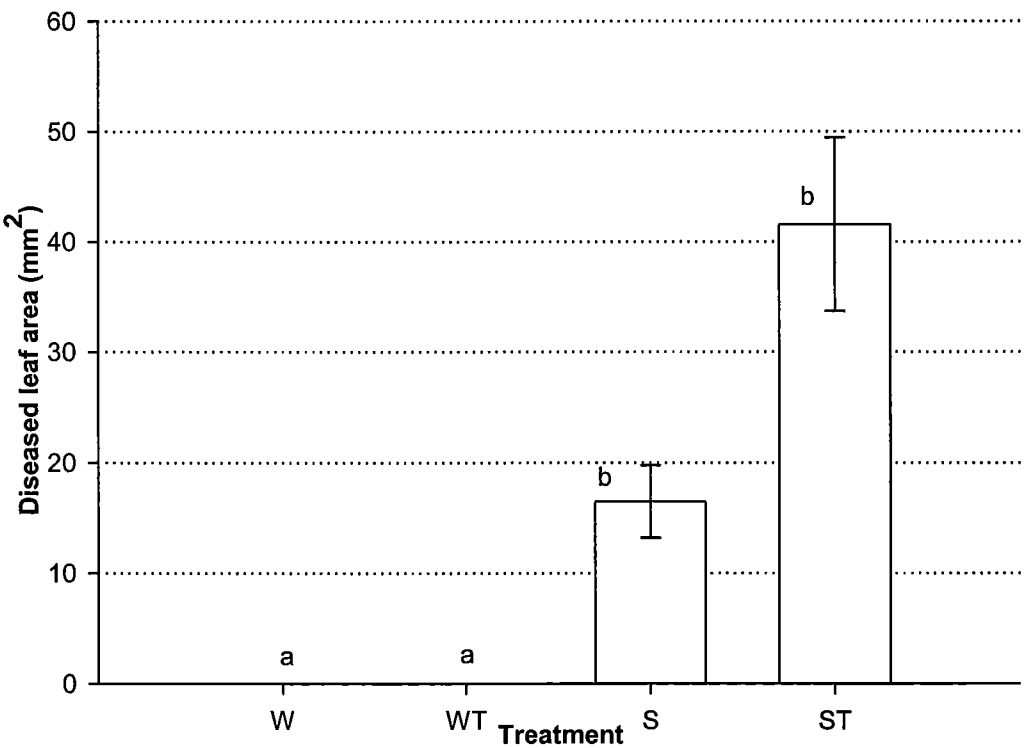


Figure 5.4 Area of diseased leaf tissue of pea seedlings 18 days after inoculation with sterile distilled (W), sterile distilled water plus Tween (WT), *S. herbarum* conidial suspension (S) and *S. herbarum* conidial suspension plus Tween (ST).

Bars represent one standard error of 6 replicates. Treatments with the same letter are not significantly different (P<0.05) according to the Mann-Whitney U test.

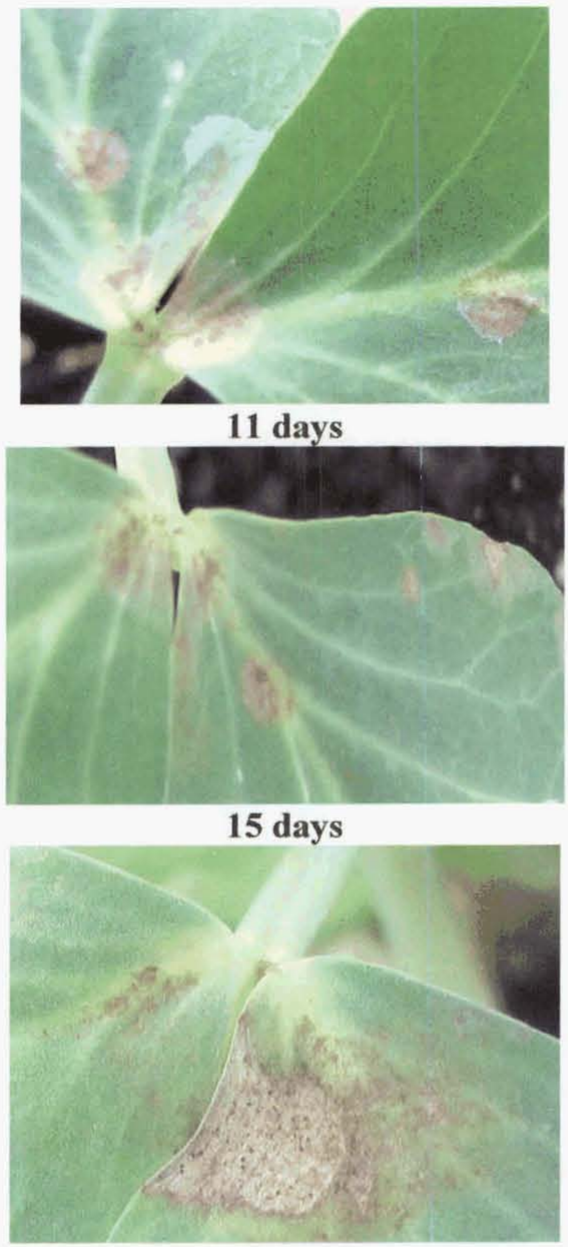


Figure 5.5 Progress of the disease observed on leaves of pea seedlings inoculated with *S. herbarum* conidial suspension (S treatment) 11, 15 and 18 days after inoculation.

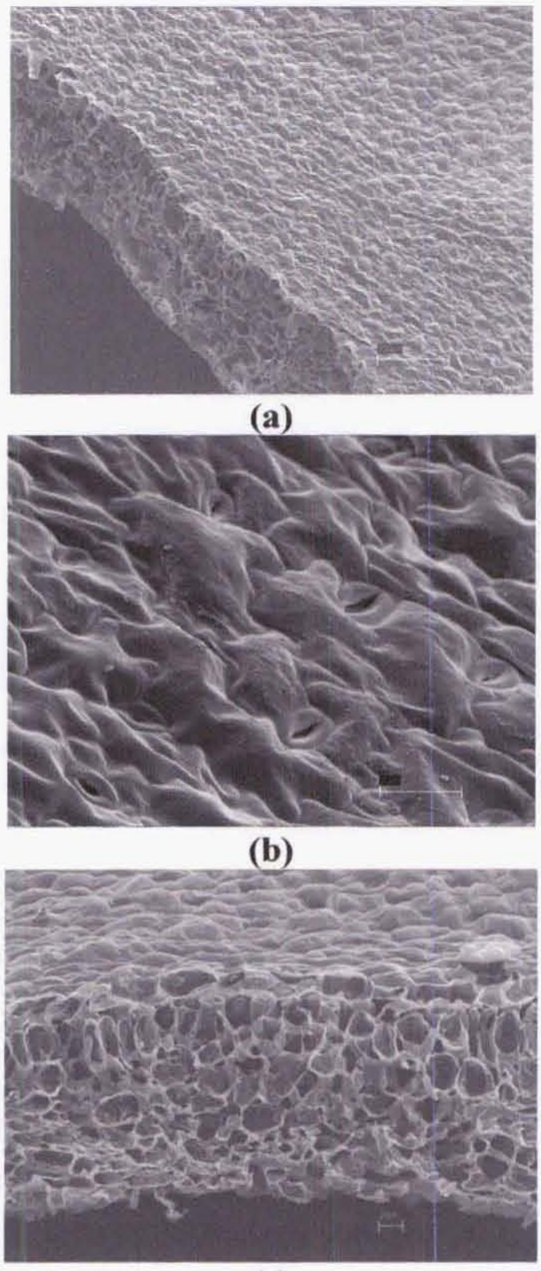
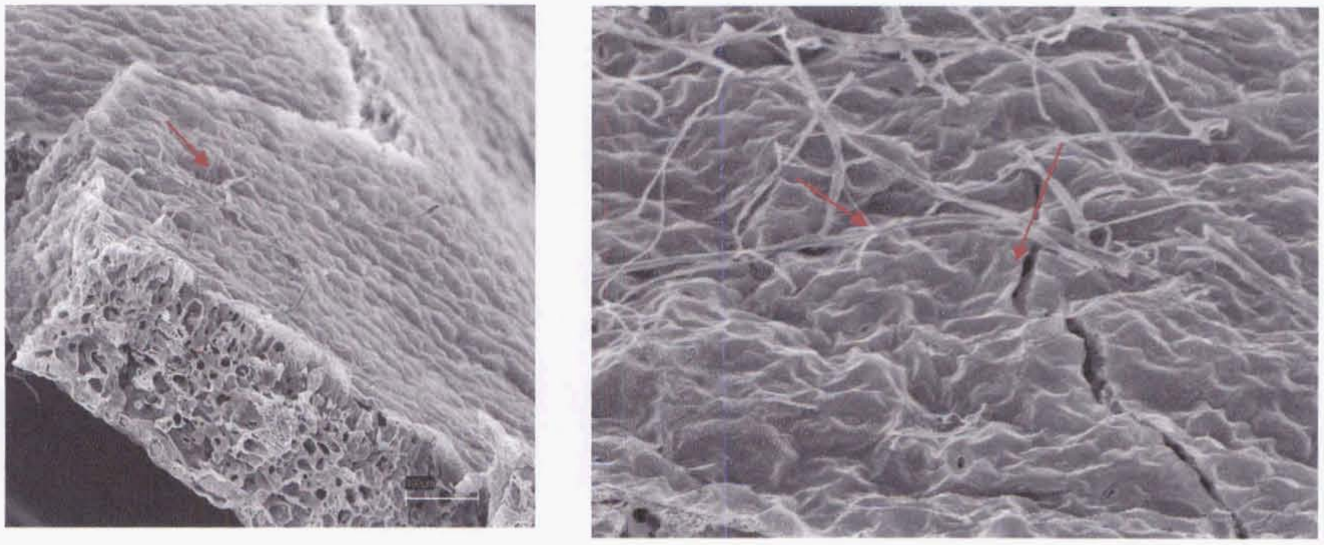
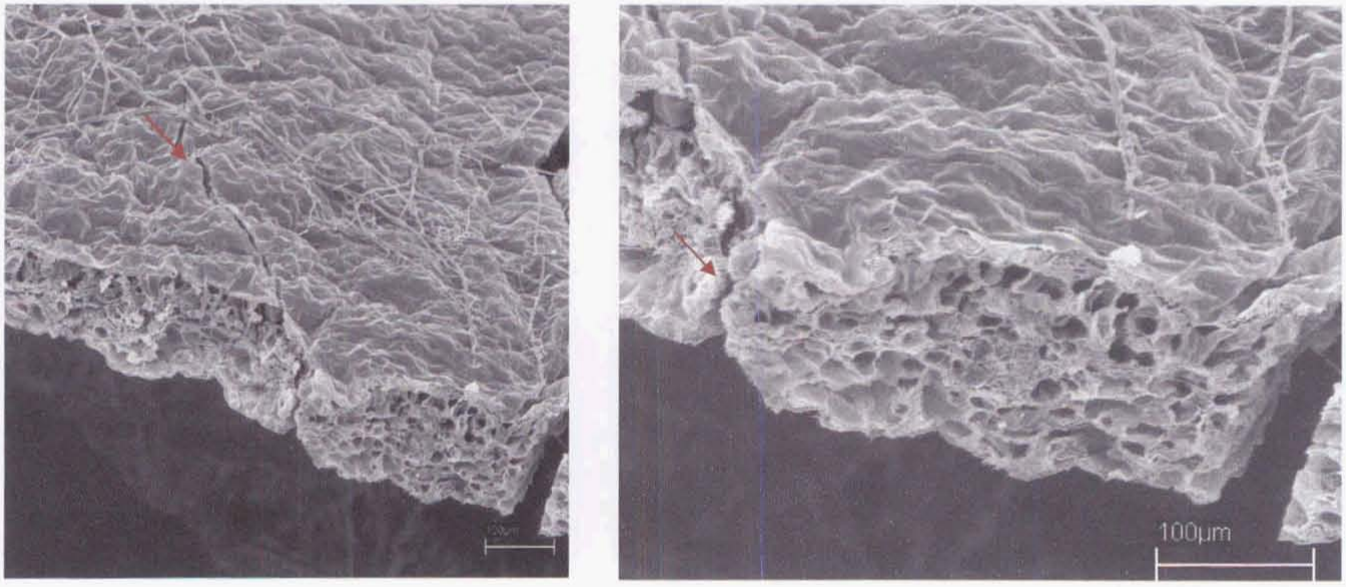


Figure 5.6 Scanning electronic microscope view of non infected leaf tissue 18 days after inoculation with sterile distilled water (W).

(a) leaf surface and mesophyl; (b) detail of stomata in a non diseased leaf surface; (c) detail of epidermis and mesophyl, (palisade, parenchyma and spongy cells).



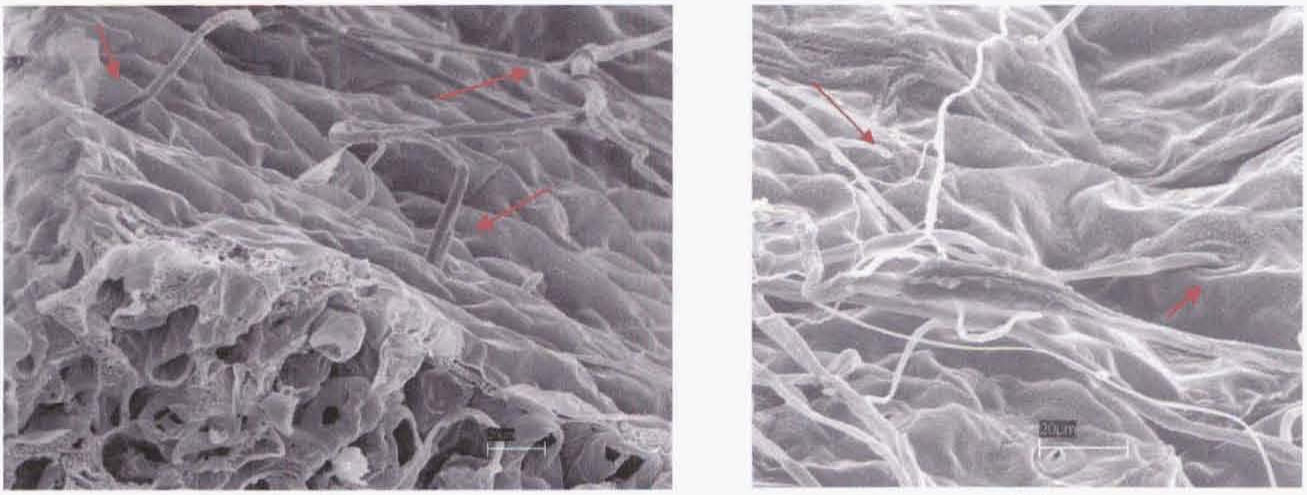
(a)



(b)

Figure 5.7 Scanning electronic microscope view of infected leaf tissue 18 days after inoculation with *S. herbarum* conidial suspension (S treatment).

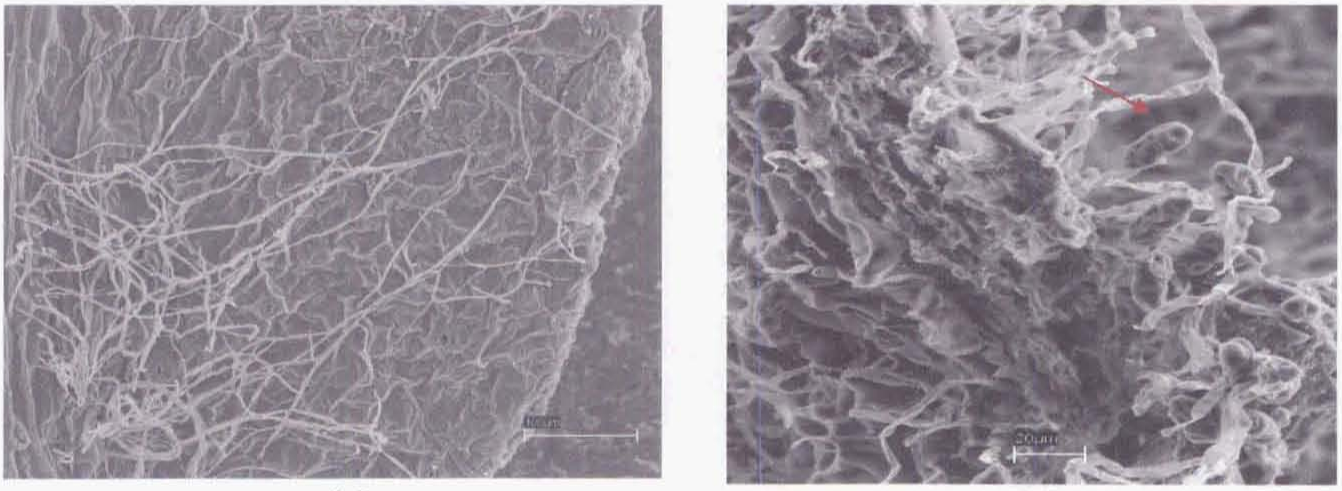
(a) leaf surface presenting initial mycelial growth; arrow indicate fungal hyphae.
(b) rupture of cuticule and epidermial cells and collapsed mesophyl.



(a) (b)

Figure 5.8 Scanning electronic microscope view of infected leaf tissue inoculated with *S. herbarum* conidial suspension (S treatment). Hyphae penetration.

(a) detail of hyphae piercing the cuticule; arrows indicate the hyphae penetration.
(b) arrows indicate hyphae penetrating leaf tissue via stomata.



(a) (b)

Figure 5.9 Scanning electronic microscope view of infected leaf tissue inoculated with *S. herbarum* conidial suspension (S treatment). Hyphae and conidia.

(a) an advanced stage of disease with complete colonization of the tissue and development of conidiophores and (b) sporulation (arrow indicates a conidium).

5.3.1.2 Senesced leaves

Leaves of plants from S and ST treatments appeared to have senesced faster than those of W and WT (Figure 5.10). Eighteen days after inoculation seedlings that had received the ST treatment had a mean number of senesced leaves of 1.75. Seedlings inoculated with S and W had an average of 1.54 and 0.97 senesced leaves, respectively. However, there was no significant ($P<0.05$) difference among these treatments. Only seedlings inoculated with WT had significantly ($P<0.05$) lower number of senesced leaves (0.67) than the treatments S and ST.

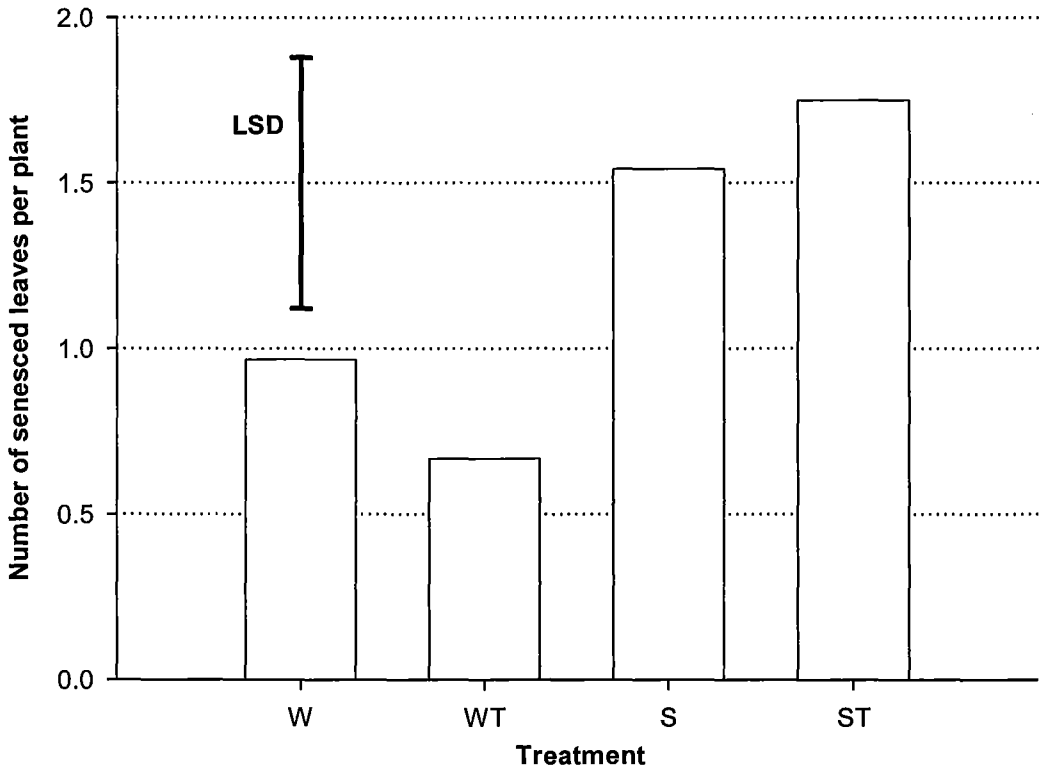


Figure 5.10 Mean number of senesced leaves of pea plants 18 days after inoculation (vegetative stage 3:2) inoculated with sterile distilled (W), sterile distilled water plus Tween (WT), *S. herbarum* conidia suspension (S); *S. herbarum* conidia suspension plus Tween (ST).

5.3.1.3 Symptoms on stems

The assessment of symptoms on stems was done only once, on the 18 th day after the inoculation. Plants sprayed with S and ST had lesions (brown spots) on the stems while no lesions were observed on treatments W and WT (Figures 5.11 and 5.12). The mean disease score of stem lesions of seedlings inoculated with ST was 2.83. Seedlings treated with S had a mean disease score of 1.83 and those values were significantly higher (P<0.01) than the untreated controls (W and WT). However, there was no significant difference (P< 0.05) between S and ST.

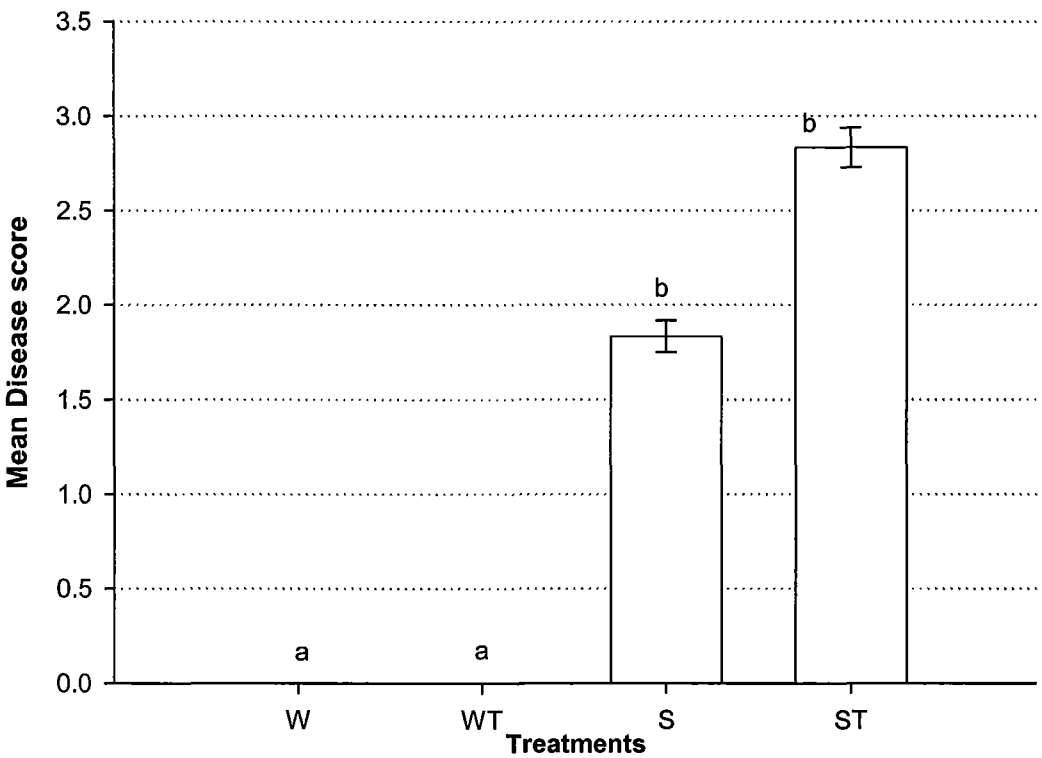


Figure 5.11 Mean disease score on stems of pea seedlings 18 days after inoculation (stage 3:2). Treatments were: sterile distilled water (W), sterile distilled water plus Tween (WT), *S. herbarum* conidial suspension (S); *S. herbarum* conidial suspension plus Tween (ST).

Bars represent one standard error for 6 replicates. Treatments with the same letter are not significantly different (P<0.05) according to the Mann-Whitney U test.

(a) infected seedling **(b) non infected seedling**

Figure 5.12 *Stemphylium herbarum* symptoms on leaves and stems of pea seedlings (stage 3:2).

(a) infected seedling inoculated with *S. herbarum* conidial suspension (S);
(b) non infected seedling inoculated with sterile distilled water (W).

5.3.2 Symptoms on flowering plants

On flowering plants the symptoms were less severe than those observed on pea seedlings (Figures 5.13 and 5.14). After the incubation period (4 days after inoculation) the leaves of plants from S and ST treatments had disease scores of 2.3 and 2.6, respectively while W and WT plants did not have any visible symptom (scores of 0 for both treatments). The treatments S and ST significantly ($P< 0.01$) increased disease score compared with seedlings sprayed with W and WT. However, there was no significant difference ($P< 0.05$) between S and ST in the disease score values. Observation of the progress of symptom development caused by *S. herbarum* was not possible because of the occurrence of other diseases such as grey mould (*Botrytis cinerea*) (R.G. Bakker, personal communication, 4 June 2004) and Ascochyta leaf blight (*Ascochyta pisi* and *M. pinodes* (Hagerdon 1984).

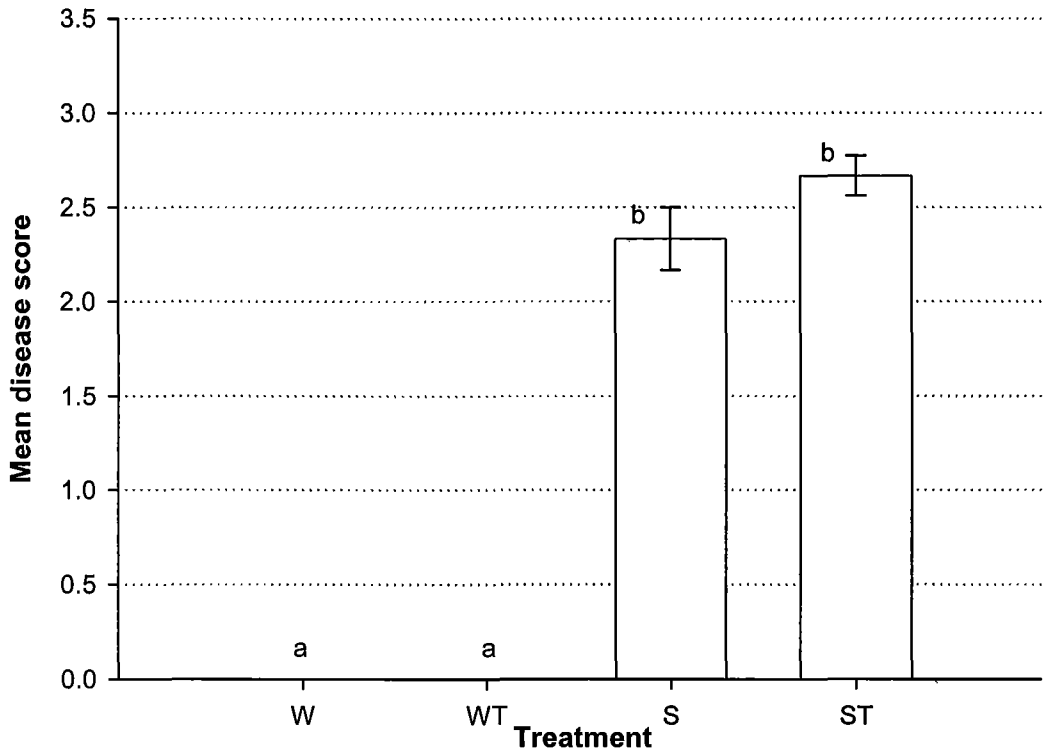


Figure 5.13 Mean disease score on leaves of pea plants (flowering stage) on the fourth day after inoculation. Treatments were: sterile distilled water (W); sterile distilled water plus Tween (WT); *S. herbarum* conidia suspension (S); *S. herbarum* conidia suspension plus Tween (ST).

Bars represent one standard error for 6 replicates. Treatments with the same letter are not significantly different ($P<0.05$) according to the Mann-Whitney U test.

Symptoms for both S and ST were similar. Figure 5.14 shows the lesions observed in the S treatment. There were no evident symptoms on stems or flowers for any of the treatments.

(a) Diseased leaves

(b) Control

Figure 5.14 Lesions caused by *S. herbarum* observed on pea leaves 11 days after inoculation at flowering.

(a) leaves inoculated with *S. herbarum* conidial suspension (S); (b) leaves inoculated with sterile distilled water (W).

5.3.2.1 Area of diseased tissue of pea plants at flowering

Pea plant leaves inoculated with W and WT had means of 3.14 and 4.0 mm^2 of lesions, respectively, which represented approximately 0.5% of the total leaf tissue damaged. Leaves inoculated with S and ST had lesions of 46.0 and 22.0 mm^2 (Figure 5.15), respectively (6.3 and 3.4% of the total leaf area with symptoms, respectively). Treatments S and ST had a significantly ($P< 0.01$) higher disease score than the controls (W and WT). Plants inoculated with S had more ($P< 0.05$) diseased tissue area than leaves inoculated with ST. The plants in all treatments

were affected by other diseases and the values may not represent the area of lesions caused solely by *S. herbarum.*

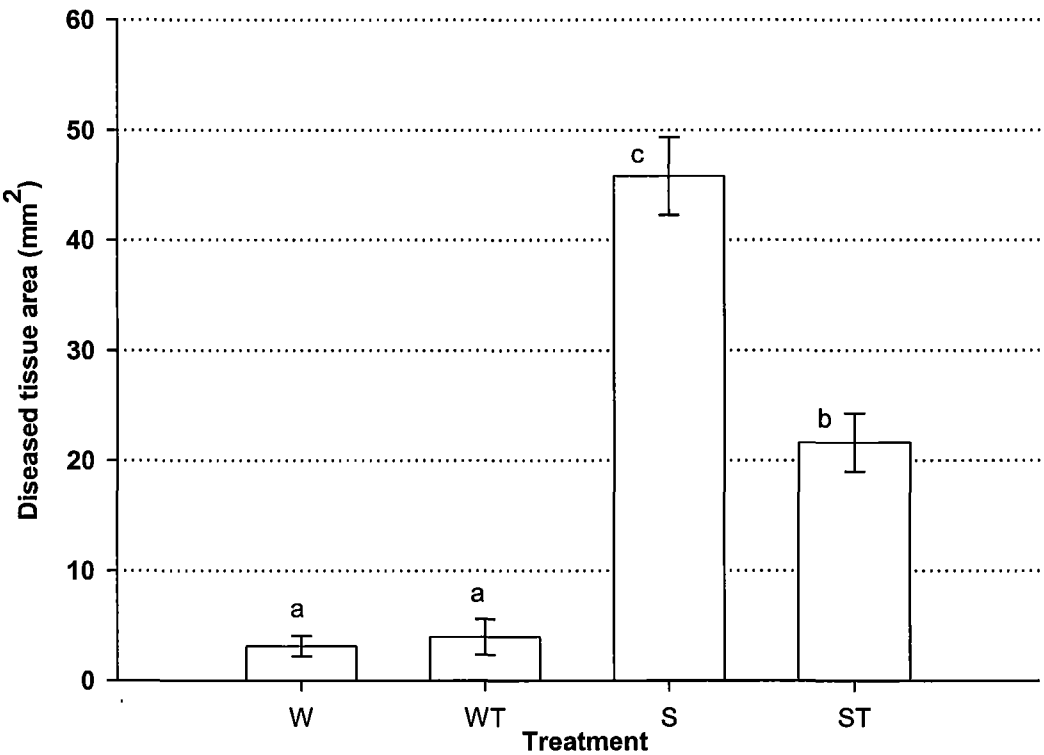


Figure 5.15 Area of diseased leaf tissue on pea plants 18 days after inoculation at flowering. Treatments were: sterile distilled (W), sterile distilled water plus Tween (WT), *S. herbarum* conidia suspension (S); *S. herbarum* conidia suspension plus Tween (ST).

Bars represent one standard error of 6 replicates. Treatments with the same letter are not significantly different ($P<0.05$) according to the Mann-Whitney U test.

5.3.2.2 Senesced leaves

There was no significant effect of inoculation on the leaf senescence of pea plants inoculated at the flowering stage ($P<0.05$).The mean number of senesced leaves per plant varied from 5 to 6 (data not presented).

5.3.3 Symptoms on plants bearing pods

Similar to plants at the flowering stage, the symptoms were less severe than those observed on seedlings. At this stage, other diseases were also occurring simultaneously and therefore the score of lesions caused only by *S. herbarum* over 18 days was not recorded.

5.3.3.1 Diseased tissue area on pea plants (pod stage).

Plants inoculated with WT had the lowest leaf diseased tissue area (23 mm^2) which was significantly lower ($P<0.05$) than the other three treatments, whereas plants inoculated with S had the highest value for diseased tissue area (about 110 mm^2). The lesion area of plants inoculated with ST was 69 mm^2 and this value was not different from the W treatment (57 mm^2) ($P< 0.05$) (Figure 5.16). Because other diseases also occurred on the leaves, as stated previously, the data presented do not only represent the area infected by *S. herbarum*. No symptoms were observed on stems or pods.

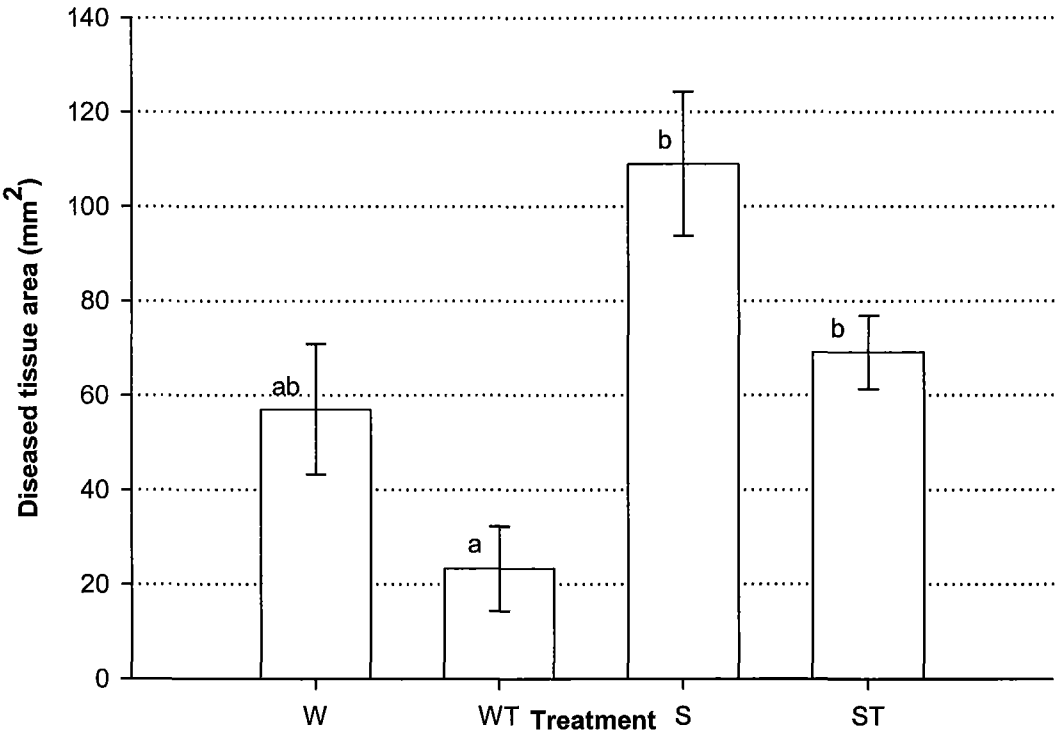


Figure 5.16 Area of diseased leaf tissue on pea plants in the pod stage 18 days after inoculation with sterile distilled (W), sterile distilled water plus Tween (WT), *S. herbarum* conidial suspension (S) or *S. herbarum* conidial suspension plus Tween (ST).

Bars represent one standard error. Treatments with the same letter are not significantly different ($P<0.05$) according to the Mann-Whitney U test.

5.3.4 Re-isolation of *S. herbarum* from leaves with symptoms

Seedlings

Stemphylium herbarum was re-isolated from 100 % of the leaves collected from seedlings inoculated with S and ST.

Flowering

Stemphylium herbarum was re-isolated only from 67 % of the leaves which were inoculated with S and ST.

Pod bearing plants

Stemphylium herbarum was re-isolated from 75 to 80 % of diseased leaves collected from plants inoculated with S and ST at the pod stage.

The pure cultures of *S. herbarum* re-isolated from inoculated tissue were similar to those before inoculation (Figure 5.17). They had rapid growth on MEA medium. The re- isolated colonies were circular in shape, had a downy texture and the mycelial colour ranged from brownish orange to dark brown. As observed in the second experiment (Chapter 4, section 4.4.3) the re-isolated *S. herbarum* produced immature fruiting bodies and many conidia. Spores were similar to those previously described (dimension range: max. min. length = 30-20 μm; max. min. width= 15-10 μm). DNA sequencing (of the *gpd* gene) performed by the Molecular Plant Pathology Laboratory, USDA/ARS, Beltsville, MD, USA also confirmed that the re-isolated fungus (isolates number 11 and 12) was *S. herbarum* (as shown in Appendices 8 and 9).

(a) (b) (c)

Figure 5.17 Re-isolation of *Stemphylium herbarum* from diseased leaves: (a) Lesions on a leaf caused by *S. herbarum*. (b) *S. herbarum* colony growing from incubated leaf tissue; (c) pure colony on MEA after 10 days under fluorescent light at 20°C.

5.4 Discussion

The *S. herbarum* isolated from marrowfat peas and nominated as isolate 7 (Chapters 3 and 4) was pathogenic on pea plants (cultivar Midichi), although the symptoms were more severe on plants at the seedling stage than later in plant growth. Leaves were the main tissue affected.

Leaves of pea plants are usually waxy (Rutledge & Eigenbrode 2003) and to ensure that conidia of *S. herbarum* would not run off after spraying, the surfactant Tween 20 was used. In this experiment, the addition of Tween 20 did not have any phytotoxic effect on the pea plants (section 5.3). Although not significantly different, the addition of Tween 20 to the conidial suspension contributed to *S. herbarum* infection, especially when applied to the seedlings. But, in adult plants the addition of the surfactant did not increase the infection or symptoms. Because young leaves are more prone to penetration of conidia (Vanderplank 1984; Guest & Brown 1997) the surfactant may increase the probability of infection due to greater adherence of conidia on the leaf surface. On the other hand, adult plants have thicker leaves and were equally resistant to a lower (no surfactant) or a higher (plus surfactant) adherence of conidia to the leaves.

Leaves had more lesions than other plant tissue. Roger and Tivoli (1995) reported faster appearance of diseases on pea leaflets and stipules than on stems. Characteristic symptoms observed on seedlings were similar to those cited on lucerne, clovers and spinach (Blancard 1992; Christensen & Wysong 1997; Raid & Kucharek 2003). Lesions were brown, irregular to oval shaped spots and approximately 6 mm in diameter (range from 4 to 8 mm). Symptoms were evident on leaves of seedlings, but this does not necessarily mean that there was no interaction between *S. herbarum* and adult pea plants. Symptoms, detected by the

naked eye, in fact represent a relatively late stage in the process of infection and colonization by a pathogen (Gaumann 1950; Fox 1993b).

Stemphylium biotypes that occur during warm (23-27 °C) wet conditions are capable of blackening the stem of lucerne plants (Stuville & Erwin 1990). Lesions observed on seedling pea stems were not as severe, more closely resembling the leaf spots and those caused by *S. loti* in Chinese vetch (Anonymous 2003c) (section 2.3.1.2). The infected seedlings were able to continue their development under the conditions of the experiment. However, it was observed that seedlings which were treated with *S. herbarum* conidia (S and ST treatments) had about 50% more senesced leaves than control seedlings.

Fungal penetration occurred directly via the epidermis, as described in onion leaves (Suheri & Price 2000) and clover (Berg & Leath 1996). Toxins excreted by the fungus (Heiny & Gilchrist 1989; Mehta & Brogin 2000; Bradley et al. 2003) may facilitate the entrance into the epidermis in seedling plants. However, stomata were also observed to serve as a route for hyphal penetration. Wilkinson and Millar (1978) also reported a similar mechanism for infection of clover plants by *S. sarciniforme*. In adult plants the fungus only developed superficially and the lesions were grey-white in colour, whereas in seedlings the destruction of cells lead to necrosis on the leaves.

In seedlings the infection was more severe perhaps because of the tender tissue of leaf surface and stems (Oku 1994b; Guest & Brown 1997). As the plant develops, the epidermis become more resistant to fungal invasion. Thicker cell walls and wax substances in the leaf surface may create difficulties for direct hyphal penetration (Bradley et al. 2003) and the fungus may penetrate mostly via the stomata (Oku,

1994b; Guest and Brown, 1997) . However, it is likely that infection by other pathogens, as was observed, might have interfered and accounted for the low infection seen in adult pea plants. In addition, the concentration of spores used for inoculation of plants at the pod stage was lower (18 x 10^3 spores /ml) than for inoculation of seedlings and flowering plants, which may have also been a factor involved in the unsuccessful invasion by *S. herbarum* on those plants.

High humidity (above 80%) and a temperature around 20°C were found to be suitable for *S. herbarum* infection of seedling leaves. The lesions observed in the inoculated plants progressed, causing leaf senescence. However, the disease did not progress to other new leaves, perhaps because of the low humidity provided in the glasshouse, and to some extent by the temperature fluctuation in the case of the seedlings. In nature conidia are disseminated by rain splash or wind (Ligero et al. 2003). Conidia germinate and infect susceptible tissue when there is free moisture, for example on a leaf surface (Ligero et al. 1998). This occurred when conidia of *S. herbarum* were sprayed on the pea leaves and plants were maintained inside plastic bags for 96 hours in a controlled environment (20 -22°C). Inside the plastic bags the humidity was 100% (Appendix 11) and spores were able to germinate and infect the foliar tissue.

After the incubation period plants were placed back on the glasshouse bench and the plastic bags removed. Lesions developed and new conidia were observed to be produced on the leaf tissue (Figures 5.3 and 5.5). These new conidia were probably released by air currents and water drops during routine watering. However, as the plants were no longer enclosed in plastic bags the humidity at the plant surface varied. So, although the conidia were spread to new leaves the conidia were not able to germinate because the period of leaf wetness may not have been long

enough to do so. Therefore, no new infection on young leaves was observed and as the disease did not progress on the new leaves, the plants continued developing normally. For the adult plants, the occurrence of other foliar pathogens appeared to limit *S. herbarum* development and infection.

Plants at the seedling stage were susceptible to *S. herbarum* infection, as confirmed not only by the observation of lesions, but also by the recovery of *S. herbarum* from infected leaves. Even though the symptoms appeared, the infected plants continued to grow. However, the presence of the fungus was associated with early leaf senescence. According to Hay and Walker (1989) and Garry et al., (1996), fungal infection does have negative effects on the partitioning and translocation of nutrients. As a result diseased plants are less efficient in capturing light and have a retarded development compared with healthy plants (Goodman et al. 1986a; Campbell 1993c). During seed formation and maturation, heavy infections may lead to seed infection and seed stalk fall (Grinstein et al. 1988; Aveling & Snyman 1993).

Even though *Stemphylium* spp. are often reported to be saprophytes, this experiment demonstrated that *S. herbarum* was able to penetrate healthy leaf tissue of pea seedlings and cause lesions. Mortensen and Bergman (1983) suggested that in such cases the fungus can be classified as a weakly pathogenic or facultative saprophytic fungus. The genus *Stemphylium* is often associated worldwide with decomposing vegetation (Basset et al. 1978) but it is now confirmed that *S. herbarum* is pathogenic on young pea plants.

5.5 Summary

- *S. herbarum* caused disease symptoms in marrowfat pea seedlings inoculated with conidia, while no symptoms were observed in plants inoculated with sterile water.

- The symptoms were brown, irregular to oval shaped spots approximately 6mm in diameter, which resulted in the premature senescence of the leaves.

- *S. herbarum* infected the young leaf tissue by penetrating directly into the epidermis and through stomata.

- The fungus was re-isolated from diseased leaves that had been inoculated with conidia. *S. herbarum* was recovered from all diseased pea seedling leaves. However, infection was less severe in adult plants.

6 Seed treatment

6.1 Introduction

Stemphylium spp. are reported to be seed borne in several vegetable species and forage legumes (Koike et al. 2001; Wu 2001) and have also been confirmed as seed borne in pea seeds (K.D.R. Wadia and R.G. Bakker, personal communication, 10 February 2003; Chapter 3). Seed germination and emergence have been negatively correlated with the presence of *Stemphylium* spp. on seeds of forage legumes (Lamprecht & Knox-Davies 1984) and other crops such as carrots (Coles & Wicks 2001). Seedlings produced from infected seeds are usually lesioned and develop poorly compared with those from healthy seeds (Lamprecht & Knox-Davies 1984). Harvey (1986) states that the use of clean seed and planting in a disease free area are the primary means for obtaining a healthy pea crop. Seed treatment is also a means of ensuring desirable seedling establishment and reducing the risk of disease spread, especially if weather conditions during sowing are favourable for microorganism development (Copeland & McDonald 2001b). Pea growers have used chemically treated seeds for the control of fungal diseases such as downy mildew, *Pythium* spp., *Fusarium* spp. and fungi of the Ascochyta complex (Jermyn 1986; Freeman 1987; Sheridan 2000; Kraft & Pfleger 2001). However, the effectiveness of pea seed treatment against *Stemphylium* spp. has not been assessed. Even chemically treated seeds may carry viable microorganisms (Kulik 1995) since the products may control only a specific but limited range of fungi. Furthermore, there is a necessity for evaluating non chemical alternatives for controlling fungi on seeds, especially for organic seed production (VanderErg 1995; Banks 1998; Lennartsson & Roberts 2005).

The objective of this work was to analyse the efficiency of thermotherapy (hot water soak) treatment and of the three commercial fungicide products registered in New Zealand for use as pea seed treatments. In the case of chemical control the opportunity was also taken to examine the effects on seeds treated using under (x 0.5) or over (x 2) the dosage recommended by the manufacturer. In all cases the aim was to control *Stemphylium* spp. in/on seed and potentially to enhance seed performance.

6.2 Material and methods

Two pea seed lots (one each of the cultivars Meteor and Midichi) and provided by the New Zealand Seed Technology Institute Plant Diagnostic Laboratory (BioLinc) were used in this experiment. These two lots were harvested in 2004 and were selected because of the high probability of *Stemphylium* spp. infection (Table 3.3).

The laboratory tests were arranged as a completely randomised design of 4 replicates containing 50 seeds each for cultivar Meteor. For cultivar Midichi 3 replicates of 35 seeds each were used due to limited seed availability. The treatments were as follows:

i. Control: untreated seeds

ii. Hot water treatment: the water temperature in which seeds were kept for 30 minutes was 50 ± 0.5°C. This temperature and time combination was chosen based on previous work in controlling seed borne pathogens on peas (Boettinger & Bowers 1975; Grondeau et al. 1992; Begum et al. 2004) and attempts to eliminate seed borne *Stemphylium* spp. described in detail in sections 2.3.5.1 and 2.3.6.2.

Seeds were immersed in a glass container with heated water (50°C) and placed in a water bath (Semco Ltd., New Zealand) to keep a constant temperature for 30 minutes. The water temperature of the container with seeds was precisely monitored using a data logger (Squirrel SQ32-3U/L-A5; Grant-Cambridge Ltd). After treatment, seeds were placed on filter paper and air dried at room temperature (18- 20°C) for 24 hours (Aveling & Snyman 1993; Bae et al. 2002).

iii. Chemical treatments: seeds were treated with three systemic fungicides registered for pea seed treatment and available in New Zealand (Table 6.1). Seeds were treated according to manufacturer guidelines. Seeds of cultivar Meteor were treated with half, normal and double the commercially recommended rate. For cultivar Midichi only the commercially recommended rate was used due to limited seed availability.

Table 6.1 Fungicide products used for pea seed treatment.

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Source: (Anonymous 2002b; Anonymous 2003a).

Suspension of Apron XL and slurries of Aliette super and Wakil XL were prepared by mixing wettable powders (Aliette and Wakil) or emulsifiable concentrate

(Apron) in water. The mixture was placed in a plastic bag with the seeds and mixed by shaking manually to provide thorough seed coverage. All treatments were applied 24 hours prior to placing the seeds for germination and health tests. Germination tests and health assessments were carried out as described in section 3.2.1 and 3.2.2. The number of normal seedlings, abnormal seedlings, dead seeds and fresh ungerminated seeds were recorded on the fifth and eighth days as prescribed in the ISTA rules for seed testing (ISTA 2003) and presented in sections 2.2.4 and 2.2.6. Forty normal seedlings from each treatment (10 seedlings per replicate) were randomly chosen and the length (mm) of primary roots and shoots measured. *Stemphylium* spp. infection was assessed after 14 days incubation on MEA agar as described in section 3.2.1.

6.2.1 Data analysis

Results of germination tests and seed infection were reported as a percentage. Values were angularly transformed prior to ANOVA analysis (Clarke & Kempson 1997). For shoot and root length, means were calculated and then separated using the least significant difference ($P< 0.05$) (John 1998).

6.3 Results

6.3.1 *Stemphylium* spp. infection

The *Stemphylium* spp. infection recorded from untreated seeds of cultivar Meteor was 9 % (Table 6.2). This was reduced to 0% and 1.5% after thermotherapy and the application of Wakil at double the recommended rate. Seeds treated with double rates of Aliette or Apron were not significantly different from the control seeds. Half and normal commercially recommended doses of the three chemicals did not significantly reduce the percentage of seeds with *Stemphylium* spp.

Table 6.2 *Stemphylium* spp. infection in untreated and treated pea seeds (cultivar Meteor) after 14 days incubation on MEA agar, at 20 ± 1°C in NUV light. Seeds were treated with the three fungicides at half, normal and double the commercial rates.

Treatment	Application rate	*Stemphylium* spp. infection (%)
Untreated control	-	9[a] (17.33)[b] b
Hot water soak	50°C for 30 minutes	0 (0.00) a
Aliette	half	9 (16.87) b
	normal	6 (14.08) b
	double	7 (14.35) b
Apron	half	8 (15.25) b
	normal	6 (13.43) b
	double	10 (17.14) b
Wakil	half	7 (15.15) b
	normal	6 (13.42) b
	double	2 (4.92) a
LSD (32 df) [c]		5.563

Means from 4 replicates per treatment containing 50 seeds.
[a]Non-transformed means rounded to whole numbers.
[b]Values in parentheses are means after angular transformation of percentage data for each replicate. Treatments with the same letter are not significant different.
[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from ANOVA analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

Stemphylium spp. infection of untreated seeds of cultivar Midichi was 35 % (Table 6.3). All treatments significantly ($P<0.001$) reduced seed borne *Stemphylium* spp. The hot water soak and Apron treatments completely eradicated the fungus. Seeds treated with Aliette and Wakil reduced the percentage of infection by *Stemphylium* spp. from 35 % to 12% and 20%, respectively.

Table 6.3 *Stemphylium* spp. infection in untreated and treated pea seeds (cultivar Midichi) after 14 days incubation on MEA agar at 20 ± 1°C in near NUV light. Seeds were treated with hot water or the three fungicides at the normal commercial rate.

Treatment	*Stemphylium* spp. infection (%)
Untreated control	35[a] (36.36)[b] d
Hot water soak	0 (0.00) a
Aliette	12 (19.42) b
Apron	0 (0.00) a
Wakil	20 (26.41) c
LSD (14 df) [c]	5.951

Means from 3 replicates per treatment containing 35 seeds.
[a]Non-transformed means rounded to whole numbers.
[b]Values in parentheses are means after angular transformation of percentage data for each replicate. Treatments with the same letter are not significant different.
[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SD = standard error of the difference between the means derived from ANOVA analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

6.3.2 Normal seedlings

The normal seedlings percentage was significantly ($P<0.001$) affected by some of the treatments (Table 6.4). The germination of untreated seeds of cultivar Meteor was 68%. Seed treatment with Apron (at half and at the commercial rates) significantly improved the percentage normal seedlings. Germination was reduced after hot water treatment to 44 % and by the double rate of Aliette to 59%. Germination for seeds treated with the other chemical products and rates did not differ from the control.

Table 6.4 Normal seedlings percentage from pea seeds untreated (control), treated with hot water and fungicides at different rates. Seeds were treated with three fungicides with half, normal and double the commercial rates. Seed lot cultivar Meteor.

Treatment	Application rate	Germination (%)
Untreated control	-	68[a] (55.48)[b] cde
Hot water soak	50°C for 30 minutes	44 (41.72) a
Aliette	half	64 (53.45) cd
	normal	63 (52.47) bc
	double	57 (49.04) b
Apron	half	75 (60.42) fg
	normal	80 (63.51) g
	double	73 (58.56) ef
Wakil	half	68 (55.33) cde
	normal	70 (56.76) def
	double	62 (52.15) bc
LSD (32 df)[c]		4.133

Means from 4 replicates per treatment containing 50 seeds.
[a]Non-transformed means rounded to whole numbers.
[b]Values in parentheses are means after angular transformation of percentage data for each replicate. Treatments with the same letter are not significantly different.
[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from ANOVA analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

Germination was 71 % for untreated seeds of cultivar Midichi and this was significantly reduced ($P< 0.001$) after hot water treatment and Apron at the normal rate (26% and 53% normal seedlings, respectively) (Table 6.5). The percentage normal seedlings after treatment with either Aliette or Wakil did not differ significantly from the control.

Table 6.5 Normal seedlings (%) from pea seeds untreated (control), treated with hot water or three fungicides at normal commercial rate. Seed lot cultivar Midichi.

Treatment	Germination (%)
Untreated control	71[a] (57.51)[b] cd
Hot water soak	26 (30.52) a
Aliette	78 (61.80) d
Apron	53 (46.91) b
Wakil	64 (53.27) bc
LSD (14 df)[c]	6.775

Means from 3 replicates per treatment containing 35 seeds.
[a]Non-transformed means rounded to whole numbers.
[b]Values in parentheses are means after angular transformation of percentage data for each replicate. Treatments with the same letter are no significantly different.
[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from ANOVA analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

6.3.3 Abnormal seedlings

The abnormal seedlings percentage for cultivar Meteor increased significantly ($P< 0.001$) after treatment with hot water to 53 % (Table 6.6) and Aliette at the double rate to 43 % compared with the control (30 %). The treatment with Apron at the normal rate significantly reduced the percentage of abnormal seedlings (19 %) compared with the untreated control. The percentage of abnormal seedlings for all the other treatments was not statistically different from the control.

Table 6.6 Percentage of abnormal seedlings in untreated and treated pea seeds (seed lot cultivar Meteor) after 14 days incubation on MEA agar, 20 ± 1°C, NUV light. Seeds were treated with three fungicides with half, normal and double the commercial rates.

Treatment	Application rate	Abnormal seedlings (%)
Untreated control	-	30[a] (33.27)[b] cd
Hot water soak	50°C for 30 minutes	53 (46.56) f
Aliette	half	32 (34.75) cd
	normal	36 (36.63) de
	double	43 (40.96) e
Apron	half	23 (28.52) ab
	normal	19 (25.61) a
	double	27 (31.44) bc
Wakil	half	32 (34.36) cd
	normal	29 (32.63) bcd
	double	36 (36.96) de
LSD (32 df) [c]		4.550

Means from 4 replicates per treatment containing 50 seeds.
[a]Non-transformed means rounded to whole numbers.
[b]Values in parentheses are means after angular transformation of percentage data for each replicate. Treatments with the same letter are not significantly different.
[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from ANOVA analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

For cultivar Midichi, the percentage of abnormal seedlings also increased significantly ($P<0.001$) after treatment with hot water and Apron at the normal rate (Table 6.7). Treatments with Aliette and Wakil did not significantly alter the percentage of abnormal seedlings compared with the untreated seeds.

Table 6.7 Percentage of abnormal seedlings from pea seeds untreated (control), treated with hot water or three fungicides at normal commercial rate. Seed lot cultivar Midichi.

Treatment	Abnormal seedlings (%)
Untreated control	27[a] (31.60)[b] ab
Hot water soak	68 (55.70) d
Aliette	22 (28.20) a
Apron	46 (42.50) c
Wakil	35 (36.10) bc
LSD (14 df) [c]	7.160

Means from 3 replicates per treatment containing 35 seeds.
[a]Non-transformed means rounded to whole numbers.
[b]Values in parentheses are means after angular transformation of percentage data for each replicate. Treatments with the same letter are not significantly different.
[c]Significant differences between any treatment means given by the least significant difference (LSD) (= t_v x SED) where SED = standard error of the difference between the means derived from ANOVA analysis and t = critical value ($P = 0.05$) of Student's t distribution for v degrees of freedom (df).

6.3.4 Dead seeds

There was no significant difference in the percentage of dead seeds among the treatments and the untreated seeds for seed lot cultivar Meteor ($P< 0.05$) or for cultivar Midichi ($P < 0.05$). The average percentage of dead seeds was 1% and 2%, respectively.

6.3.5 Fresh ungerminated seeds

There was no significant effect of the treatments on the percentage of fresh ungerminated seeds for both seed lots. The average of fresh ungerminated seeds was 1% and 0% for seed lots cultivar Meteor and Midichi, respectively.

6.3.6 Shoot and root length

The seeds of cultivar Meteor treated with Apron (at half and normal rates) and Wakil (at double rate) produced seedlings with significantly ($P< 0.05$) longer shoots than the control seeds (Figure 6.1). For cultivar Midichi all the chemical treatments significantly ($P< 0.001$) reduced shoot length whereas hot water treatment had no effect on the shoot length (Figure 6.2).

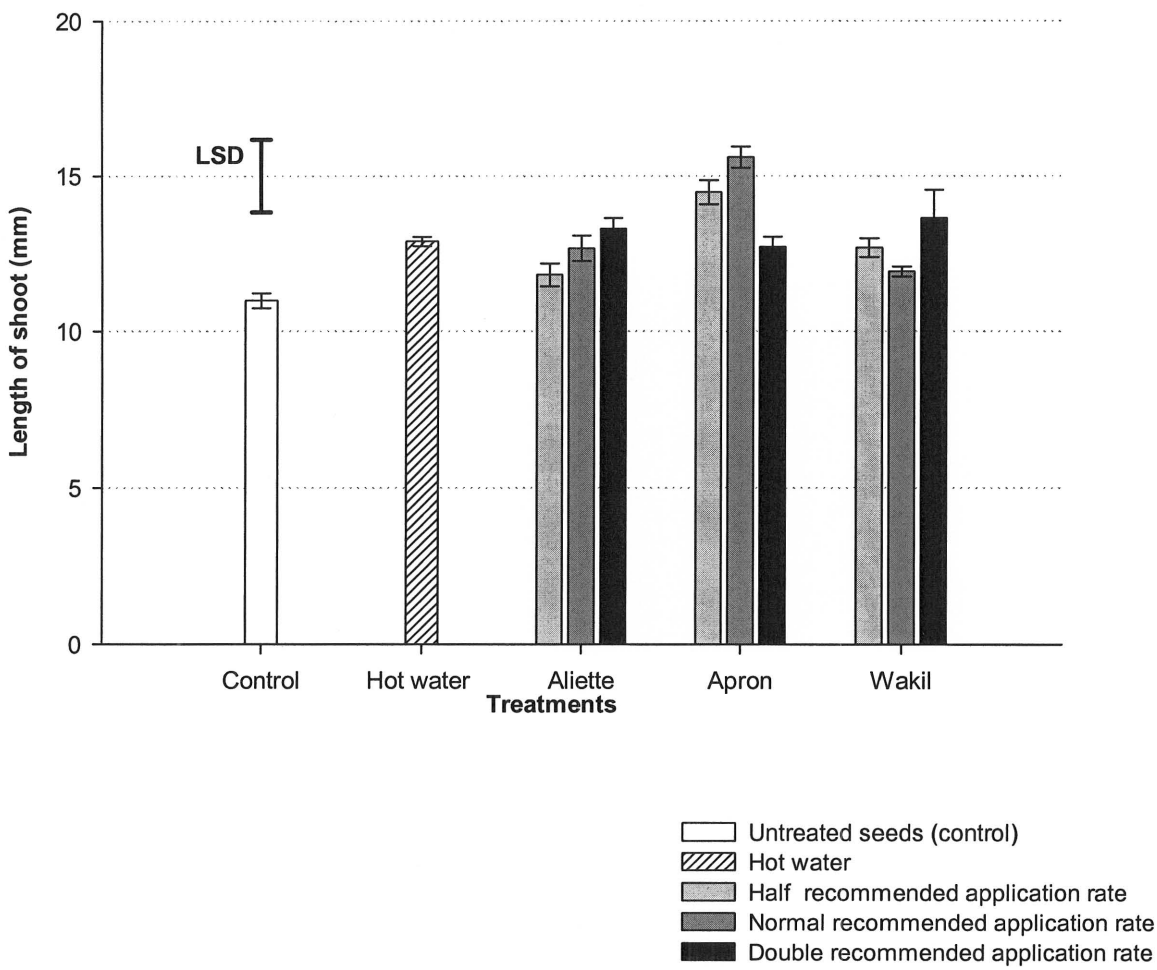

Figure 6.1 Length (mm) of normal seedlings shoots from pea seeds untreated (control), treated with hot water and with fungicides at different rates. Seed lot cultivar Meteor.

Means of 4 replicates per treatment, with bars representing one standard error. LSD = 2.329 (P <0.05).

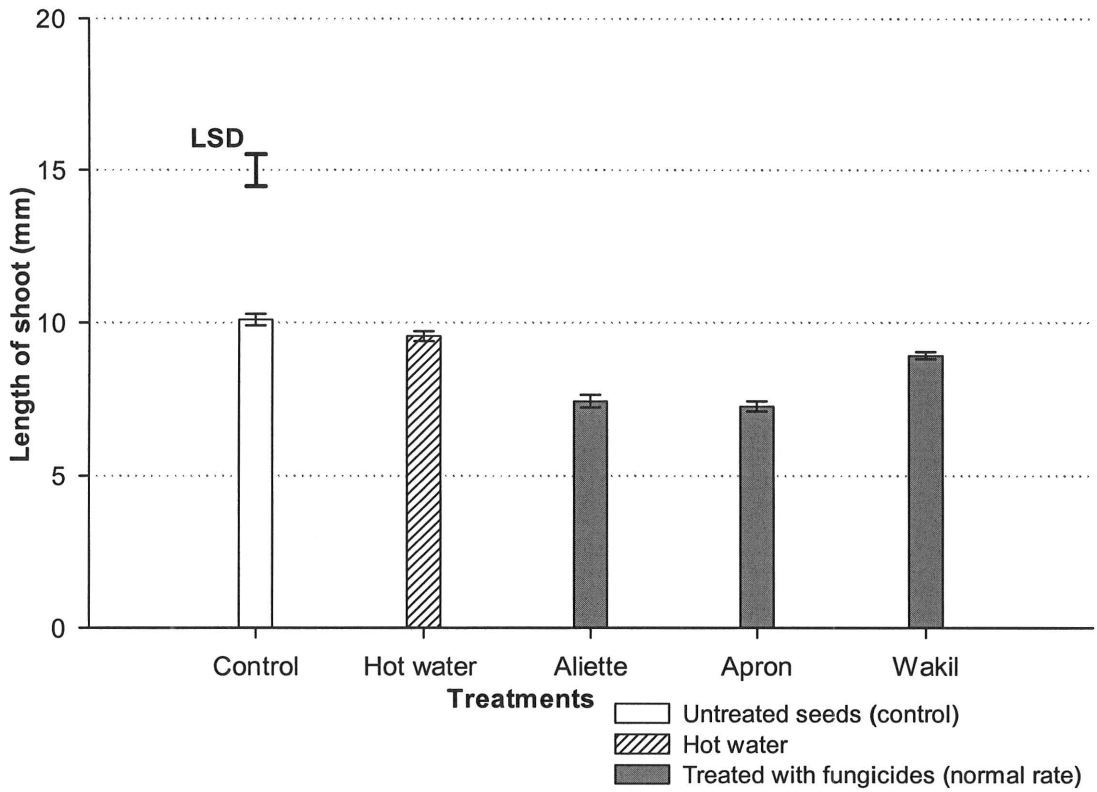

Figure 6.2 Length (mm) of normal seedlings shoots from pea seeds untreated (control), treated with hot water and with fungicides at normal rates. Seed lot cultivar Midichi.

Means of 3 replicates per treatment, with bars representing one standard error. LSD = 1.070 ($P< 0.001$).

The root length of seedlings of cultivar Meteor was increased significantly by Aliette application at all rates, by Apron application at half and normal rates, and by Wakil application at the half rate (Figure 6.3). Only treatment with Apron at the double rate decreased root length, from 44 mm to 37 mm. None of the other treatments, including the use of hot water, differed significantly from the untreated seeds.

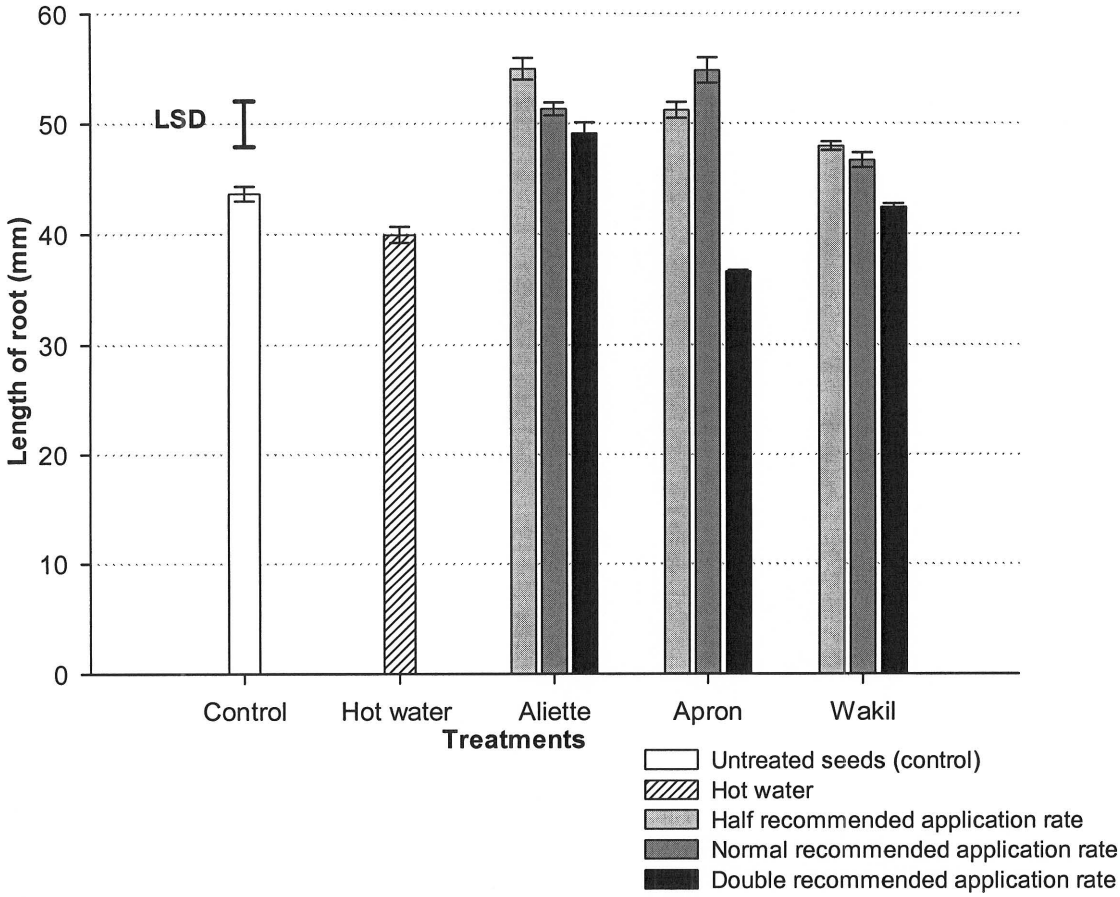

Figure 6.3 Length (mm) of primary roots of normal seedlings from pea seeds untreated (control), treated with hot water and with fungicides at different rates. Seed lot cultivar Meteor.

Means of 4 replicates per treatment, with bars representing one standard error. LSD = 4.195 ($P< 0.001$).

For cultivar Midichi the treatment with Apron and Wakil at commercial rates and hot water all significantly ($P<0.001$) reduced the length of the primary roots (Figure 6.4). There was no significant difference in root length between untreated seeds and seeds treated with Aliette.

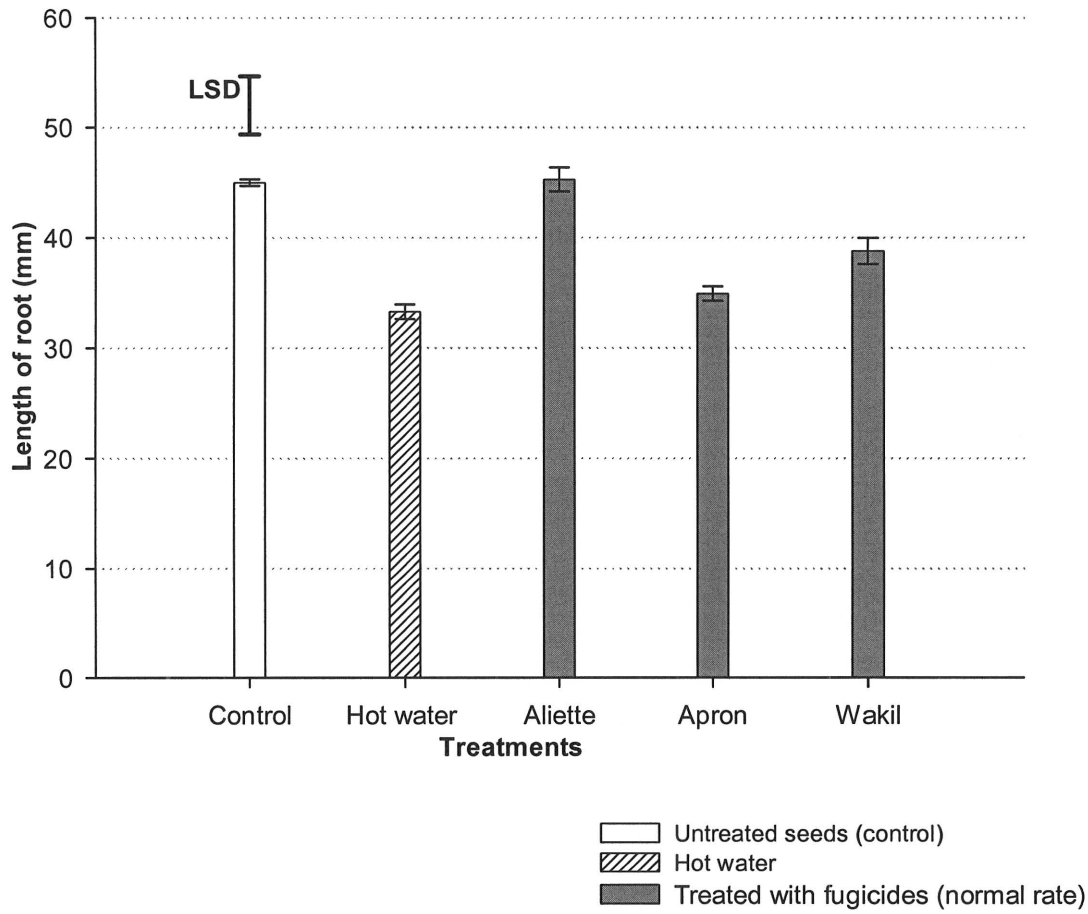

Figure 6.4 Length (mm) of primary roots of normal seedlings from pea seeds untreated (control), treated with hot water and with fungicides at normal rates. Seed lot cultivar Midichi.

Means of 3 replicates per treatment, with bars representing one standard error. LSD = 5.299 ($P< 0.001$).

6.4 Discussion

6.4.1 *Stemphylium* spp. infection

6.4.1.1 Hot water soak treatment

Success in controlling seed borne fungi depends on finding conditions lethal to the pathogen but which cause minimal damage to seed quality (Halmer 2000; Nesmith 2004). In this experiment, complete control of *Stemphylium* spp. was obtained

using the hot water treatment (50°C for 30 minutes). Aveling & Snyman (1993) also reported similar results. They observed that a hot water soak completely eradicated *S. vesicarium* from onion seeds. For peas, Boettinger and Bowers (1975) reported that a hot water soak treatment (30 minutes at 50°C) was effective in controlling seed borne bacterial blight (*Pseudomonas syringae* pv. *pisi*). For small-seeded legume species the hot water treatment (50 – 60 °C at 30 minutes) has been successful in reducing seed borne pathogens such as *Stemphylium* spp. (Lamprecht & Knox-Davies 1984; Aveling & Snyman 1993; Halmer 2000).

Heat treatments are effective in eliminating seed borne diseases because high temperature inhibits fungal growth and reproduction. Growth and asexual reproduction of *Stemphylium* spp. occur over a broad temperature range (from 18 to 42 °C) (Neergaard 1945; Bashi et al. 1973; Ligero et al. 1998). The development of warm temperature strains of *Stemphylium* spp. occurs even at temperatures around 40°C (Bashi et al. 1973; Stuville & Erwin 1990; Mehta & Brogin 2000). But high temperature, for example the 50°C used in this experiment, may directly inhibit fungi by inactivating important enzyme systems, resulting in disruption of the plasma membranes and cytoplasm of the hyphae (Campbell 1993b).

Unfortunately this same mechanism also acts against the cell structure of the seeds causing damage. Consequently, there is a decrease in seed vigour and germination (Nesmith 2004) as will be discussed in section 6.4.2.

Complete control of *Stemphylium* spp. was obtained with the hot water soak. Hot water is used widely to control pathogens that are not or are inadequately controlled by fungicides. However, the method presents limitations for commercial application to large amounts of seed (Grondeau et al. 1992). Critical points are the

precise temperature control during the treatment and the seed species type (Agarwal & Sinclair 1997).

6.4.1.2 Fungicides treatment

Chemicals were not as efficient as hot water treatment in reducing *Stemphylium spp.* infection. The systemic fungicides used in this trial failed to eradicate *Stemphylium* species, with the exception of the Apron application on the Midichi seed lot.

Systemic fungicides are highly selective and as demonstrated in this experiment the three chemicals registered for pea seed treatment do not effectively control *Stemphylium* spp., being originally developed to control *Pythium* spp., *Fusarium* spp., *Ascochyta* root rot, *Septoria* and downy mildew.

In vitro studies have shown that fungicides such as tebuconazole, carbendazim/flusilazole mixture and procymidone were effective in inhibiting *S. vesicarium* growth in potato dextrose agar culture (Aveling & Snyman 1993). Ureba et al. (1998) also reported successful control of *S. vesicarium* in garlic plants with tebuconazole, a mix of procymidone and chlorothalonil. None of the systemic fungicides used in the experiment contained these active ingredients, which may explain why *Stemphylium* spp. total control was not achieved using the fungicides tested.

The exception was the application of Apron in Midichi seeds which completely eradicated the fungus. Additionally, it was observed that the application of Wakil at a double rate did reduce the percentage infection in the Meteor seed lot. Those two products have the metalaxyl ingredient in common. Apron is purely metalaxyl

whereas Wakil is a mixture of metalaxyl, fludioxonil and cymoxanil. It is reported that the primary effect of metalaxyl is the impaired biosynthesis of fungal RNA and consequently inhibition of protein, nucleic acid production and mitosis (Fisher & Hayes 1982). Studies conducted in onion leaves also showed that *S. botryosum* was susceptible to the application of metalaxyl (commercial product Ridomil) and infection was reduced by 53 % (Brar et al. 1991). But *S. vesicarium* was not successfully controlled in onion leaves by metalaxyl, the pathogen being more sensitive to chlorothalonil or iprodione (Srivastava et al. 1995; Gupta et al. 1996).

In this study only Apron (at the commercial rate) successfully eradicated *Stemphylium* spp. but only from Midichi pea seeds. Apron was not able to eliminate *Stemphylium* spp. from seeds of the Meteor cultivar. There might be several factors involved in the response of each seed lot to the treatments used. One of them may be the *Stemphylium* species occurring in those seed samples.

As demonstrated in chapter 4, in the Canterbury region at least 5 species (Table 4.3) may occur alone or in combination. It is possible that a differing susceptibility of each *Stemphylium* spp. in the pea seed lots used may be the reason for complete control in seed lot of Midichi seeds and no significant reduction in *Stemphylium* spp. for the Meteor seed lot. Another possible reason is that the inoculum may have been located in different parts of the seed. The seeds were all surface sterilized prior to plating and thus the seed borne inoculum could only be embedded in internal tissues or in the embryo (Brown 1997). It may be that in the Meteor seed lot the inoculum was positioned in the embryo whereas in the Midichi seed lot *Stemphylium* spp. were located in seed tissues more easily accessable by the fungicide.

6.4.2 Normal germinated and abnormal seedlings

For both seed lots the percentage of normal seedlings was substantially reduced using hot water treatment as the percentage of abnormal seedlings increased after the hot water soak.

According to Boettinger and Bowers (1975) cultivars vary in their tolerance to hot water treatment. Here the two seed lots had already a relative low germination (around 70%) indicating that seed deterioration already had started and presumably, the hot water treatment just hastened the process (Agarwal & Sinclair 1997; J.G. Hampton, personal communication, 24 March 2005). In this study it was observed that Midichi seed germination declined more drastically than Meteor seeds after hot water treatment (45% and 24% decrease, respectively). Grondeau et al. (1992) also reported a reduction in germination percentage by 30% after hot water treatment. These authors also reported differences in susceptibility between cultivars, with cultivar Solara being more sensitive to heat damage than cultivar Belinda.

Heat may have a negative effect on normal seedling production. At a temperature of 50°C, seed cells may have experienced disruption of membranes. The consequences of those changes are cracked seed coats, a high number of dead and abnormal seedlings in laboratory germination tests and poor emergence in the field (Boettinger & Bowers 1975). In this study the hot water treatment significantly increased the percentage of abnormal seedlings of both seed lots.

Chemical treatment may have toxic effects on seed germination, particularly for damaged or deteriorating seeds. For Meteor, germination was reduced when double the recommended rate of chemicals was used. Seeds of Midichi treated with Apron (at the commercial rate) also had lower germination. Such chemicals and dosages

were phytotoxic, resulting in abnormal seedlings (twisted roots and distorted and small shoots) (Appendices 12 and 13). Detrimental effects of fungicides in legumes were also reported by Zhang and Hampton (1999). These authors worked with legume species (peas, soybean and beans) and reported that electrical conductivity of seeds (an indicator of cell membrane integrity) treated with recommended application rates did not differ from untreated controls. Thus, the fungicides did not affect the membrane integrity of seed cells. However, at double rates some of the chemicals used had a negative effect on the cell integrity. Apron (35 SD) significantly increased the conductivity of garden peas and soybean seeds. Apron TZ significantly increased the conductivity of soybean and Aliette Super significantly increased the conductivity of garden peas, soybean and broad bean.

As demonstrated for seed lot Meteor, double recommended rates of the three fungicides decreased the percentage of normal seedling and increased the percentage of abnormal seedlings, indicating increased seed deterioration. Characteristic defects, as a consequence of physiological disturbances, may include retarded growth of the seedlings as a whole or of individual parts of it, and short, stubby or spindly primary or seminal roots (Halmer 2000; Schmitt 2000). Even registered formulations may slow germination and emergence of some seed lots because the active ingredient is slightly phytotoxic and it is difficult to avoid some detrimental effect (Halmer 2000). This is particularly evident when fungicides are applied to physically damaged seed lots or physiologically deteriorating seed lots, when the chemical can gain access to the embryo (J.G. Hampton personal communication, 24 March 2005). Murray & Kuiper (1988) demonstrated that fungicides (commercial names Baytan and Erex) applied at twice their recommended rates decreased emergence of damaged wheat seeds by 20 %,

whereas non damaged seeds dressed with the same treatments had a reduction of 12% in emergence.

6.4.3 Shoot and root length

For both cultivars, the hot water treatment did not affect the formation of the shoots, but primary root length was decreased after the hot water soak treatment. The negative effect of the hot water treatment may be related to damage to embryo cell structures, that are responsible for radicle formation, protusion and elongation (Copeland & McDonald 2001a) as mentioned previously in section 2.3.5.1.

Different cultivars and seed lots may react differently to the same seed treatment (Murray & Kuiper 1988; Agarwal & Sinclair 1997). Overall, the seed lot of cultivar Midichi was more sensitive to the chemical treatment than the seed lot of cultivar Meteor. A general trend was observed for seed lot cultivar Meteor: an increase in root length followed by an increase in shoot length after treatments with fungicides, mainly at half and normal recommended application rates. For the seed lot of cultivar Midichi all chemical treatments significantly reduced shoot length and two (Apron and Wakil) reduced primary root length. Similar observations were made by Murray & Kuiper (1988) on cereals. They stated that damaged wheat seeds produced seedlings with shorter coleoptiles (27.5 mm) than undamaged seeds (32.5mm) after dressing with Erex (a.i. triadimefon and lindane).

Among the chemicals used in this experiment, Aliette and Wakil are absorbed mainly through the roots of the germinating seeds and then translocated. Apron acts by penetrating the seed coat and is then translocated (Anonymous 2002b; Anonymous 2003a). For Meteor seedlings, the action of the chemicals against the

fungi and the non toxic effects may have favoured root elongation and consequently shoot growth. For Midichi however, the chemicals may have affected normal cell division, elongation and differentiation (Campbell 1993c). A toxic effect in such cells results in shortened seedling radicles and shoots (Halmer 2000).

The different responses post-treatments between seed lots observed in this experiment also may be due to differences in genetics, conditions of growth in the field and during harvesting, processing and storage and level of fungal infection (Brown 1997). The conditions in which the seeds were produced and processed were unknown. They may certainly interfere with the degree of deterioration (Powell et al. 1997; Siddique & Wright 2003) of the seed lots used, as well as the intensity and the type of fungal infection (Bashi et al. 1973; Christensen & Wysong 1997). For instance, it is possible that Midichi seeds were exposed to environmental conditions that favoured greater *Stemphylium* spp. infection (35%) compared with the seed lot of Meteor which had 9% *Stemphylium* infection. Adittionally, stress caused by several factors, for example excess or deficit of moisture or temperature when seeds were still in the parent plant, may have also conferred a degree of damage to the seeds (Powell et al. 1997; Copeland & McDonald 2001a). But one factor that may account for those differences is that Midichi seeds are generally larger seeds than Meteor. Having a bigger surface area than Meteor seeds, Midichi seeds are more likely to be physically damaged (Vanderberg 1995). Damaged seeds are more likely to have reduced germination, delayed emergence and shorter or deformed seedlings and stresses caused by seed dressings seem to be additive (Murray & Kuiper 1988). This was also observed in this experiment after treatments were applied.

6.5 Summary

- The hot water soak treatment eradicated *Stemphylium* spp. from infected pea seeds for both pea seed lots (cultivars Meteor and Midichi). But there was a reduction in seed germination because of an increase in the percentage of abnormal seedlings. For normal seedlings, both shoot and primary root length were negatively affected by the hot water soak treatment.

- The three systemic fungicides used in this study were less effective in controlling *Stemphylium* spp. infection compared with the hot water treatment, even though they were less harmful to seeds.

- For the seed lot of cultivar Meteor, Apron at the commercial rate was the best treatment, considering percentage of normal/ abnormal seedlings and length of shoot and primary roots. Half rates of the products had a similar effect to the commercial rate; however, the use of reduced doses should be avoided due to the possible development of resistance to that chemical by the pathogen. With the exception of Wakil, double rate application of chemicals did not improve *Stemphylium* spp. control. Additionally, double rates were phytotoxic to seeds, generally reducing germination, increasing the percentage of abnormal seedlings and decreasing the length of primary roots.

- The seed treatment response was slightly different for the seed lot of cultivar Midichi. *Stemphylium* spp. infection was eradicated with the use of Apron and substantially reduced by the Aliette and Wakil treatments. The occurrence of different *Stemphylium* species might be the reason for such diverse results. However, seeds of this cultivar were more vulnerable to chemical treatment compared with the seed lot of cultivar Meteor. There was a reduction in germination after all chemical seed treatments. Aliette had fewer negative effects on shoot and root length, as well as germination and abnormal seedling production, compared with the other chemical treatments.

7 General discussion

The objective of this thesis was to test the null hypothesis that *Stemphylium* spp. were not pathogens of peas. The results gathered in this research provide information about the significance of *Stemphylium* infection on peas in New Zealand and may be of importance to future seed/plant pathology studies.

7.1 *Stemphylium* spp. are seedborne pathogens of pea

Experiment 1 (Chapter 3) was performed to quantify the occurrence of *Stemphylium* spp. from New Zealand grown pea seed lots and to assess the possible effects on seed germination and emergence under laboratory and glasshouse conditions.

The experimental results showed that *Stemphylium* spp. infection of pea seed in the seed lots tested varied from 0 to 46%, with seed lots harvested in 2004 having the highest percentage of the fungi. Two hypotheses may explain this high percentage of infection in the 2004 seed lots:

1. Environmental conditions in the seasons 2003-2004 were more favorable for *Stemphylium* spp. infection ;

2. *Stemphylium* spp. are not able to survive in the seeds for long periods, and therefore more recently harvested seed lots have a higher percentage infection.

Fungal infection on or in seeds depends on several factors, such as the presence of inoculum and agronomic practices during seed crop development (section 2.4.1). Environmental conditions have been reported to play a major role in favoring the occurrence of *Stemphylium* spp. on crops (Oku 1994a; Martiniello & Porta-Puglia 1995; Maude 1996). Several experiments and field surveys have investigated the relationship between environmental factors (mainly humidity and temperature) and *Stemphylium* spp. infection of plants and seeds, e.g. in onion (Langston 2001; Stivers 2004), garlic (Ligero et al. 2003) and leeks (Suheri & Price 2001). In all the cases, leaf wetness was the main factor contributing to *Stemphylium* spp. infection of leaf tissue.

Thus, there could be an expectation to find a high percentage of *Stemphylium* spp. infection in those years with more rainfall during the growing season. However, no correlation was found between the rainfall during (spring/summer) 2001-2002 (368 mm), 2002-2003 (259 mm) and 2003-2004 (217 mm) and the occurrence of *Stemphylium* spp. in the seed lots assessed (section 3.3.1). This fact may suggest that the persistence of *Stemphylium* spp. in seeds may be restricted. Also, the interaction between temperature and rain, the length of leaf wetness and the susceptibility of plants (Suheri & Price 2000; Bradley et al. 2003) occurring in the crops would favour the infection. For instance, it could be possible that in 2003-2004, despite the low rainfall, there was less evaporation and hence, the chances for spore germination and infection were increased (E. E. Jones, personal communication 26 June 2005).

Fungi such as *Alternaria* spp., *Fusarium* spp. and *Phoma* spp. that are present in/on freshly harvested seeds may disappear over a storage period of 6 months (Bankole et al. 1995-1996) for instance, due to the short life span of conidia that are

the active colony forming units (Jensen et al. 2002). Shrestha et al. (2003) reported that seed borne *Alternaria brassicae* (Berk. Sacc.) survived in the seeds at least one year (at temperatures 5 and 10°C) but this period may be shorter depending on the storage temperature. This could be the reason for the higher percentage of *Stemphylium* spp. observed in the 2004 seed lots. Thus it would be expected that seeds recently harvested and which would be sown in the consecutive season, could have more *Stemphylium* than those stored for one or more years.

The occurrence of *Stemphylium* spp. in these commercial pea seed lots indicates that pea seeds are a possible source of *Stemphylium* spp. inoculum and a means of pathogen spread to new non infected paddocks. It is important, from an agricultural point of view, to consider pea crops as a possible source of *Stemphylium* spp. when planning crop rotations. Peas, which are commonly used to break diseases cycles (White 1987), may introduce a pathogenic *Stemphylium* spp. for other plant species commonly grown in Canterbury and which are susceptible to *Stemphylium* spp. (such as onion, garlic, lucerne, carrots and clovers).

7.1.1 Effects of *Stemphylium* spp. on pea seed germination and seedling emergence

The results obtained in the first experiment indicate that *Stemphylium* spp. did not affect seed germination. Pea seed lots with more than 20% *Stemphylium* spp. had a germination of close to 90% (Tables 3.3 and 3.4). The presence of the fungi did not result in the production of abnormal seedlings. *In vitro* observations confirmed that *Stemphylium* spp.were present in both normal and abnormal seedlings.

Stemphylium spp. infection had a significant effect in reducing the number of seedlings per pot (Figure 3.2) and primary root length (Figure 3.3) by 60% in the

cv. Rondo 95 seed lot. The primary root is the main structure affected by the fungus. The fungal colonization of the radicle may have prevented the absorption of water and nutrients for the seedling, which was reflected in poor establishment and shorter roots of infected seedlings compared with those from non infected seeds.

However, no significant effects were observed for the other seed lot (cv. Midichi) which had the highest percentage of infection (Figure 3.2). This suggests that, under the conditions provided in the trial, the infection caused by *Stemphylium* spp. did not interfere drastically in seedling emergence. There is also the possibility that not all *Stemphylium* spp. are pathogenic to peas. So, one seed lot may have high infection by a non pathogenic isolate/species and have high germination. Additionally, a number of different *Stemphylium* spp. (pathogenic and non pathogenic) may infect a single seed lot, which may produce abnormal (pathogenic strain infection) and normal (non pathogenic strain infection) seedlings (E. E. Jones, personal communication 26 June 2005).

7.2 *Stemphylium* species isolated from pea seeds

The work with *Stemphylium* spp. isolated from marrowfat pea seeds (Chapter 4) revealed that species infecting the seed lots tested are commonly pathogenic to other legume plants, and belonged to the groups C and E according to the classification proposed by Camara et al. (2002): *S. astragali, S. herbarum /vesicarum/ alfalfae* (group C) and *Stemphylium* spp. close to *S. loti / S. sarciniforme* (group E).

From the quantitative (growth rates) and qualitative data (visual characteristics and reproductive structures) obtained by growing the isolates on artificial media (sections 4.3.1- 4.3.4) it was possible to distinguish more than one *Stemphylium* spp. However, comparing the morphological and physiological features with the keys and literature available was not sufficient to draw solid conclusions about the identity of all the isolates. Some of the difficulties encountered when trying to identify the species by microscopic observations only were:

- Isolates from the same species can grow and reproduce differently depending on the media used, as observed previously for *S. radicinum (Alternaria radicina)* (Curren 1968). This was the case in experiment 2 (Chapter 4).

- Conidial morphology is one of the main criteria to distinguish *Stemphylium* from other similar genera, such as *Alternaria* and *Ulocladium*, and to some extent to distinguish different *Stemphylium* species. However, the genus *Stemphylium* is extremely complex in terms of conidial shape and dimension, as some characteristics overlap among species (Neergaard 1945; Ellis 1971; Camara et al. 2002). During the experiment it was clear that the species isolated were closely related, and morphological data would not be sufficient to clarify which *Stemphylium* species were seed borne.

- The variable nomenclature of the *Stemphylium* genus and the different media used in many studies made comparisons from the literature difficult. For example in 1986, Simmons segregated *S. botryosum* (with its slow maturing teleomorph *P. tarda*) from the somewhat similar *S. herbarum* (with its more rapidly maturing teleomorph *P. herbarum*) (Stuville & Erwin

1990). Some of the literature used to distinguish the *Stemphylium* isolates did not consider those changes and the use of keys was somewhat limited. Isolate 7 for example, which was chosen for further studies, could be classified as *S. botryosum* as well as *S. herbarum* due to similarity in the conidial dimensions between the two species (33-35 x 24-26 µm for *S. botryosum* and 35-45 x 20-27 µm for *S. herbarum*).

The phylogenetic relationships of these species and other *Stemphylium* species already catalogued and fully identified by the Molecular Plant Pathology Laboratory, USDA/ARS, Beltsville, MD (USA) was used to support and clarify the microscopic observations. Isolate 7 for instance, was classified as *S. herbarum* due to its grouping with *S.herbarum* isolates on the phylogenetic tree (Appendix 8), and being distant from the *S. botryosum* branch.

Stemphylium herbarum has been identified as seed borne in wheat (*Triticum aestivum*) seeds (Khanzada et al. 2002) and its anamorph (*P. herbarum*) catalogued as a non regulated pest on peas (Anonymous 2004b). Other species, such as *S. vesicarium* and *S. sarciniforme* have already been isolated from pea seeds elsewhere (Simmons 1969; Wegrzycka 1990). No information on the occurrence of *Stemphylium astragali* on pea seeds was found.

The aims of this study were achieved, with the study providing more detailed information about:

i. the *Stemphylium* spp. which can be seed borne in/on peas;
ii. how they grow in different media;
iii. the conditions which favour the production of conidia (section 4.3.4) especially of isolate 7 (*S. herbarum*), which was used for the pathogenictiy study.

7.3 *Stemphylium herbarum* as a pathogen of pea

The third experiment was designed to test the pathogenicity of *S. herbarum* (isolate 7) and characterize symptom development in pea seedlings. The pathogenicity study confirmed that the marrowfat pea cv. Midichi was a host of *S. herbarum* (isolate 7). Conidia (3 x 10^4 conidia ml^{-1}) were able to infect tender tissues when mild temperatures (20 ± 1°C) and free moisture (100%) was available. Once conidia germinated, penetration occurred via the stomata and directly through the cuticle. *S. herbarum* produces toxins (stemphyloxin II and stemphyperylenol) (Andersen et al. 1995) which may have acted by collapsing the leaf cells, thereby assisting the conidium germ tube to reach the mesophyl cells. These findings show that *S. herbarum* may cause both necrotrophic and biotrophic infections. The symptoms developed were similar to those caused by *Stemphylium* spp. in other legume plants such as lucerne (Stuville & Erwin 1990; Christensen & Wysong 1997), clover (Bradley et al. 2003; Anonymous 2003c) and Chinese vetch (Anonymous 2003c). Leaf spots caused by *S. herbarum* did not progress to new leaves in this experiment, probably because of the lack of extended periods of moisture that are vital for spore germination (Bradley et al. 2003).

7.3.1 Mechanisms of pea seed infection

The occurrence of other diseases was a problem following inoculation of adult plants. It was not possible to assess whether plants infected by *S. herbarum* would produce infected seeds and which infection mechanism is involved (direct penetration or systemic).

Systemic infection seems to be the most widely accepted means by which *Stemphylium* spp. infect plants. Although the way of penetration into seeds was not totally identified in this study, two possible paths can be suggested :

i. Spores are spread by wind and water splash reaching floral tissues.

ii. Direct penetration.

The first alternative seems to be reasonable because other pea pathogens such as *M. pinodes* use this infection route. Infections by ascospores of *M. pinodes* at the flowering stage constitute a major threat for the crop because of risks of pod infection and seed abortion (Roger & Tivoli 1995). However, instead of ascospores, *Stemphylium* spp. conidia could affect flower tissues. Additionally, because *Stemphylium* spp. have a primary saprophytic habit, it is likely that premature senescence of pea plants, i.e. starting at stage R4 and R5 (Table 2.1) would enhance infection of flowers. In this case, husbandry practices reducing premature senescence, such as avoiding moisture stress, could be considered as part of disease control (Roger & Tivoli 1995).

In the case of peas, the direct penetration pathway appears to be improbable because seeds are enclosed in the pod. It is unlikely the conidia would overcome the pod barrier and reach the seeds, as occurred with leaf tissues (Figures 5.3 and 5.5), unless the pods were cracked and seeds were exposed to airborne conidia.

The results and observations made in this study are insufficient to identify accurately how *S. herbarum* reaches the seeds. A detailed study of the mechanisms of seed infection by *Stemphylium* spp. is needed, so that methods for reducing and even preventing the fungus from sporulating and the disease from spreading could be developed. No evidence was found in the literature that *Stemphylium* spp. could systemically infect seeds. Only a few fungi are known to be transported passively through the xylem, and these are basically vascular pathogens. Conidia of *Fusarium* and *Verticillium*, for example, are transported in the xylem of

watermelon and tomato (Goodman et al. 1986c). Could this be another route of infection by *Stemphylium* spp. spores?

7.3.2 Proposed *S. herbarum* disease cycle on peas

There is limited information in the literature about the epidemiology of *Stemphylium* spp. on peas. Based on observations from the third experiment (section 5.3), a part of the disease cycle of *S. herbarum* on peas can be proposed. As only conidia of *S. herbarum* (strain 7) were tested, the cycles proposed in Figure 7.1 relates only to this species attacking marrowfat peas.

In the first part of the cycle (Figure 7.1 A) external airborne conidia (1) coming in from outside (i.e. a neighbouring paddock) reach susceptible tissues (2) and, under suitable environmental conditions, more conidia are produced from infections during the season (3). As already mentioned (section 4.3.4) this isolate of *S. herbarum* (7) produced abundant conidia on artificial media, suggesting that it also would have great ability to reproduce in nature, attacking leaves (4, 5, 6) and floral tissues.

In the second part of the cycle (Figure 7.1B) the fungus uses the seed as a means to perpetuate itself to be transported to other areas (1). When infected seed is sown, a proportion of those seedlings emerge diseased (2).Conidia produced on the lesions can spread from plant to plant, by rain-splash or wind (3 and 4). Throughout the season, conidia are successively produced, causing many infections, including of floral tissue, with the consequence of producing infected seeds (5).

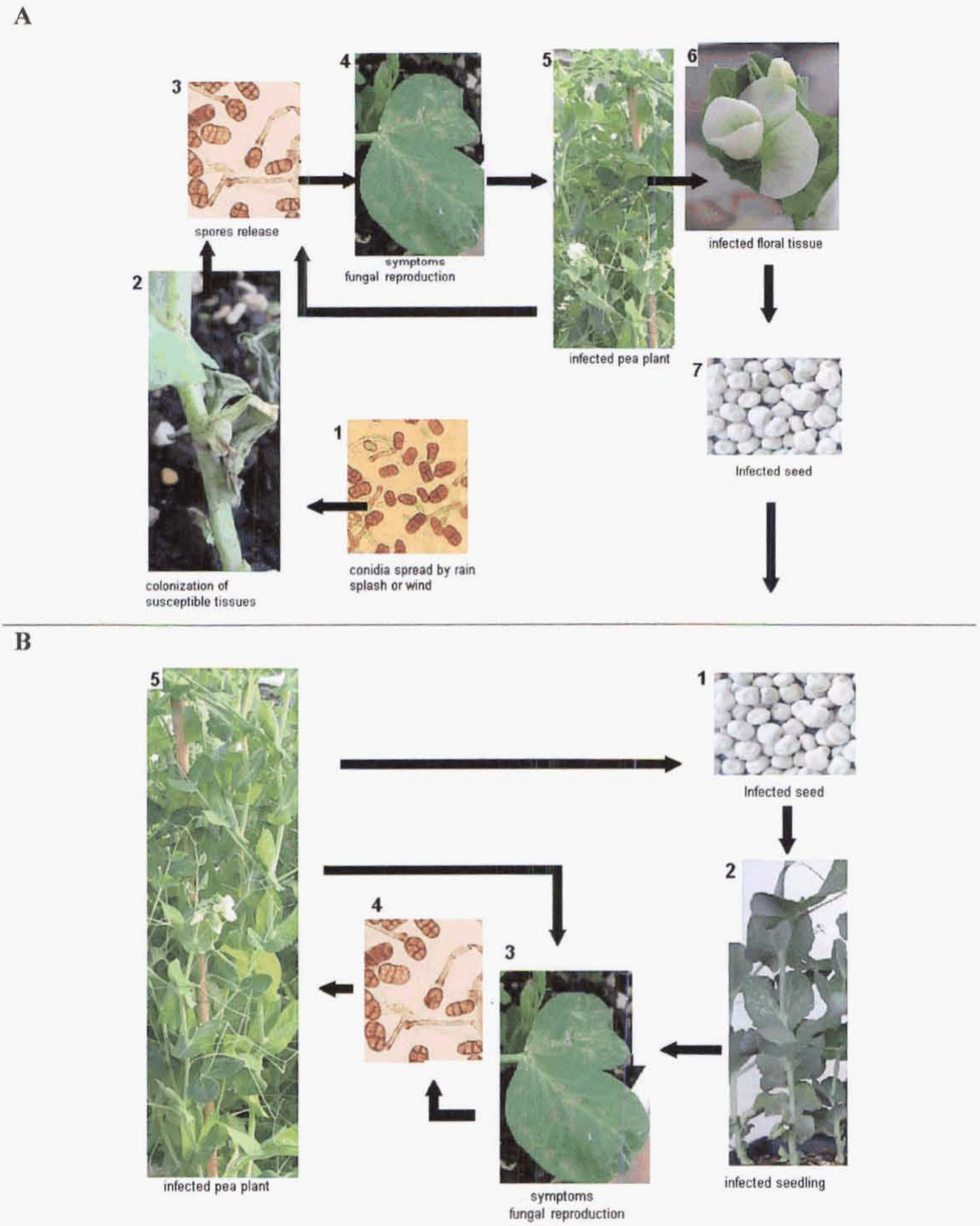

Figure 7.1 Proposed asexual disease cycle of *Stemphylium herbarum* on pea plants. (A) Externally sourced spores to seed. (B) Seed to seed.

In the present study with peas, only conidia were tested as an inoculum source. However, it is probable that the New Zealand climate would favor the full pathogen life cycle, including both conidia and ascospores of *Stemphylium* spp., as has been already studied in crops such as lucerne (Stuville & Erwin 1990), asparagus (Hausbeck 2003) and onion (Langston 2001). Ascospores of *Stemphylium* spp. (*Pleospora* spp.) are usually produced on dead plant tissue during moist weather and are carried by rain splash or wind to plants (Ligero et al. 1998).

7.3.3 Potential for the occurrence of *Stemphylium* spp. on pea crops grown in Canterbury

Canterbury is particularly privileged as a seed production region because of the warm and dry summers (Anonymous 2000; MetService 2005). Such conditions usually maintain low levels of fungal disease pressure and prevent fungal colonization of seeds (Martiniello & Porta-Puglia 1995). However, the survey conducted in the first experiment revealed that pea seed lots from three seasons (2002-2004) were infected with *Stemphylium* spp., indicating that the fungus had attacked the parent plants.

Stemphylium spp. are well known pathogen of other legumes, onion and asparagus but have not been identified as a problem on peas. The question therefore is how those pea seeds were infected by those *Stemphylium* species. A possible explanation could be that these *Stemphylium* spp. may have expanded their host range to peas as a result of increasing use of irrigation on Canterbury pea seed crops. *Stemphylium* spp. need temperatures around 20°C and high relative humidity, with periods of leaf wetness longer than 16 hours to successfully infect plants (Rizvi & Nutter 1993; Suheri & Price 2001; Bradley et al. 2003). The use of

a dense plant population associated with irrigation may create a crop microclimate which favours opportunist fungi, such as *Stemphylium* spp.

7.4 Evaluation of seed treatments for reducing *Stemphylium* spp. in pea seeds

In the fourth experiment the effects of seed treatments on the *Stemphylium* spp. occurrence, and the germination of pea seeds were evaluated. The magnitude of the effects varied between the seed lots. Hot water treatment was undoubtedly the most efficient method to eliminate *Stemphylium* spp. seed borne infection (Tables 6.2 and 6.3). However, it also significantly decreased the percentage seed germination (Tables 6.4 and 6.5).

The results obtained testing the three fungicides Aliette Super, Apron XL and Wakil XL, applied at half, normal and double the commercially recommended rate, were not so conclusive. For one seed lot (Meteor), the fungicides did not have any significant effect in reducing the percentage of *Stemphylium*, while for another cultivar (Midichi) all the chemicals reduced *Stemphylium* spp. infection and Apron completely eliminated *Stemphylium* spp. The products which contained metalaxyl as the active ingredient appeared to give better results in controlling the fungus, but this need to be investigated in more detail. Chlorothalonil, prochloraz, captafol, maneb (Grinstein et al. 1988), iprodione (Wu et al. 2001), tebuconazole and carbendazim/flusilazole have been reported to give satisfactory control of *Stemphylium* spp. (Aveling & Snyman 1993).

The effect of the treatments on germination and abnormal seedlings percentage were variable between the seed lots, probably due to the intrinsic characteristics of each seed lot. Both seed lots had a low germination percentage (68 and 71%) which indicates that the seeds were already deteriorating physiologically. After

application of the treatments the trend was to decrease the percentage of normal seedlings and increase the abnormal seedling percentage, especially after the hot water soak and application of double rate of fungicides. These observations are in agreement with several other similar seed treatment evaluations (Murray & Kuiper 1988; Grondeau et al. 1992; Aveling & Snyman 1993).

7.5 Approach for future research

There is an urgent need to catalogue, assess and communicate the nature and threats posed by new and or re-emerging fungal pathogens to agriculture due to the international seed trade and the economic aspects in controlling crop diseases (Wu 2001; Mathur & Kongsdal 2002; Ophel Keller 2003). This research has introduced the first evaluation of pea seed borne *Stemphylium* spp., and *Stemphylium herbarum* as a pathogen of pea plants. Most of the objectives of this project were accomplished. However, many questions have also been raised regarding the significance of *Stemphylium* spp. occurrence in peas. Some of the limiting points found during the experiments which could be improved in future investigations have already been discussed. In addition, there are several hypotheses and assumptions that could be covered in future research and which would complement this primary study. Based on this thesis, future research could focus on the follow questions:

1. Does *Stemphylium* spp. infection interfere with pea seedling emergence in the field?

Information about the significance of *Stemphylium* spp. infection emergence of pea seeds in the field is lacking in the literature. In this work, no correlation was found between *Stemphylium* spp. infection and seed germination, but *Stemphylium* spp.

appeared to have a negative effect on seedling emergence as assessed in pot trials. It is also important to point out that experiments were small scale, due to the limited number of seeds available for the tests. However, the information generated may help to rationalize future agronomic experiments.

2. How does *S. herbarum* (and/or the other *Stemphylium* spp. isolated) reach and infect the pea seeds?

There is much more to be understood about the relevance of the propagation of *Stemphylium* spp. through pea seeds and the mechanism of seed infection. No studies have addressed the infection mechanisms by *Stemphylium* spp. in pea seeds.

3. Do *Stemphylium* spp. persist for long periods on or in pea seeds?

As proposed previously (section 7.1) the percentage of seeds infected by *Stemphylium* spp. may decrease with time as occurs with other seed borne fungi. No information regarding the survival period of *Stemphylium* spp. in pea seeds has been found.

4. Are other pea cultivars susceptible to *S. herbarum* (isolate 7)? Are the other *Stemphylium* spp. isolates (table 4.3) pathogenic to peas ?

Under controlled environment conditions *S. herbarum* (strain 7) was pathogenic to marrowfat peas. However, other species were found (Table 4.3). Similar tests, as presented in this thesis, are required to assess the pathogenicity of these other isolates. The results from these experiments could serve as a guide for conditions required for obtaining the inoculum and promoting infection (Chapter 4). One of

the major difficulties would be to prevent other diseases that occur under similar conditions as *Stemphylium* spp.

5. Does the complete asexual-sexual cycle of *Stemphylium herbarum* develop on pea plants?

Information about diseases caused by *Stemphylium* spp. in temperate crops indicates that the sexual spores (ascospores) are the primary source of inoculum, for example in early spring (Ligero et al. 1998; Suheri & Price 2001). By providing the appropriate conditions isolate 7 should produce ascospores (Ligero et al. 1998; Gilchrist et al. 1982). These could then be assessed for their ability to infect peas, in a similar way as was tested for conidia (section 5.3.1). This would supplement the disease cycle suggested (Figure 7.1) and clarify if the fungus could overwinter in pea debris, producing ascospores as the source of primary inoculum for susceptible pea or other crops.

6. In New Zealand pea crop/seed systems, can the disease caused by *Stemphylium herbarum* (or other *Stemphylium* spp.) be recognized and, does it represent a significant problem?

Symptoms of *S. herbarum* (isolate 7) in the foliage of young pea plants were characterized under glasshouse conditions (Figure 5.3), and this could be used as a basis for recognition of *S. herbarum* occurrence in the field. Also, field surveys and monitoring health status of pea seed lots could show if the occurrence of *S. herbarum* (and/or the other species) in 2002-2004 was sporadic, or if the fungus is consistently infecting peas each season. Information about the relevance of diseases and the economic impact in the pea seed/crop

production is still limited for important pathogens (Maude 1996) and non existent for *Stemphylium* spp. At the present, it is known that *S. herbarum* can cause lesions on pea foliage, but it is important to quantify any effects on plant and seed production.

7. Would the responses to hot water soak (Chapter 6) be the same using another set of time and temperature combinations or testing seeds with higher germination?

Hot water (50°C for 30 minutes) was successful in eliminating *Stemphylium* spp. (Tables 6.2 and 6.3).However, the effects on the germination percentage were variable, with hot water treatment definitely negatively affecting the seeds. A recent study demonstrated that a hot water soak (52°C for 12 minutes) did not cause significant effect on pea seed germination (88% untreated seeds and 89% after treatment) (Begum et al. 2004). Further studies in controlling *Stemphylium* spp. could be performed using pea seeds with higher germination and vigour than those used in the present experiment, and by testing a larger number of pea seed lots.

8. Is there variation in the susceptibility of different *Stemphylium* species and isolates to the different fungicide active ingredients used as seed dressings?

Fungicides were somehow limited in controlling *Stemphylium* spp. However, due to the limited number of seeds available for these tests, they should be redone with more seeds and using seed lots which carry *Stemphylium* spp. but which also have high ($\geq$ 90%) germination, in order to assess the effects of the seed treatments.It would be also necessary to not only quantify but also to identify the *Stemphylium*

spp. occurring in specific pea seed lots and determine which species are most susceptible to the chemicals registered for pea seed treatment.

8 Conclusions

- *Stemphylium* spp. incidence in the pea seed lots ranged from 0 to a maximum of 46% for the pea seed lots assessed.

- *In vitro* the fungi was present in seeds that produced both normal and abnormal seedlings.

- *Stemphylium* spp. infection was not correlated with germination of the pea seed lots tested.

- In glasshouse conditions, seed borne *Stemphylium* spp. reduced seedling emergence, root length and root weight of a seed lot cv. Rondo 95. No differences were observed in cv. Midichi.

- Cultivation of the nine isolates of *Stemphylium* spp. showed that for most isolates, mycelial growth was promoted on OMA and PSA. Conidial production was achieved only on OMA, MEA and PEA.

- DNA sequencing of the *gpd* gene identified two isolates as *S. astragali*, one isolate as *Stemphylium* spp. close to *S. astragali*, two isolates as belonging to the *S. herbarum / vesicarium / alfalfae* group, and three isolates as *Stemphylium* spp. close to the *S. loti / sarciniforme* group. All these species are pathogenic to legume species.

- Isolate 7 (*S. herbarum*) had the greatest ability to produce conidia under NUV light conditions. This isolate was able to infect pea seedlings through stomata and direct penetration of the conidium germ tube into epidermal cells. Symptoms were subsequently observed in the leaves and stems.

- Hot water treatment completely eliminated *Stemphylium* spp. from pea seeds, but drastically reduced seed germination due to the increase of abnormal seedlings.

- Double rates of the fungicides applied to the seed lot of cv. Meteor did not reduce *Stemphylium* spp. compared with the untreated seeds. The double rates were phytotoxic to seeds, reducing germination by increasing the percentage of abnormal seedlings, and decreasing the length of primary roots. For cv. Midichi, the fungicide Apron completely eliminated *Stemphylium* spp., but germination was significantly reduced.

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"...and some of the seed fell into good soil, where it came up and grew and bore fruit, and the yield was hundredfold (Mark, 4, 1-9)"

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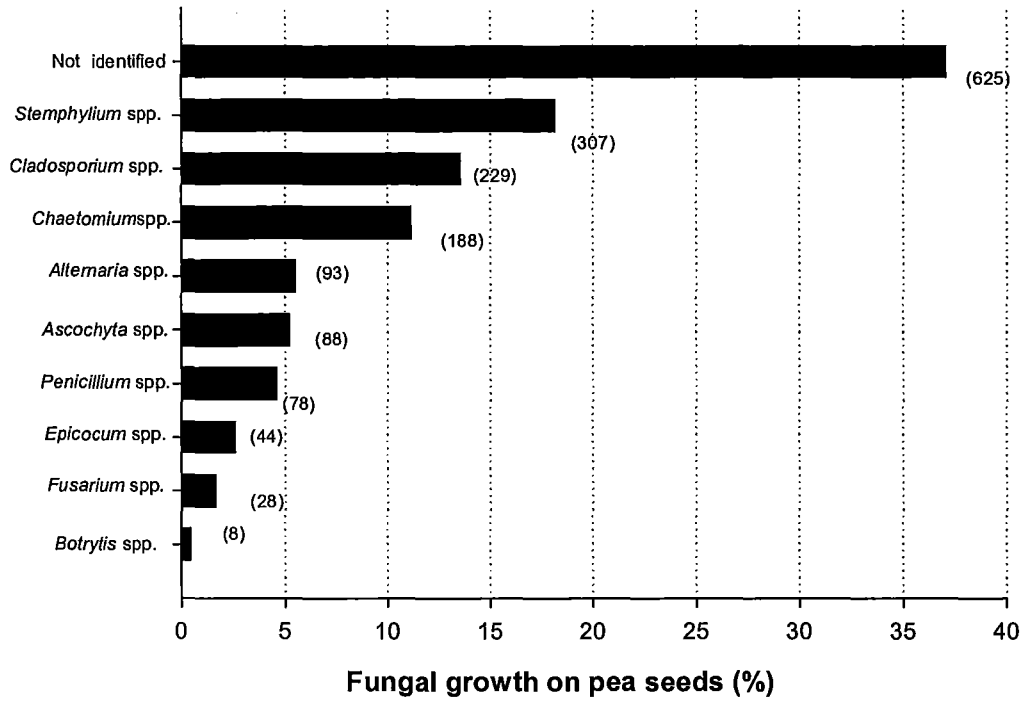
Appendices

Appendix 1 Presence of *Stemphylium* spp. on surface sterilised pea seeds (10 minutes in 1% NaOCl) on MEA (malt extract agar plus 0.1% chloramphenicol).

Plates were incubated at 20°C under 12 hours dark and 12 hours NUV light for 14 days. (a) non infected seeds; (b) seeds with fungal infection. Arrow indicates a *Stemphylium* spp. colony.

(a) non infected seeds **(b) infected seeds**

Appendix 2 Variability of pea seed mycobiota assessed in eighteen pea seed lots after 21 days of incubation on MEA (malt extract agar plus 0.1% chloramphenicol) at 20°C for 21 days under 12 hours dark and 12 hours NUV light.



Genera identified using dichotomous key (Malloch 1981): *Stemphylium* Wallr, *Cladosporium* Link, *Chaetomium* Kunze ex Fries, *Alternaria* Nees ex Wallr, *Ascochyta* spp., *Penicillium* Link, *Epicocum* Link ex Schlect, *Fusarium* Link, *Botrytis* Pers. ex Fries.

Bars represent the percentage of colonies of each genus from 1688 colonies. Values in parentheses indicate the total number of colonies of each genera.

* "Not identified portion" represents genera of fungi for which mycelial growth was observed but the incubation conditions were not adequate to allow identification.

Appendix 3 Seedling establishment (cv. Rondo 95) in glasshouse trial after 30 days.

(a) Non infected seeds

(b) Infected seeds

Appendix 4 Media used for growth of *Stemphylium* spp. isolates.

Potato Dextrose Agar (PDA)

Item	Quantity
Potato Dextrose Agar (Merck; KGaA/Germany)	39 g
Distilled Water	1000 ml

Preparation: PDA powder was mixed with distilled water and made up to 1 litre. This was autoclaved for 15 minutes at 121°C.

Malt Extract agar (MEA)

Item	Quantity
Malt extract Agar (Merck; KGaA/Germany)	48 g
Distilled Water	1000 ml

Preparation: MEA powder was mixed with distilled water and made up to 1 litre. This was autoclaved for 15 minutes at 121°C.

Oat Meal Agar (OMA)

Item	Quantity
Oat meal Flemings "Cremoata" fine ground (14/6/2000)	100 g
Agar (Bacteriological/Germantown)	15g
Distilled Water	600-700 ml

Preparation: The oat meal fine ground was boiled for 30 minutes after which the mixture was sieved, 15 g of agar added and made up to 1 litre with distilled water. This was then autoclaved for 15 minutes at 121°C.

Appendix 4 Media used for growth of *Stemphylium* spp. isolates (continued).

Pea Seed Extract Agar (PSA)

Item	Quantity
Pea seed extract	400 ml
Agar (Bacteriological/Germantown)	15g
Distilled Water	855 ml

Preparation: Pea seed extract was prepared by soaking 100 g of pea seeds in 300 ml water overnight. The seeds were boiled for 20 minutes after which the excess water was removed with a filter. The seeds were then ground with a common blender to a particle size of less than 3 mm. To this, 15 g of agar was added and made up to 1 litre with distilled water. The mixture was then autoclaved for 15 minutes at 121°C.

Prune Extract Agar (PEA)

Item	Quantity
Sucrose (Merck; KGaA/Germany)	5 g
Yeast extract	1 g
Agar (Bacteriological/Germantown)	30g
Prune extract	100 ml
Distilled Water	900 ml

Preparation: Prune extract was prepared by destoning and chopping 50 g prunes and adding 100ml of distilled water. The mixture was boiled for 30 minutes and filtered through a Whatman n.3 filter paper and then through a Whatman n.1 filter paper. Aliquots of the extract were stored at 5°C until use. For the agar, all the ingredients were mixed and boiled to dissolve after which they were autoclaved for 15 min at 121°C.
Source: Plant Diagnostic Laboratory -BioLinc (2003).

Appendix 5 Colour and shape of nine *Stemphylium* isolate colonies on MEA, OMA, PDA, PSA and PEA.

Isolate	Colour of colony surface *	Colour of colony base*	Texture	Shape**
MEA				
1	LB	YB and DB	downy	C
2	OG and G	BO and DB	cottony	I
3	OG	O	downy	C
4	Bo	DB	downy	C
5	OG and G	DB	cottony	I
6	OG and G	BO	cottony	I
7	GB	DB	downy	C
8	W and OG	GY	downy	C
9	W (margin) and GO	GY	downy	C
OMA				
1	Yb in the centre and W margin	O B and YG	downy	I
2	Lb (centre) W margin	BY	downy	C
3	W	BY and GY	cottony	C
4	a mix W and b	BY and b(1)	downy	C
5	OG and G	BY	downy	C
6	bO (centre) and W (margin)	BY	downy	C
7	W and B	BY and b(1)	downy	C
8	OB and w	BY and b (1)	cottony	C
9	W	GO	cottony	C

* See Key of colour in Appendix 6

** C= circular; I=irregular

Appendix 5 Colour and shape of nine *Stemphylium* isolate colonies on MEA, OMA, PDA, PSA and PEA (continued).

Isolate	Colour of colony surface *	Colour of colony base*	Texture	Shape**
PDA				
1	bo (centre) and W(margin)	bo (in the centre) and W (margin)	effuse	C
2	bo	DB and DO	downy	I
3	GO centre and margins and lb (intermediate area)	DB(centre) ; Ob and GO (margins)	downy (presenting concentric rings in obverse view)	C
4	BO (1) and lb	DB and BO (1)	downy	C
5	Bo(1) in the centre and LO	bO (1)	effuse	I
6	bo	DB (centre) and LB (1)	effuse (rings visible in obverse view)	C
7	B(1)	DB and B (2)	downy	C
8	Bo (1)	DB	downy	C
9	Bo (1)	DB (centre) ; BY margins and Go	downy (forming rings visible in obverse view)	C
PSA				
1	W	go	downy	I
2	OG	Y B	downy	C
3	W	BY	downy	C
4	W with patches of BY and DB	Db	downy	C
5	W and OG	Db	downy	C
6	PO	Db	downy	C
7	bO to Lb (1)	Db	effuse	C
8	W	Db	downy	
9	W	BY	downy	C

* See Key of colour in Appendix 6

** C= circular; I=irregular

Appendix 5 Colour and shape of nine *Stemphylium* isolate colonies on MEA, OMA, PDA, PSA and PEA (continued).

Isolate	Colour of colony surface*	Colour of colony bottom*	Texture	Shape**
PEA				
1	OG and W	Go	effuse	C
2	OG and W	Lb	downy	C
3	OG	GO (1)	downy (similar to 2)	C
4	yb (centre) and W (margins)	Db	effuse (fruiting bodies forming rings visible in obverse view)	C
5	bO	Y B (centre) and bo	downy	C
6	bO	Go	downy	C
7	YB (1)	YB (1)	effuse with visible concentric rings in obverse view	C
8	bo (1)	PO and GO	downy	C
9	OW	Lo	downy	C

* See Key of colour in Appendix 6
** C= circular; I=irregular

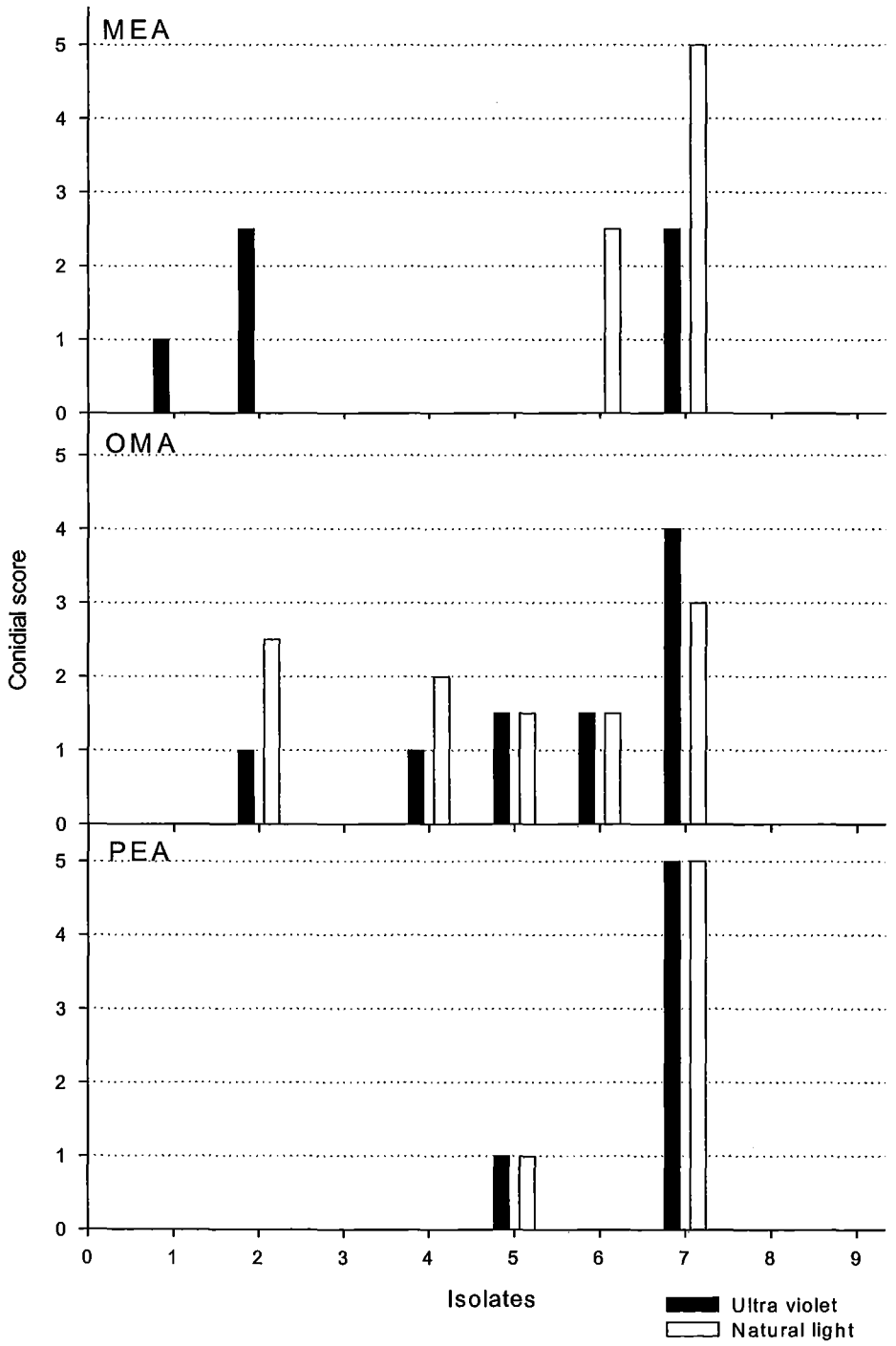
Appendix 6 Key of colours based on Kornerup and Wanscher (1968).

Symbol	Colour name	Code *	Plate**	Symbol	Colour name	Code *	Plate**
B	brown	F7	5	GO (1)	greyish orange	B5	5
b	brown	F4	5	GY	golden yellow	B7	5
b(1)	brown	F5	5	LB	light brown	D4	5
B (1)	brown	E8	5	Lb	light brown	D6	5
B(2)	brown	E6	5	lb	light brown	D7	5
BO	brownish orange	C8	6	LB (1)	light brown	D8	5
Bo	brownish orange	C3	6	Lb (1)	light brown	D5	5
bO	brownish orange	C5	5	LO	light orange	B4	5
bo	brownish orange	C6	5	Lo	Light orange	A4	5
BO (1)	brownish orange	D5	5	O	orange	B8	6
Bo (1)	brownish orange	C4	5	OG	orange grey	B2	5
bO (1)	brownish orange	C7	6	OW	orange white	A2	5
bo (1)	brownish orange	C3	5	OB	olive brown	F4	4
BY	brownish orange	C7	5	Ob	olive brown	F5	4
DB	dark brown	F8	5	PO	pale orange	B3	5
Db	dark brown	F7	5	W	white	A1	5
DO	deep orange	B8	5	Y B	yellowish brow	E8	5
G	grey	B1	5	Yb	yellowish brown	E4	5
GB	greyish brown	D3	5	yb	yellowish brown	F6	5
GO	greyish orange	B3	5	YB (1)	yellowish brown	E6	5
Go	greyish orange	B4	5	YG	yellowish grey	B2	4
go	greyish orange	B6	5				

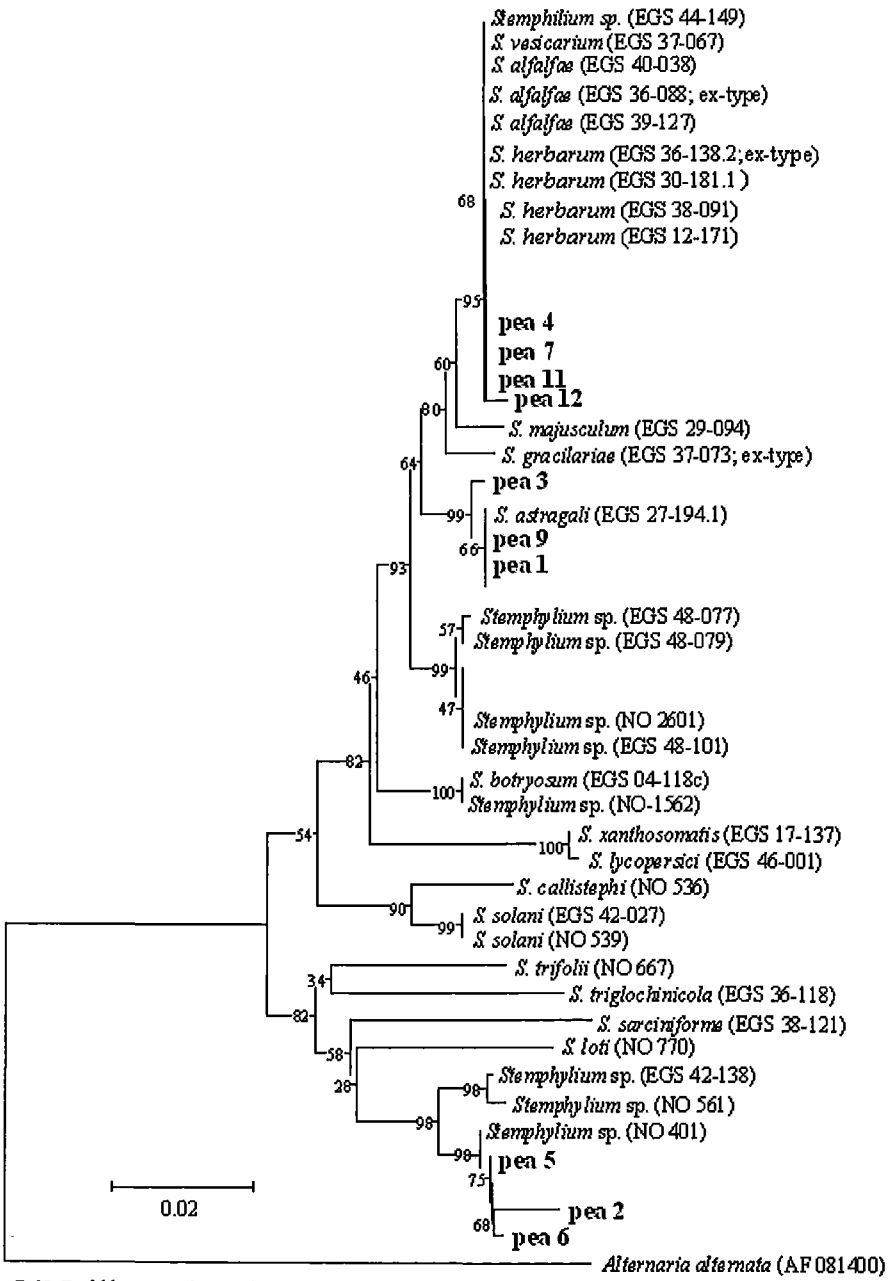
*Code: refers to the system of colour identification in Kornerup and Wanscher (1968).

** Plate: according to the colour samples reference in the Colour Dictionary (Korneup & Wanscher 1967).

Appendix 7 Score of conidial production (0= absent; 5= abundant) of *Stemphylium* spp. isolates after exposure under natural light and NUV light on three different media (MEA, OMA and PEA).



Appendix 8 Phylogenetic relationships of *Stemphylium* species and strains inferred from DNA sequencing *gpd* (glyceraldehyde phosphate dehydrogenase) locus. The phylogenetic tree was generated after distance analysis and bootstrap values (> 50%) from 1000 replicates are indicated at the branches.



Source: O'Neill, Molecular Plant Pathology Laboratory, USDA/ARS, Beltsville, MD, USA.

Appendix 8 Phylogenetic relationships of *Stemphylium* species and strains inferred from DNA sequencing *gpd* (glyceraldehyde phosphate dehydrogenase) locus. The phylogenetic tree was generated after distance analysis and bootstrap values (> 50%) from 1000 replicates are indicated at the branches (continued).

The reliability of phylogenetic relationships is commonly assessed by performing a bootstrap analysis. Bootstrap analysis serves to test the robustness of the sequence relationships as if scanning along the alignment. The bootstrap proportions show how many times given branches come from a given node, and are then interpreted as confidence levels. Normally, values above 50% are regarded as significant (Emelyanov 2003). In the tree provided by the Molecular Plant Pathology Laboratory, USDA/ARS isolates 1 and 9 that had similar morphology had also similar *gpd* sequences and formed a group (bootstrap value 66%) with *S. astragali.* Isolate 3 was also close related with this group (99%) however, its location in the phylogenetic tree suggests slight differences from isolates 1 and 9. Isolates 2, 5 and 6 were grouped together (bootstrap value 75%) forming a branch of *Stemphylium* spp. related to *S. loti* (28%) and *S. sarciniforme.*

Isolates 4 and 7 were included in the same branch (95%) with S. *vesicarium, S. alfalfae* and *S. herbarum* and one *Stemphylium* sp. (isolate EGS 44 149). Despite the fact that they were included in the group with several species, the location on the tree suggests that these isolates are more closely related to *S. herbarum* (68%) than to the other species. Isolates 11 and 12 are in fact identical to isolate 7. However, they were re isolate from pea leaves after inoculation with conidial suspension (section 5.3.4). The DNA sequencing of the gene encoding *gpd* confirmed that the symptoms observed on pea plants were caused by *S. herbarum* (isolate 7 = 11 = 12).

Appendix 9 List of isolates used in the phylogenetic analysis of *Stemphylium* species and strains.

Species	Isolate designation	Host	Geographic region	Collector or Contributor
S. astragali	Pea 1	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
Stemphylium sp. (close to *S. loti/sarciniforme* group)	Pea 2	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
S. astragali	Pea 3	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
S. herbarum	Pea 4	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
Stemphylium sp. (close to *S. loti/sarciniforme* group)	Pea 5	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
Stemphylium sp. (close to *S. loti/sarciniforme* group)	Pea 6	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
S. herbarum	Pea 7	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
S. astragali	Pea 9	*Pisum sativum*	Canterbury New Zealand	K.D.R. Wadia
S. herbarum	Pea 11	*Pisum sativum*	Canterbury New Zealand	Carmen Teixeira
S. herbarum	Pea 12	*Pisum sativum*	Canterbury New Zealand	Carmen Teixeira
Stemphylium sp.	EGS 44-149	*Malus* sp.	Hastingo, New Zealand	H.M. Dance
S. vesicarium	EGS 37-067	*Medicago* sp.	South Africa	E.Simmons

Source: Camara et al. (2002); O'Neill (unpublished).
EGS: Emory Simmons Culture Collection; NO= Nichole O' Neill Culture Collection, Beltsville.
- : unknown

Appendix 9 List of isolates used in the phylogenetic analysis of *Stemphylium* species and strains (continued).

Species	Isolate designation	Host	Geographic region	Collector or Contributor
S. alfalfae	EGS 40-038	*Medicago sativa*	Kansas,USA	C. Chairisook
S. alfalfae	EGS 36-088	*Medicago sativa*	Australia	E.Simmons
S.alfalfae	EGS 39-127	*Medicago sativa*	California, USA	E.Simmons
S. herbarum	EGS 36-138.2	*Medicago sativa*	India	H. Joshi
S. herbarum	EGS 30-181.1	*Medicago sativa*	Palmerston North, New Zealand	K.S. Milne
Stemphylium sp.	EGS 38-091	*Lens culinaris*	Washington, USA	E.Simmons
S. vesicarium	EGS 12-171	*Pisum sativum*	Canada	J.W.Groves
S. majusculum	EGS 29-094	*Lathrynus maritimus*	New York, USA	C. T. Rogerson
S. gracilariae	EGS 37-073	*Gracilaria* sp.	Israel	S. Schatz
S. astragali	EGS 27-194.1	*Astragalus sinicus*	Tokushima, Japan	T. Tominaga
Stemphylium sp.	EGS 48-077	*Medicago sativa*	Auckland, New Zealand	H.M. Dance
Stemphylium sp.	EGS 48-079	*Medicago sativa*	Auckland, New Zealand	H.M. Dance
Stemphylium sp.	NO 2601	*Clematis* sp.	Netherlands	N. O'Neill

Source: Camara et al. (2002); O'Neill (unpublished).
EGS: Emory Simmons Culture Collection; NO= Nichole O' Neill Culture Collection, Beltsville.
- : unknown

Appendix 9 List of isolates used in the phylogenetic analysis of *Stemphylium* species and strains (continued).

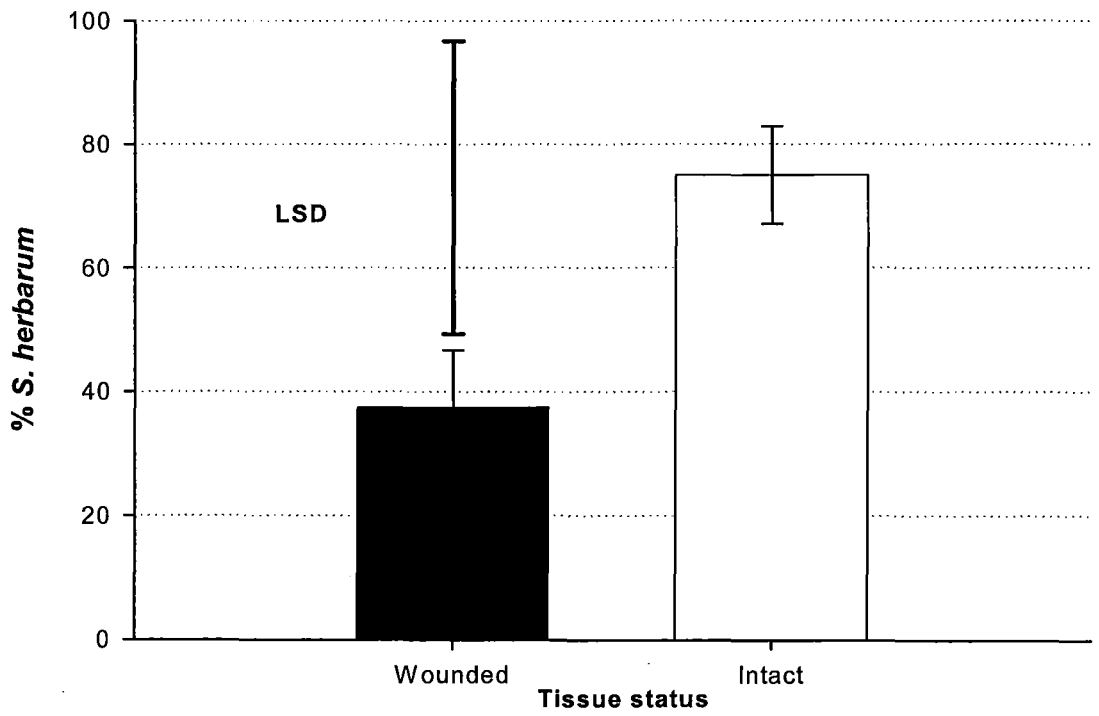
Species	Isolate designation	Host	Geographic region	Collector or Contributor
Stemphylium sp.	EGS 48-101	-	-	E.Simmons
S. botryosum	EGS 04-118c	*Medicago sativa*	Ontario, Canada	W. Benedict
Stemphylium sp.	NO 1562	*Medicago sativa*	California, USA	E.Simmons
S. xanthosomatis	EGS 17-137	*Xanthosoma sagittifolium*	New Caledonia	B. Huguenin
S. lycopersici	EGS 46-001	*Lycopersicon esculentum*	Dominican Republic	B. Pryor
S. calistephi	NO 536	*Calistephus chinensis*	California	K. Baker
S. solani	EGS 42-027	*L. esculentum*	Indiana, USA	E. Simmons
S. solani	NO 539	*Lupinus sp.*	Georgia, USA	H. D. Wells
S. trifolli	NO 667	*Trifolium repens*	USA	-
S. triglochinicola	EGS 36-118	*Triglochin maritima*	Devon, UK	J. Webster
S. sarciniforme	EGS 38-121	*Trifolium pratense*	Massachusetts	E. Simmons
S. loti	NO 770	*Trifolium pratense*	New York, USA	S. Braverman
Stemphylium sp.	EGS 42-138	*Malus silvestris*	Western Australia	C. Robertson
Stemphylium sp.	NO 561	*Trifolium repens*	New Jersey, USA	E. Simmons
Stemphylium sp.	NO 401	*Spinacia oleracea*	California, USA	E. Simmons

Source: Camara et al. (2002); O'Neill (unpublished).
EGS: Emory Simmons Culture Collection; NO= Nichole O' Neill Culture Collection, Beltsville.
- : unknown

Appendix 10 Percentage of *S. herbarum* recovered from pea leaves artificially inoculated with *S. herbarum* conidia.

White column represents re-isolation from non wounded leaves and black column from wounded tissue.

Bars on columns represent one standard error. LSD= 47.4 ($P<0.05$) is represented by bar outside the columns.



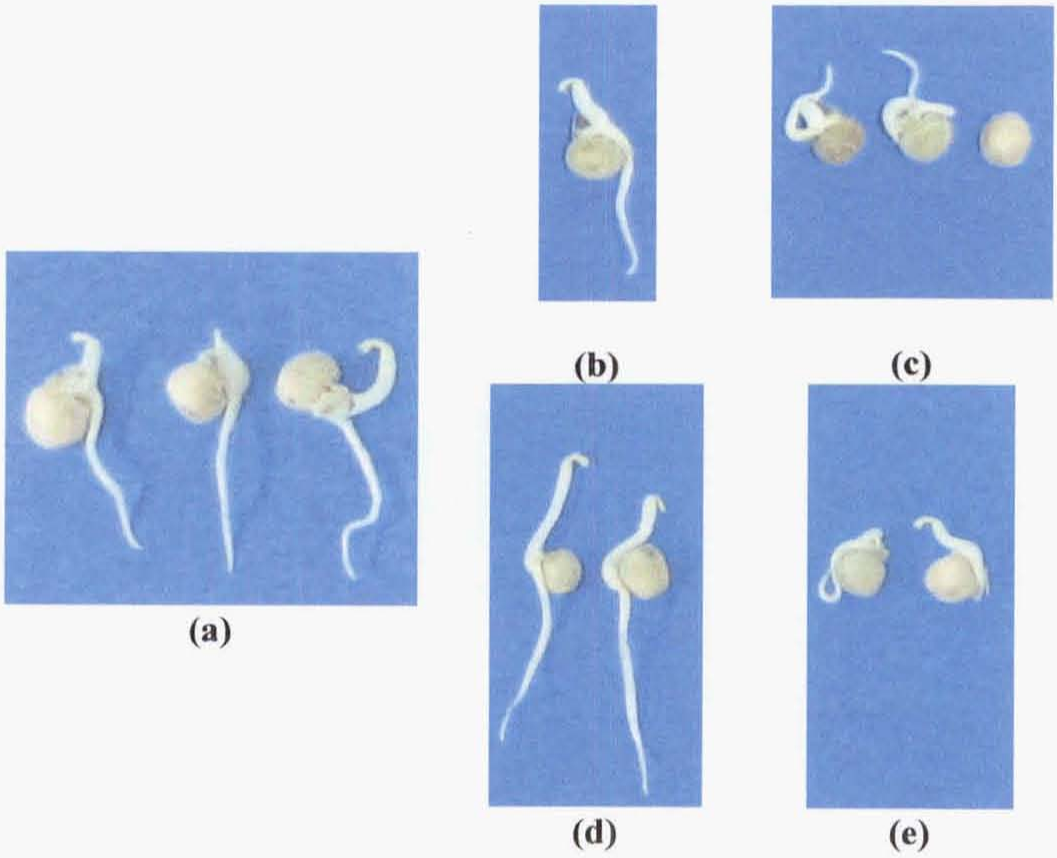
Appendix 11 Inoculation and incubation of pea plants during pathogenicity studies.

(a)Plants covered by plastic bags (96 hours incubation period).
(b)Water drops on the pea seedling leaves.

(a) **(b)**

Appendix 12 Aspects of seedlings of cultivar Meteor.

(a) Normal seedlings from untreated seeds (control).
(b) Normal seedling from seeds treated with hot water;
(c) Seedling abnormalities caused by hot water treatment.
(d) Normal seedlings treated with Aliette;
(e) Abnormal seedlings treated with double rate of Aliette.



Appendix 13 Aspects of seedlings of cultivar Midichi.

(a) Normal seedlings from untreated seeds (control).
(b) Normal seedling from seeds treated with hot water;
(c) Aspect of seedling abnormalities caused by hot water treatment.
(d) Normal seedlings treated with Aliette ;
(e) Abnormal seedlings treated with Aliette.
(f) Normal seedlings treated with Apron;
(g) Abnormal seedlings treated with Apron;
(h) Normal seedling treated with Wakil;
(i) Abnormal seedlings treated with Wakil.