Studies on biological control of *Chenopodium album* by *Ascochyta caulina*

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Studies on biological control of *Chenopodium album* by *Ascochyta caulina*

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STELLINGEN

1. De conclusie dat de schimmel *Ascochyta caulina* weinig perspectief biedt bij de bestrijding van het onkruid *Chenopodium album* in de Westeuropese akkerbouw (Eggers & Thun, 1988. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 11: 225-237) is onjuist.

2. Het toedienen van een sublethale dosis van een herbicide aan een plant verlaagt het afweermechanisme van de plant tegen schimmelaantasting (Sharon *et al.*, 1992. Plant Physiology 98: 654-659).

3. Het aanbrengen van bacteriën in substomatale holtes van een waardplant (stomatale infiltratie) verhoogt de kans op infectie (Zidaek and Backman, 1991. Phytopathology 80: 1016). Dit principe geldt waarschijnlijk ook voor schimmels en biedt mogelijkheden voor verhoging van de effectiviteit van mycoherbiciden.

4. De vergelijking van Bastiaans om de relatie tussen aantasting van een rijstblad en bladfotosynthese te beschrijven (Bastiaans, 1991. Phytopathology 81: 611-615) is alleen bruikbaar bij een lage aantastingsgraad.

5. De planteresten van groenbemesters die in de bouwvoor worden ingewerkt kunnen gebruikt worden ter vermeerdering van micro-organismen die selectief onkruiden of gewasbeschadigers aantasten.

6. Bij de teelt van aardappelen biedt rassenkeuze een mogelijkheid om de kans op doorwas te verkleinen (Beukema & van der Zaag, 1990. Introduction to potato production).

7. Publiceren over een potentieel bestrijdingsmiddel zonder vooraf een patentaanvraag ingediend te hebben verkleint de kans op vermarkten van het bestrijdingsmiddel; geheimhouding echter staat op gespannen voet met de openbaarheid van onderzoeksresultaten behaald met gemeenschapsgelden.

8. Het huidige tekort aan menselijke organen voor transplantatie komt vooral door de hoge kosten die daar mee gemoeid zijn en in mindere mate door een tekort aan donoren.

9. Gezien het besteedbaar inkomen per huishouden (Statistisch Jaarboek 1995, Centraal Bureau voor de Statistiek) wordt de provincie Flevoland ten onrechte door de Europese Unie beschouwd als een achtergebleven gebied.

10. Natuur in Nederland is herkenbaar aan een bezoekerscentrum.

Stellingen behorend bij het proefschrift van C. Kempenaar:
'Studies on biological control of *Chenopodium album* by *Ascochyta caulina*'.

Wageningen, 26 juni 1995.

AUTHOR'S ABSTRACT

Studies were carried out to assess the perspectives of the plant pathogenic fungus *Ascochyta caulina* (P. Karst) v.d. Aa & v. Kest. as a mycoherbicide against *Chenopodium album* L., world-wide a weed in arable crops. *A. caulina* is associated with necrosis of leaves and stems of plants belonging to genera of *Chenopodium* L. and *Atriplex* L. The studies were focused on recognition of factors that may limit disease development and on relationships between disease development and control. Three control strategies of *C. album* were studied: application of *A. caulina* to the soil, to young plants and to flowering plants. Disease development of plants after the application of the fungus was quantified for a broad range of conditions. Relationships between disease development and control were demonstrated. The perspectives of *A. caulina* as a mycoherbicide against *C. album* were discussed. It was concluded that the fungus has a good potential to be further developed into a mycoherbicide against the weed.

Additional index words: weed, weed control, biological weed control, mycoherbicide, disease, disease assessment, necrosis, photosynthesis, perthotrophic, annual plant, host specificity, pycnidiospore, competition, propagation, life cycle.

Pictures on the cover:
Maize and *Chenopodium album* in plots of experiment '93M 40 days after treatment. The picture at the top represents a plot of treatment S0 (control treatment), the picture at the bottom a plot of treatment S1 (treated with *Ascochyta caulina*). Treatments are further explained in Chapter 6.

Foto's op de omslag:
Maïs en melganzevoet in veldjes van experiment '93M, 40 dagen na bespuiting met een sporenvrije oplossing (bovenste foto) of een sporensuspensie van *Ascochyta caulina* (onderste foto).

CONTENTS

GENERAL INTRODUCTION

Context

Weed control has always been a concern of man. Through the ages he has developed various mechanical, cultural, biological and chemical methods to control weeds. During the past 50 years, the use of chemical methods has increased rapidly, mainly because these methods were effective and relatively inexpensive. However, man has become aware of negative side-effects of intensive use of chemical methods. This awareness resulted in a shift in the approach of crop protection. It was decided by governments represented in the United Nations that Integrated Pest Management (IPM) must be the standard in crop protection (UNCED, 1992). IPM is the control of harmful organisms in crops utilizing all suited technology and methods that keep populations of the harmful organisms below the economic threshold levels in an environmentally sound way (*e.g.* Oudejans, 1991). The Dutch government decided that the use of and the dependency on chemical methods must be reduced (MJP-G, 1991). To reach this goal, alternative methods to control weeds, pests and diseases have to be further developed. The research in the present thesis fits in that context.

Biological weed control

The term weed has been used to indicate a harmful plant species as well as a harmful vegetation of several plant species (Van der Zweep, 1979). In the present thesis a weed is defined as a plant species of which individuals often occur at localities where they interfere with the objective of the manager. A weed population is a group of individuals of the weed, occupying a particular area (Odum, 1971). A weed population at a specific locality may be characterized by its density, expressed as the number of individuals or the amount of biomass per unit area. The density of a weed population at a specific locality will be between zero and a level at which all resources are utilized by the individuals of the species, the carrying capacity. With time, the density of a weed population at a specific locality will vary as it is affected by natural control and control carried out by man. Natural control is the maintenance of population density within certain limits by the action of the whole environment, including both stochastic and density-dependent (regulation) elements (Huffaker *et al.*, 1971; Frantzen, 1994). Control carried out by man is any deliberate action to reduce the population density of a weed.

Biological weed control is an approach utilizing living organisms to reduce the population density of a weed or of several weeds at a specific locality. Biological weed control may

be selective or non-selective, depending on the control organism utilized. Principles and strategies of biological weed control were reviewed by *e.g.* Huffaker (1970) and Wapshere *et al.* (1989). Biological weed control may be categorized according to the way the biological control organism harms the weed population: by feeding on the weed, by being competitive to the weed or by being pathogenic to the weed. The use of endemic, plant pathogenic fungi as mycoherbicides to control weeds has received much attention in recent years.

The mycoherbicide approach

The use of plant pathogenic fungi as mycoherbicides is a specific approach to biological weed control. The potential of this approach was demonstrated some 20 years ago by Daniel *et al.* (1973) for the control of the weed *Aeschynomene virginica* L. in rice crops. Plants of the weed were controlled by spray application of a high density of conidia of the fungus *Colletotrichum gloeosporioides* (Penz) Sacc. f. sp. *aeschynomene*. The mycoherbicide approach may be defined as the use of a plant pathogenic, endemic fungus in an inundative strategy to reduce the population density of a weed at a specific locality (Templeton, 1979; Charudattan, 1991). Mycoherbicides are mass-cultured, and applied periodically, just as herbicides.

Success of a mycoherbicide approach is determined by scientific, technical, economical and practical factors. Among these, the amount of disease that can be incited within the weed population targeted is a key factor. A disease is any disorder of a living organism, or part of a living organism, that is harmful to the organism. Diseases caused by fungi are characterized by symptoms such as blights, rots, wilts and anthracnoses, or by causal organisms such as rusts, mildews and smuts. The amount (or level) of disease at a specific moment may be quantified by severity (amount of disease per unit area of a plant) or incidence (frequency of diseased plants). The harmful effect of a disease on an individual plant may vary from a small reduction of the vigour of the plant to death of the plant, depending on the type of symptoms, time of onset of disease and subsequent disease development (course of amount of disease with time). Disease development may be quantified by the integral of the curve describing the course of amount of disease with time. The harmful effect of a disease on a weed population is the sum of the effects of the disease on the individual plants. Holocomb (1982) listed factors that may influence disease development (Table 1.1). The importance of the factors mentioned differs with each pathosystem.

Table 1.1. Factors that may influence disease development, after Holocomb (1982)

Host-related factors

1. Degree of host resistance or susceptibility to a pathogen
2. Physical and physiological barriers to infection in the host
3. Abundance and distribution of the host
4. Age and vigour of the host
15. Predisposition of host by environmental factors
6. Host effects on environment
7. Diversity in host's genetic base

Pathogen-related factors

1. Host range
2. Virulence
3. Types and numbers of propagules produced by the pathogen
4. Mechanisms for genetic variability of the pathogen
5. Vector relationships and other means of pathogen dissemination
6. Means of pathogen survival during a host-free period

Environmental factors that affect host and pathogen

1. Temperature
2. Moisture (precipitation, relative humidity, dew)
3. Other weather conditions (*e.g.* wind, radiation)
4. Form and availability of nutrients
5. Oxygen, carbon dioxide, pH

Time factor

1. Duration as it affects sequences and interactions

Management practices that affect host, pathogen and environment

Objectives

World-wide three mycoherbicides are now available on a commercial basis, and five mycoherbicides on a non-profit basis (Powell and Jutsum, 1993; Scheepens and Lotz, 1994). In the forthcoming years, several more mycoherbicides may be marketed in view of their stage of development. In Europe the first mycoherbicide has now past registration. This mycoherbicide was developed for the control of *Prunus serotina* Erhr. in Dutch forests using the fungus *Chondrostereum purpureum* Pers. ex Fr. (De Jong *et al.*, 1990). The aim of the present thesis was to assess the perspectives of the fungus *Ascochyta caulina* (P. Karst) v.d. Aa & v. Kest. as a mycoherbicide against *Chenopodium album* L., world-wide a weed in arable crops. The research was focused on recognition of factors that may limit disease development (Table 1.1) and on relationships between disease development and control.

Manipulation of a tri-partite system

The annual plant species *Chenopodium album* L. is a common weed world-wide (*e.g.* Holm *et al.*, 1977, Van den Brand, 1985). Trivial names are common lambsquarters, fat hen, or melganzevoet (Dutch). Propagation is always from seeds, which may survive in soil for many years. There is no special seed dispersal mechanism. *C. album* exhibit great plasticity to the environment. In crops *C. album* plants may reduce yields by competition depending on characteristics of the crop and the weed population, densities and arrangements of plants, relative emergence dates and environmental conditions (*e.g.* Zimdahl, 1980). The life cycle of *C. album* is completed in about 4 months, but it varies with the photoperiod of the season and the location. The life cycle is depicted in Fig 1.1.
The perthotrophic fungal species *Ascochyta caulina* (P. Karst.) v.d. Aa and v. Kest. is associated with necrosis of leaves and stems of various *Chenopodium* L. and *Atriplex* L. species (Van der Aa and Van Kesteren, 1979). It is endemic in Europe and Siberia. Unfortunately, the life cycle of *A. caulina* is known only in part. A saprophytic phase in the life cycle is expected. *A. caulina* reproduces asexually by the formation of pycnidiospores, which may be splash-dispersed during rain showers. The asexual life cycle can be completed within a week. Sexual reproduction of *A. caulina* has not been observed (yet).
Considering the life cycles of both the weed and the fungus, control of *C. album* in crops may be achieved by the application of pycnidiospores of *A. caulina* to young plants. The competitive ability and the reproductive capacity of the plants will be reduced depending on the amount of necrosis that can be incited within a certain period of time. Considering life cycles of related *Ascochyta* Lib. species (Maden *et al.*, 1975; Hagedorn, 1984; Gossen and Morrall, 1986), the application of pycnidiospores of *A. caulina* to the soil and to flowering plants may also be a way to control *C. album*.

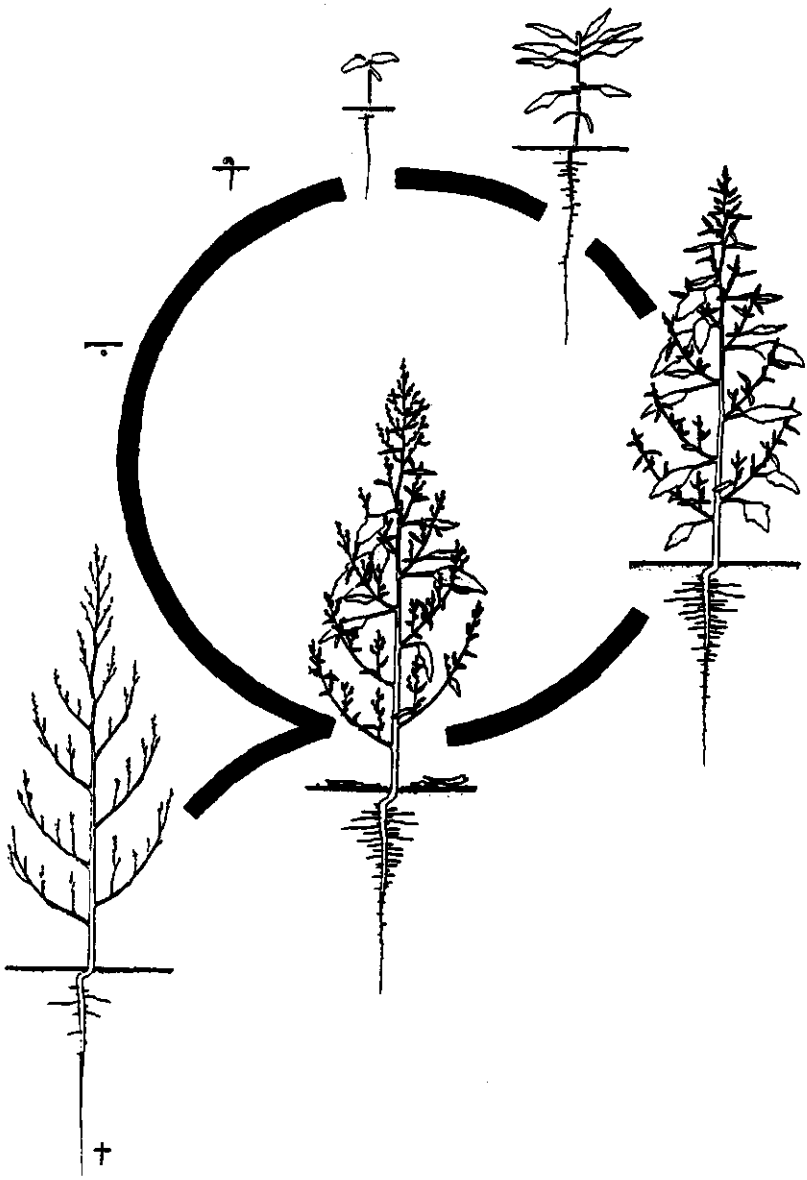

Fig 1.1. Life cycle of *Chenopodium album* L.

Outline

Chapters 2 and 3 of this thesis deal with methodology. Production of pycnidiospores of *A. caulina* on artificial media is described in Chapter 2, followed by a description of a method to obtain uniform emergence of weeds (Chapter 3). A study on factors that may influence disease development of young *C. album* plants after application of *A. caulina* is described in Chapter 4. The effect of leaf necrosis caused by *A. caulina* on photosynthesis of *C. album* leaves is described in Chapter 5. Chapters 6 through 8 deal with control of *C. album*. Control of *C. album* in crops by application of *A. caulina* to young plants is described in Chapter 6. Control of *C. album* by pre-emergence application of *A. caulina* to the soil is described in Chapter 7. Experiments on propagation of *C. album* after application of *A. caulina* to flowering plants is described in Chapter 8. Results are integrated and discussed in Chapter 9.

CHAPTER 2

PRODUCTION AND STORAGE OF PYCNIDIOSPORES OF *ASCOCHYTA CAULINA*, A POTENTIAL MYCOHERBICIDE AGAINST *CHENOPODIUM ALBUM*

C. Kempenaar, P.J.F.M. Horsten and P.C. Scheepens

ABSTRACT

Experiments were carried out to determine the effect of age of cultures of *A. caulina* on production and germination capacity of pycnidiospores of the fungus. Cultures of *A. caulina* were grown on an oat meal agar in small Petri dishes (Ø 9 cm; height 1.3 cm) or a wheat bran medium in larger Petri dishes (Ø 20 cm; height 4 cm). The media were inoculated with a suspension of pycnidiospores of *A. caulina*. At different dates after inoculation, cultures were assessed on the number of pycnidiospores produced. Harvested pycnidiospores were plated onto water agar in Petri dishes and assessed on germination. The course of spore production with time could be described by a Gompertz model. Under growth conditions of 20 °C and continuous light, maximum numbers of spores produced on cultures were reached within 14 days after inoculation of the media. A negative relationship was demonstrated between the age of cultures and spore germination. Addition of nutrients to suspensions of harvested pycnidiospores restored germination at high levels, even when pycnidiospores were stored for longer than a year.

INTRODUCTION

Ascochyta caulina (P. Karst.) v.d. Aa & v. Kest. is a plantpathogenic fungus that causes necrotic spots on leaves and stems of plants of *Chenopodium* L. and *Atriplex* L. species (Van der Aa and Van Kesteren, 1979). The fungus reproduces asexually by production of pycnidiospores in pycnidia. Scheepens (1979) suggested that pycnidiospores of *A. caulina* (P. Karst.) v.d. Aa & v. Kest. could be used as a mycoherbicide to control *Chenopodium album* L, world-wide a common weed. To test control of *C. album* by application of pycnidiospores of *A. caulina*, large numbers of spores with predictable germination have to be produced. Several artificial media and production methods can be used for this purpose (Churchill 1982). In general, liquid and solid media are distinguished. We observed that solid media were better suited for production of pycnidiospores of *A. caulina* than liquid media (Horsten and Kempenaar, 1994). Rao and Haware (1991) drew the same conclusion for *Ascochyta rabeie* (Pass.) Lab. This *Ascochyta* species also produced more pycnidiospores on solid media than in liquid media.

In this paper we describe a study on production, storage and germination of pycnidiospores of *A. caulina*. Further on, the term spore(s) is used to indicate pycnidiospore(s). Spores were grown in small fermentation units on an oat meal agar medium or a wheat bran medium. The effect of age of cultures on the number of spores produced and on germination of harvested spores was determined.

MATERIALS AND METHODS

Seed inoculum preparation. A. caulina was isolated from a leaf of a *C. album* plant found in an arable field at Wageningen in 1990. The isolate was axenically transferred to oat meal agar slants in tests tubes. The tubes were kept at room temperature (18-24 °C) till some mycelium had appeared, and subsequently stored at 5 °C in the dark. Oat meal agar was made by suspending 60 g oat meal (Quaker Oats B.V., The Netherlands) in 1 l demineralized hot water. The suspension was heated to 100 °C and subsequently strained over a mesh wire with holes of 2 by 2 mm. Finally, 15 g agar (Oxoid Agar Bacteriological, Unipath LTD, England) was added to 1 l oat meal suspension. The oat meal agar was sterilized at 120 °C for 30 min.

Each spore production experiment was started with a standardized procedure. Some fungal mycelium was taken from stock cultures stored at 5 °C, and axenically transferred to oat meal agar in plastic Petri dishes (Ø 9 cm). The Petri dishes were placed in a climate cabinet at 20 °C in continuous light (75 $\mu mol.m^{-2}.s^{-1}$; Philips TL 13W/83). The growth conditions were considered optimal for production of spores of *A. caulina* (Horsten and Kempenaar, 1994). After an incubation period of circa 3 weeks, cultures on the oat meal agar were flooded with 10 ml sterile demineralized water per Petri dish. After 3 h, supernatants with suspended spores were poured into a flask. Three h of inundation of the fungal cultures were sufficient to release most of the spores from the pycnidia (Horsten and Kempenaar, 1994). Spore density of the collected suspension was determined by means of a haemocytometer under a light microscope (Tuite, 1969). The suspension was diluted to 10^6 $spores.ml^{-1}$, and used as seed inoculum.

Production of spores on oat meal agar. In experiments 1 and 2, production of spores on oat meal agar in plastic Petri dishes (Ø 9 cm; height 1.3 cm) and on germination of harvested spores were studied. In experiment 1, twenty-five Petri dishes with 40 ml oat meal agar each, were inoculated by spreading 1 ml seed inoculum over the surface of each plate using a Drigalski spatula. The Petri dishes were placed in the climate cabinet (continuous light and 20 °C). Five Petri dishes were randomly taken from the climate chamber at different intervals within a period of a month. The cultures in the Petri dishes were flooded with 10 ml distilled water with 0.05% Tween 80 (v/v) surfactant per Petri dish. The Petri dishes were regularly shaken. Supernatants with suspended spores were poured into test tubes after 3 h. Volume and spore density of the collected spore suspensions were determined to calculate the number of spores produced per Petri dish. Samples of 1 ml were taken from each spore suspension, and spread over the surfaces of 1% (w/v) water agar plates in Petri dishes (Ø 9 cm). The Petri dishes were placed in the

climate cabinet (continuous light and 20 °C) for 20 h. Proportions of germinated spores were determined by observing 200 spores per Petri dish under a light microscope. A spore was considered germinated when the germination tube was longer than the width (circa 5 µm) of the spore.
Experiment 2 was a replication of experiment 1, but we added a second experimental factor to the experiment. Each collected spore suspension was divided into two equal parts. Three and a half g Czapek-Dox broth.l^{-1} spore suspension and 0.4 g yeast extract.l^{-1} spore suspension (media of Difco, USA and Merck, Germany, respectively) were added to one part, whereas the other part was used as the addition-free control. Germination of spores of suspensions with and without added nutrients was determined as described for experiment 1.

Production of spores on wheat bran. In experiment 3, spore production on a wheat bran medium in glass Petri dishes (∅ 20 cm; height 4 cm; content 1 l) was studied. Wheat bran medium was prepared by wetting a mixture of 30 g wheat bran (Zonnatura B.V., The Netherlands) and 90 g dry quartz sand with 60 ml distilled water in a Petri dish. The medium was sterilized at 120 °C for 30 min. Five Petri dishes with wheat bran medium were inoculated with 10 ml seed inoculum per Petri dish. The Petri dishes were thoroughly shaken, placed in the climate cabinet (continuous light and 20 °C), and subsequently shaken every two to three days to prevent agglomeration of medium by fungal growth. Six g of wheat bran medium inclusive fungal biomass were taken out of each Petri dish at different intervals within a period of a month. The samples were divided into two parts, 5 g was used to determine spore production and 1 g was used to determine the moisture content. The samples of 5 g were transferred to flasks, and flooded with 20 ml distilled water with 0.05% (v/v) Tween 80 per flask. The flasks were regularly shaken. Supernatants with suspended spores were poured into test tubes after 3 h. Volume and spore density of collected suspensions were determined. The samples of 1 g were dried at 70 °C for 2 days. Numbers of spores produced per gram dry medium (including fungal dry matter) were calculated.

Effect of storage of spores on germination capacity. In experiment 4, Petri dishes with wheat bran medium were inoculated and incubated as described for experiment 3. After 11 days, the contents of the Petri dishes were placed between two layers of blotting paper in the laboratory at 18-24 °C and 30-60% RH for three to four days. Subsequently the cultures (inclusive medium) were transferred to glass jars, and stored at 5 °C in the dark. The cultures in cold storage had a moisture content of 2 to 4%. Samples of 5 g were taken from the cultures in cold storage at different intervals over a period of 385 days. Spores

were harvested from the samples, and the number of spores harvested per gram dry medium was determined as described for experiment 3. Spore germination was determined as described for experiment 2.

Data analysis. Spore production was analysed with a Gompertz model for sigmoidal growth (*e.g.* Campbell and Madden, 1990):

$$y = c^{*}\exp(-\exp(-b^{*}(t-m))) \quad (1)$$

where y is the number of spores produced per unit medium at time t, c is the maximum spore production per unit medium, b is a shape parameter, t is the day number after inoculation of the medium (age of the culture), and m is a location parameter.
Spore germination was analysed by means of analysis of variance and by an exponential model:

$$y = a * b^{t} \quad (2)$$

where y is the proportion of germinated spores at time t+20 h (spore germination was assessed 20 h after spores were plated onto water agar), parameters a and b are shape parameters, and t is the day number at which the spores were harvested.
The models were fitted to the data by means of non-linear regression procedures of Genstat 5 (Payne *et al.*, 1987). Proportions of germinated spores were angular-transformed prior to the analysis of variance.

RESULTS

Spore production. New spores were harvested from cultures on oat meal agar and wheat bran medium 4 days after the media had been inoculated. During the next 6 to 10 days, numbers of spores produced increased rapidly till a maximum spore production level was reached. The course of the numbers of spores produced with time on the two media could be described by the Gompertz model. Parameters c, b and m of the Gompertz model describing spore production on oat meal with time, assessed by fitting the Gompertz model to the data sets of both experiment 1 and 2, were $2.0*10^{8}$ spores.plate^{-1} (s.e. = $0.2*10^{8}$), 1.2 day^{-1} (s.e. = 3.7) and 7.5 days (s.e. = 1.5), respectively ($P<0.05$; $R^{2}_{adj} = 78$). S.e's are estimates of the standard errors. R^{2}_{adj} is the percentage of variance explained by the regression. Parameters c, b and m of the Gompertz model describing spore production on wheat bran medium with time, assessed by fitting the model to the data of experiment

3, were $4.0*10^7$ spores.gram^{-1} dry medium (s.e. = $0.4*10^7$), 2.3 day^{-1} (s.e. = 4.0) and 7.1 days (s.e. = 1.6), respectively ($P<0.05$; $R^2_{adj} = 67$). In Fig. 2.1, normalized treatment averages of numbers of spores produced on the media, and curves of the two normalized Gompertz models are shown. The treatment averages and the Gompertz models were normalized by dividing them by the corresponding value of parameter *c*.
The numbers of spores that were harvested from cultures stored at 5 °C in experiment 4, was not significantly affected by the duration of the storage of the cultures.

Spore germination. The effects of harvest time and nutrients on spore germination are shown in Fig. 2.2. Spores harvested from relatively young cultures almost all germinated within 20 h. Proportions of germinated spores decreased with age of cultures when no nutrients were added to the spore suspensions. The decrease in germination for the data of the experiments 1 and 2 could be described by the exponential model ($P<0.05$; $R^2_{adj} = 79$). The parameter estimates were $a = 1.3$ and $b = 0.9$. No significant exponential nor linear decrease in germination was observed when nutrients were added to the spore suspensions ($P>0.05$). Analysis of variance of the data of experiment 2 showed a significant interaction ($P<0.001$) between harvest date and nutrients in spore suspensions.
The effect of duration of storage of cultures at 5 °C on spore germination is shown in Fig. 2.3. Spores that were harvested from cultures stored less than 40 days almost all germinated within 20 h even without nutrients added. Proportions of germinated spores decreased with duration of storage when no nutrients were added. The decrease in germination could be described by the exponential model ($P<0.05$; $R^2_{adj} = 69$). The parameter estimates were $a = 0.8$ and $b = 0.996$. However, the model underestimated germination of spores that were stored less than 40 days. No significant exponential nor linear decrease in germination was observed when nutrients were added to the spore suspensions ($P>0.05$).

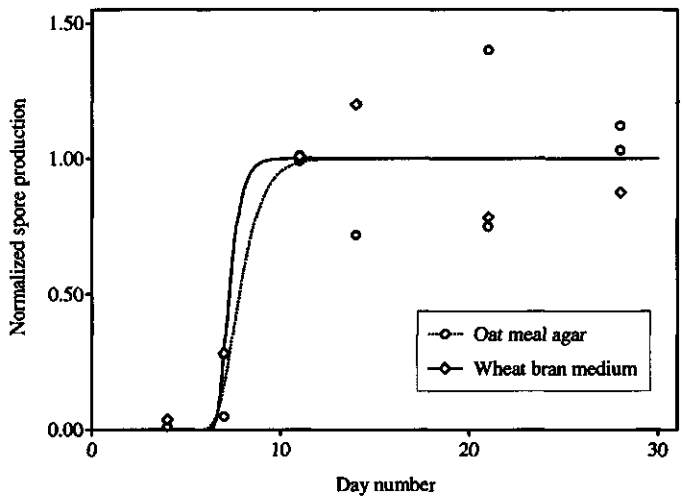

Fig. 2.1 Experiments 1, 2 and 3. Normalized production of spores of *Ascochyta caulina* on two growth media and Gompertz curves fitted to data. Day number is the time after inoculation of the medium. Entries are treatment averages.

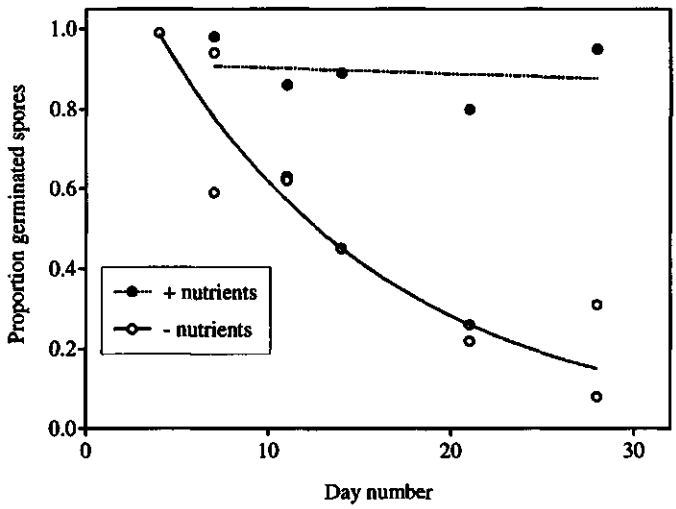

Fig 2.2 Experiments 1 and 2. Germination of spores of *Ascochyta caulina*, with or without added nutrients, as a function of culture age. Proportions were assessed 20 h after the spores were plated on water agar. Entries are treatment averages. The exponential decrease as described by the curves was only significant when no nutrients were added.

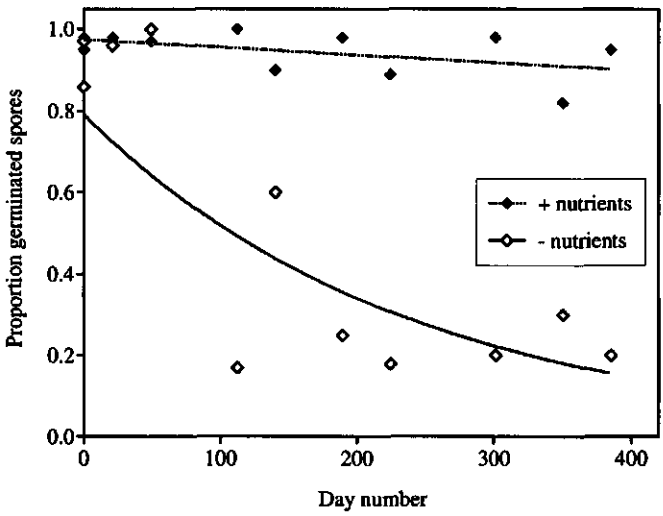

Fig 2.3 Experiment 4. Effect of long-term storage at 5 °C of cultures of *Ascochyta caulina* on spore germination in the presence or absence of added nutrients. Entries are single observations, assessed 20 h after the spores were plated on water agar. The exponential decrease as described by the curves was only significant when no nutrients were added.

DISCUSSION

Oat meal agar is a commonly used medium in microbiology. It was suited for small scale spore production. In preliminary experiments, the highest numbers of spores of *A. caulina* were produced on oat meal agar medium when compared to 5 other agar media, malt agar, V8-juice agar, rye agar, Czapek-Dox agar and Czapek-Dox/yeast extract agar (Horsten and Kempenaar, 1994). The wheat bran medium was suited for large scale spore production. Originally described by Schmitz-Elsherif (1990), it was slightly adjusted to a composition which was optimal for production of spores of *A. caulina*. The numbers of spores produced on oat meal agar medium and wheat bran medium showed a sigmoidal course with time, and could be described by the Gompertz model. Under conditions that we considered optimal for spore production, the maximum level of production was reached within two weeks after inoculation of the medium. The relatively high values that were estimated for the shape parameter (*b*) of the Gompertz model indicate that high spore production rates occurred in the period between 3 and 11 days after inoculation of media. The time period in which spore production occurred corresponded with observations of fungal development on the media. Early pycnidia were observed on the

media three days after inoculation. In the following days more pycnidia developed. Spore production reached its maximum level at the end of the second week after inoculation of the media. At this time, surfaces of inoculated media were almost completely covered with mature pycnidia. We tested only one *A. caulina* isolate on spore production. We assume that spore production will vary between isolates of *A. caulina*. Indications for this assumption are derived from studies on other *Ascochyta* species (Webb and Lindow, 1987; Kaiser *et al.*, 1994). For instance, Webb and Lindow reported that spore production between isolates of *Ascochyta pteridis* (Bres.) Sacc. varied a factor 100.

Eggers and Thun (1988) showed that spore germination of *A. caulina* was affected by availability of water, temperature, nutrients in the spore suspension, and spore density. Our study showed that the age of a culture, and thus the age of spores, had a large effect on germination too. The decrease in germination rate was probably due to internal nutrient exhaustion by maintenance respiration of the spores during ageing. This conclusion was based on increased spore germination after addition of nutrients to spore suspensions. The nutrients used to stimulate spore germination were selected after Scheepens (unpublished data). He observed high germination rates of *A. caulina* spores in a Czapek-Dox broth supplemented with yeast extract. Czapek-Dox broth consisted of sugars and minerals, the yeast extract of vitamins and proteins.
A predictable germination and a long shelf life are characteristics desired for microbial control agents. A high and predictable germination rate of spores of *A. caulina* can only be guaranteed when nutrients are added to spore suspensions. Spores of *A. caulina* maintained viability for a period longer than 1 year when stored in cultures on wheat bran at 5°C. By storage of cultures of *A. caulina* on wheat bran medium at 5 °C, and the use of nutrients, we disposed of large numbers of spores with high and predictable germination rates, which enabled us to carry out field experiments on hundreds of square meters.

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CHAPTER 3

A METHOD TO OBTAIN FAST AND UNIFORM EMERGENCE OF WEEDS FOR FIELD EXPERIMENTS

C. Kempenaar and B.J. Schnieders

ABSTRACT

In studies on crop-weed competition it may be desirable to manipulate time of emergence, density and spatial arrangement of weeds. This requires a method to obtain a fast and uniform emergence of weeds. Two planting methods were compared that differed in the medium (agar or blotting paper) on which seeds were germinated, and in the way of transplanting the seeds (with or without the medium). In wet soil, emergence started earlier when seeds were germinated on agar and subsequently planted with agar. Root tips of seeds germinated on blotting paper may have been more damaged during transplantation, because rot tips tended to grow into the blotting paper. The duration, heterogeneity and final level of emergence were not affected by the planting method. In dry soil in a greenhouse experiment, the final level of emergence was much less when seeds were planted using the blotting paper method than using the agar method. The agar may have provided a small source of water which reduced dehydration of emerging plants. The results were similar for the three species studied, *Chenopodium album* L., *Senecio vulgaris* L. and *Solanum nigrum* L.

INTRODUCTION

Time of emergence, spatial arrangement, and density of weeds in a crop are factors that largely determine the outcome of competition (Harper, 1977; Kropff *et al.*, 1992; Lotz *et al.*, 1993). To study these factors and their interactions with weed control measures the weeds in the crop have to be manipulated in order to meet the experimental demands. In some experiments weeds that emerged from the seed bank were thinned to desired densities (Kropff *et al.*, 1984). If required, seeds were added to the seed bank (Peterson and Nalewaja, 1992; Zanin *et al.*, 1993). In other experiments germinated seeds (Dunan and Zimdahl, 1991; Kropff and Spitters, 1992; Lotz *et al.*, 1994) or seedlings (Perera *et al.*, 1992) were planted at specific positions in the crop. Transplantation of weeds into the crop allows manipulation of the time of emergence, the spatial arrangement and the plant density, but it is laborious.

Methods to plant germinated weed seeds often include the following steps in time, (1) germination of seeds on wet blotting paper, (2) planting of the germinated seeds in the soil and (3) thinning of the emerged plants to desired densities. When we used this method to plant germinated weed seeds in crops, we observed that root and shoot tips of germinated

seeds were easily damaged and that emergence was poor when the soil was dry. To overcome these limitations we developed a planting method which consisted of (1) germination of seeds on gelatinized water agar, (2) planting of the germinated seeds with water agar in the soil and (3) thinning to desired densities. The method developed is similar to fluid drilling techniques for crops, where seed germination is also induced prior to planting with a gel carrier (Gray, 1981). The objective of this paper is to compare the effect of the new 'agar' method and the standard 'paper' method on emergence of three annual weed species, under extreme moisture conditions in a greenhouse and under field conditions.

MATERIALS AND METHODS

Plant material. Mature plants of *Chenopodium album* L. from two populations (*C. album*_1 and *C. album*_2), *Senecio vulgaris* L. (groundsel), and *Solanum nigrum* L. (black nightshade) were collected from arable fields in the autumn of 1992. After drying the plants in a greenhouse for approximately one month, the seeds were harvested, stored in glass jars and kept in the dark at room temperature (*C. album*_1, *S. vulgaris* and *S. nigrum*) or at 4 °C (*C. album*_2) until they were used in the experiments.

Planting methods. The planting methods using blotting paper (paper method) or water agar (agar method) both included the following steps in time, (1) germination of the seeds, (2) planting of the seeds in the soil and (3) thinning to desired densities. The present study was confined to steps 1 and 2.

Seeds were germinated on media in plastic Petri dishes (Ø 9 cm). In the paper method the germination medium was blotting paper (Ø 8.5 cm, weight 3 g) wetted with 10 ml demineralized water. In the agar method the germination medium was 10 ml of 2% gelatinized agar (Oxoid Agar Bacteriological, Unipath LTD, England). A template was used to allow quick and standardized placement of groups of seeds on the media. The template was a plastic tray (Ø 8.5 cm; height 2 cm; thickness of the bottom 1 mm) with 20 holes (Ø 3 mm) in the flat bottom. The template was placed into the Petri dish, circa 1000 seeds were dispersed on the template resulting in 5-10 seeds per hole, and subsequently the template with the remaining seeds was removed. The Petri dishes were placed in an incubator (light period 14 h, 15-20 $\mu mol.m^{-2}.s^{-1}$, 25 °C; dark period 10 h, 15 °C) for 2 days (*C. album*_1 and *S. vulgaris*) or 4 days (*C. album*_2 and *S. nigrum*). Preliminary germination tests had shown that these incubation periods were required to reach the desired germination stage, i.e. root and shoot tips just visible.

On the day of planting single groups of seeds were transferred to their positions in the soil with a small spoon. In the paper method one group of seeds was scooped off the blotting paper, placed in one hole and covered with soil. In the agar method one group of seeds and a piece of water agar (approximately 0.3 gram) was scooped out of the Petri dish, placed in one hole and covered with soil. The seeds were planted at a depth of circa 1 cm.

Greenhouse experiments. Seeds of *C. album*_1, *S. vulgaris* and *S. nigrum* were planted in a 10 cm thick layer of coarse quartz sand in trays (dimensions 2 m by 1m by 0.15 m) on 31 March 1993. The trays were placed in a greenhouse in Wageningen, under conditions of 20/30 °C (min./max. temperature) and circa 60% relative humidity. On the day of planting more than 75% of the seeds had germinated irrespective of the germination medium. Experiment 1a was done in one tray with wet sand (11% w/w). The moisture level was close to water logging, and the tray was watered daily. Experiment 1b was done in one tray with dry sand (2% w/w). Both experiments had a two-factor (planting method and weed species) randomized block design with 4 replicates. So, 24 plots (6 treatments * 4 blocks) per tray were established, with 8 planting positions per plot. The planting positions per plot with one or more emerged plants were counted twice a day until no further emergence was observed.

Field experiments. In experiment 2, seeds of *C. album*_2 were planted in a sandy soil (3.5% organic material) of an arable field at Wageningen on 3 May 1993. On the day of planting, more seeds had germinated on water agar (30%) than on blotting paper (14%) ($P<0.001$). The seeds of *C. album*_2 germinated slowly compared with *C. album*_1. The experiment had a one-factor (planting method) randomized block design with 5 replicates. Each plot had 10 planting positions. The planting positions per plot with one or more emerged plants were counted daily until no further emergence was observed.

In experiments 3a, 3b and 3c, seeds of *C. album*_1, *S. vulgaris* and *S. nigrum* were planted in arable fields with different soil types at three locations. In experiment 3a seeds were planted in a sandy loam soil (22% of the soil particles < 16 µm) at Lelystad, the Netherlands, on 12 May 1993. In experiment 3b seeds were planted in a sandy clay loam soil (29% of the soil particles < 16 µm) at Westmaas, the Netherlands, on 18 May 1993. In experiment 3c seeds were planted in a sandy soil (3.5% organic material) at Wageningen on 1 June 1993. On the days of planting more than 75% of the seeds had germinated irrespective of the germination medium. All three experiments had a two-factor randomized split-plot design with 5 replicates. The factor weed species was assigned to the main-plots and the factor planting method was assigned to the sub-plots.

Each sub-plot had 10 planting positions. The planting positions per sub-plot with one or more emerged plants were counted circa every 2 days until no further emergence was observed. Table 3.1 summarizes the actual weather conditions during the course of the field experiments.

Data analysis. Emergence was defined as the number of planting positions with one or more emerged plants per plot. The estimated parameters of a linear piecewise regression model (Montgomery and Peck, 1982) that described emergence in time were used to compare the two planting methods. The model described emergence in three distinct phases, (1) a phase in which emergence had not yet started, (2) a phase in which emergence occurred and (3) a phase in which emergence had obtained its final level (Fig. 3.1). The equations that described emergence (y) in the three phases against time after planting (t) are:

$$y = 0 \qquad \text{for } t \leq s$$

$$y = \frac{c}{d} * (t - s) \qquad \text{for } s < t \leq s + d$$

$$y = c \qquad \text{for } t > s + d$$

where s is the time until start of emergence (day), d is the duration of emergence (day) as well as a measure for heterogeneity of emergence, and c is the final level of emergence (-). Parameter b (= c/d = tanα) is the rate of emergence (day^{-1}). The time required to obtain emergence in 50% of the planting positions per plot ($t_{50\%}$) was calculated as:

$$t_{50\%} = s + 0.5 * d$$

The model was fitted to the data of emergence against time after planting using Genstat 5 software (Payne *et al.*, 1987). Analyses of variance to test the effects of the treatments on s, d, c, b and $t_{50\%}$ were also done using Genstat.

Table 3.1 Range of daily minimum (T_{min}) and maximum (T_{max}) temperatures in a 14 days period after planting, and precipitation on day t after planting in the field experiments.

	expt 2	expt 3a	expt 3b	expt 3c
T_{min} (°C)	0 - 13	5 - 14	6 - 14	7 - 18
T_{max} (°C)	13 - 26	14 - 25	19 - 26	14 - 29
Precipitation (mm)	8 (t= 0)	5 (t= 0)	7 (t= 0)	5 (t= 0)
	17 (t= 6)	5 (t= 3)	11 (t= 1)	10 (t= 2)
	3 (t=10)	5 (t= 4)	1 (t= 2)	2 (t= 3)
		5 (t= 5)	5 (t= 4)	2 (t=10)
		30 (t= 6)	5 (t= 6)	7 (t=14)

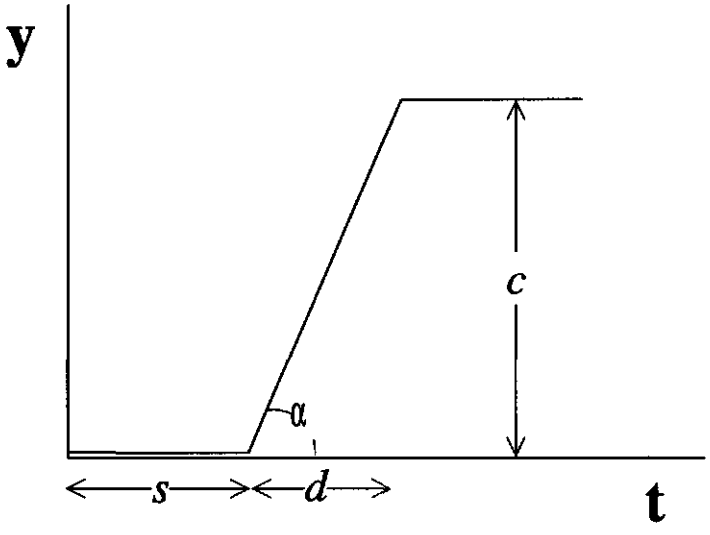

Fig. 3.1 The piecewise linear regression model for emergence (y) against time after planting (t). Parameter s is the time until start of emergence, parameter d is the duration of emergence, parameter c is the final level of emergence and parameter b (= $\tan(\alpha) = c/d$) is the rate of emergence.

RESULTS

Greenhouse experiments. In experiment 1a in wet sand, plants emerged within 4 days and emergence was well described by the model ($P<0.05$; $R^2_{adj}>90$). The time until start of emergence (*s*) of all species was shorter ($P<0.01$) when seeds were planted with the agar method (Table 3.2). No effect of the planting method on the duration of emergence (*d*) or the final level of emergence (*c*) was found for any species ($P>0.05$). The rate of emergence (*b*) of *C. album*_1 was higher when seeds were planted with the agar method, but that of *S. vulgaris* and *S. nigrum* was not affected by the planting method (interaction between planting method and species $P<0.05$). The $t_{50\%}$ of all species was shorter when seeds were planted with the agar method ($P<0.001$).

In experiment 1b in dry sand, no plants of *S. vulgaris* and *S. nigrum* and very few plants of *C. album*_1 emerged when seeds were planted with the blotting paper method. Emergence was higher but still incomplete when the agar method was used (Table 3.2). Emergence counts were stopped after five days, because the emerged plants died from drought. Emergence was still well described by the model ($P<0.05$; $R^2_{adj}>90$).

Table 3.2 Time until start of emergence *s* (day), duration of emergence *d* (day), final level of emergence *c* (-; maximum 8), average emergence time $t_{50\%}$ (day) and standard errors of the mean describing emergence of *Chenopodium album*, *Senecio vulgaris* and *Solanum nigrum*, planted with the agar method or with the paper method in two greenhouse experiments. The parameters were estimated with a piecewise linear regression model.

Expt	Species	Parameters							
		s		d		c		$t_{50\%}$	
		agar method	paper method	agar method	paper method	agar method	paper method	agar method	paper method
1a	*S. vulgaris*	0.3 ± 0.1	1.1 ± 0.2	1.6 ± 0.2	1.0 ± 0.2	7.3 ± 0.2	7.6 ± 0.2	1.2 ± 0.0	1.5 ± 0.1
	*C. album*_1	0.8 ± 0.2	1.3 ± 0.3	1.1 ± 0.3	1.9 ± 0.5	7.8 ± 0.1	7.1 ± 0.5	1.4 ± 0.2	2.3 ± 0.2
	S. nigrum	1.3 ± 0.3	2.1 ± 0.3	1.4 ± 0.3	1.8 ± 0.4	8.0 ± 0	8.0 ± 0	2.0 ± 0.1	3.0 ± 0.2
1b	*S. vulgaris*	0.4 ± 0.1	*	1.3 ± 0.4	*	5.0 ± 0.8	0.0 ± 0	1.1 ± 0.1	*
	*C. album*_1	0.8 ± 0.2	*	0.7 ± 0.3	*	2.0 ± 0.0	0.8 ± 0.2	1.2 ± 0.2	*
	S. nigrum	1.2 ± 0.5	*	0.5 ± 0.1	*	2.3 ± 0.5	0.0 ± 0	1.9 ± 0.4	*

* no average because of plots with zero emergence

Field experiments. In the field experiments the plants emerged in the first week after planting. The emergence was well described by the model ($P<0.05$; $R^2_{adj}>80$), except for some plots of experiment 3a. In these plots some plants died after emergence. Although the model did not describe the emergence in these plots so well ($P<0.05$; $R^2_{adj}>60$), the estimated parameters were used for data analysis.

In experiment 2 (Table 3.3) the time until start of emergence (s) was shorter when seeds were planted with the agar method ($P<0.01$). No effect of the planting method on the duration of emergence (d), the final level of emergence (c), or the rate of emergence (b) was found ($P>0.05$). The $t_{50\%}$ was shorter when seeds were planted with the agar method ($P<0.001$).

In experiment 3a (Table 3.3) the time until start of emergence (s) of all species was

Table 3.3 Time until start of emergence s (day), duration of emergence d (day), final level of emergence c (-; maximum 10), average emergence time $t_{50\%}$ (day) and standard errors of the mean describing emergence of *Chenopodium album*, *Senecio vulgaris* and *Solanum nigrum*, planted with the agar method or with the paper method in four field experiments. The parameters were estimated with a piecewise linear regression model.

Expt	Species	Parameters							
		s		d		c		$t_{50\%}$	
		agar method	paper method	agar method	paper method	agar method	paper method	agar method	paper method
2	*C. album*_2	2.0 ± 0.2	3.7 ± 0.3	3.4 ± 0.6	3.0 ± 0.4	10.0 ± 0	9.9 ± 0.1	3.7 ± 0.1	5.2 ± 0.1
3a	*S. vulgaris*	2.3 ± 0.1	2.5 ± 0.3	4.1 ± 2.2	3.9 ± 0.5	5.0 ± 1.3	5.7 ± 0.6	4.5 ± 1.2	4.5 ± 0.5
	*C. album*_1	2.7 ± 0.3	3.6 ± 0.5	3.0 ± 0.4	3.3 ± 0.5	6.7 ± 0.2	6.9 ± 0.5	4.2 ± 0.3	5.2 ± 0.4
	S. nigrum	3.9 ± 0.1	4.9 ± 0.3	2.3 ± 0.1	2.5 ± 0.2	9.8 ± 0.2	9.6 ± 0.2	5.1 ± 0.1	6.2 ± 0.3
3b	*S. vulgaris*	2.6 ± 0.2	3.2 ± 0.2	2.0 ± 0.5	2.0 ± 0.2	9.6 ± 0.2	9.7 ± 0.3	3.6 ± 0.1	4.0 ± 0.1
	*C. album*_1	2.7 ± 0.1	3.3 ± 0.1	2.2 ± 0.2	1.6 ± 0.2	9.3 ± 0.4	9.6 ± 0.3	3.8 ± 0.1	4.0 ± 0.1
	S. nigrum	3·0 ± 0·2	3·4 ± 0·1	2.4 ± 0.4	1.7 ± 0.2	10.0 ± 0	9.8 ± 0.2	4.2 ± 0.1	4.3 ± 0.1
3c	*S. vulgaris*	0.2 ± 0.1	0.3 ± 0.1	1.6 ± 0.2	2.1 ± 0.3	9.6 ± 0.2	9.6 ± 0.2	1.0 ± 0.1	1.3 ± 0.1
	*C. album*_1	0.8 ± 0.2	1.1 ± 0.2	1.5 ± 0.2	2.0 ± 0.4	9.1 ± 0.2	9.5 ± 0.4	1.6 ± 0.1	2.1 ± 0.2
	S. nigrum	3.4 ± 0.1	4.0 ± 0.1	1.9 ± 0.2	2.0 ± 0.2	9.9 ± 0.1	9.6 ± 0.2	4.3 ± 0.1	4.8 ± 0.1

shorter when seeds were planted with the agar method ($P<0.05$). No effect of the planting method on the duration of emergence (*d*), the final level of emergence (*c*), the rate of emergence (*b*) or $t_{50\%}$ was found for any species ($P>0.05$). Establishment after emergence was incomplete for the species *C. album*_1 and *S. vulgaris* because plants died in the second week after planting, irrespective of the planting method.

In experiment 3b and 3c (Table 3.3) the time until start of emergence (*s*) of all species was shorter when seeds were planted with the agar method ($P<0.01$ and $P<0.001$, respectively). No effect of the planting method on the duration of emergence (*d*), the final level of emergence (*c*) or the rate of emergence (*b*) was found for any species in either experiment ($P>0.05$). The $t_{50\%}$ of all species was shorter when seeds were planted with the agar method ($P<0.05$ and $P<0.01$, respectively).

DISCUSSION

The piecewise linear regression model proved to be a good tool to analyse the emergence data of the three weed species. Theoretically, logistic models with a binomial distribution are suited better to analyse emergence data (Schimpf *et al.*, 1977), but these models did not give satisfactory results in our situation. Schimpf *et al.* (1977) already mentioned that logistic models are only useful to fit data of emergence as long as rates of emergence are not too high. Our data indeed had high rates of emergence. Since emergence occurred in a relatively short period of time and appeared to be almost linear with time, it was acceptable to use the piecewise linear regression model. The piecewise linear regression model should only be used with some reservation when plants die after emergence as in experiment 3a. When plants die after emergence, and emergence and mortality data become confounded, the parameters *s*, *b* and *c* will be underestimated.

In wet soil (experiments 1a, 2, 3a, 3b and 3c), the time until start of emergence (*s*) and the average emergence time ($t_{50\%}$) were shorter when seeds were planted with the agar method. Seeds established better when planted with the agar method than with the blotting paper method and growth was resumed sooner. Indeed, we observed that some germinated seeds were damaged when they were removed from the blotting paper, due to physical contact with the spoon or due to damage of the root tips that had grown into the blotting paper. The duration of emergence (*d*), the final level of emergence (*c*) and the rate of emergence (*b*) were not affected by the planting method. The duration of emergence (*d*) is also a measure for heterogeneity of emergence and plant growth, *e.g.* a high heterogeneity

gives a large duration of emergence, and thus heterogeneity of emergence was not influenced by the planting method. The duration of emergence (*d*) in the field experiments was two to four days. In experiment 3a mortality of emerged plants of *C. album*_1 and *S. vulgaris* occurred irrespective of the planting method. This mortality may have been caused by heavy rains (>30 mm) on the experimental site on day six after planting. On this day more plants of *C. album*_1 and *S. vulgaris* than of *S. nigrum* had emerged, so the first two species may have been more affected by the rain.

In dry sand (experiment 1b), seeds planted using the agar method gave much more emergence than seeds planted using the blotting paper method. The agar possibly supplied some water to the emerging plants, so that they could actually emerge, but the total amount of water was insufficient to prevent mortality soon after emergence.

The agar method is less laborious to use than the blotting paper method since germinated seeds could be planted faster with the agar method than with the paper method. The water agar showed no negative effects on later growth and development. Several weeks after planting, the piece of water agar had disappeared or was present as a little piece of flexible organic material around the stem base of the plant, just underneath the soil surface. The number of weed seeds that have to be planted in a single planting position depends on the germinative capacity and the vigour of the seeds. In case of poor germination, germination stimuli (Anderson, 1968; Webster, 1979; Egley, 1986) can be given to the seeds.

The conclusion of this study is that the water agar method provides a fast and uniform emergence of three annual weeds and is more reliable to use under dry conditions than the blotting paper method. It is therefore a useful method to establish weed plants at specific times, densities and spatial positions in a crop.

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CHAPTER 4

APPLICATION OF PYCNIDIOSPORES OF *ASCOCHYTA CAULINA* TO *CHENOPODIUM ALBUM* PLANTS; SPORE GERMINATION AND DISEASE DEVELOPMENT

C. Kempenaar, P.J.F.M. Horsten and P.C. Scheepens

ABSTRACT

Disease development of *C. album* plants and plants of 14 other species after spray application of pycnidiospores of *A. caulina* to the plants was studied in climate chamber experiments. The experiments were carried out to analyse disease development with time and to recognise factors that may limit disease development. Two time courses of necrosis of *C. album* plants were observed, (1) an increase of necrosis followed by a decline, and (2) an increase of necrosis up to completion with subsequent plant death. Courses of necrosis with time could be described by a non-monotonic, critically damped model when plants survived infection and by a monomolecular model when plants died from infection. Disease development was influenced by interactions between wetness period, density of the spore suspension applied, plant development stage at the time of spore application, and temperature. Disease was favoured by a long wetness period, a high number of spores applied, an early plant development stage at the time of spore application, and a temperature of circa 20 °C. Disease development was limited to plant species of the genera *Chenopodium*, *Atriplex* and *Spinacia* with differences between the species. Pathogenicity differed significantly between three *A. caulina* isolates tested. Differences in resistance between four source populations of *C. album* was small.

INTRODUCTION

The mycoherbicide concept was introduced by Daniel *et al.* (1974), who demonstrated that a plant pathogenic, endemic fungus can be utilized to control its weed host by applying a massive dose of inoculum at a particular development stage of the weed. Subsequently, in the last two decades, over 100 fungi have been examined for their ability to control their weed hosts (*e.g.* Templeton, 1982; Scheepens and Van Zon, 1982, Charudattan, 1991). Scheepens (1979) suggested to study *Ascochyta caulina* (P. Karst.) v.d. Aa & v. Kest. for the control of *Chenopodium album* L., an annual plant, and word-wide an important weed in many crops (*e.g.* Holm *et al.*, 1977; Schroeder *et al.*, 1993). *A. caulina* causes necrotic lesions on leaves and stems of plants of *Chenopodium* and *Atriplex* species. The fungus is endemic in Europe and central Siberia (Van der Aa and Van Kesteren, 1979).

Eggers and Thun (1988) studied infection of *C. album* plants after application of pycnidiospores of *A. caulina* to the plants. They were not optimistic about the potential of

A. caulina as a mycoherbicide, but could not make a thorough evaluation. The objective of the study presented here is to provide a basis for the evaluation of the perspectives of *A. caulina* as a post-emergence mycoherbicide against *C. album*. Disease development of *C. album* plants after spray application of suspensions of *A. caulina* pycnidiospores was investigated. Host specificity and some factors that might limit disease development of *C. album* were studied. Holocomb (1982) listed factors that might limit disease development (Table 1.1). We studied effects of source population of *C. album*, isolate of *A. caulina*, wetness period after spore application, the density of the spore suspension applied, plant development stage at the time of spore application, and temperature on disease development. In some experiments, observations were made on spore germination and fungal development.

MATERIALS AND METHODS

Plant material. Seeds of *C. album* were collected from mature plants on different locations in Wageningen, The Netherlands, in 1991. Seed samples of four source populations were used, (1) a plant on an arable field with a sugar beet crop, (2) a plant on an arable field with a maize crop, (3) a plant on a ruderal site, and (4) plants in a weed demonstration garden (Department of Agronomy, Wageningen Agricultural University). Seeds of other weed species were harvested from plants in the same weed demonstration garden. Seeds of cultivated species were obtained from commercial seed batches. All seeds were stored in the dark at 5 °C until sowing.

Plants to be used in experiments were grown in a climate chamber on a peat soil in plastic pots. Germination of seeds was induced prior to sowing to improve uniformity of emergence as described in Chapter 3. If present, seed coatings were removed by several washings in water. Seeds were placed on 1% (w/v) water agar in Petri dishes (Ø 9 cm). Germinated seeds were planted in peat soil, which consisted of a mixture of 10 volumes of peat (Trio B.V., The Netherlands; Triomf no. 17) and 1 volume of coarse sand. Soil volume in a pot was 600 ml. The pots were placed in a climate chamber with a day-night regime of 14 h light at 18°C, 75% RH, and 10 h darkness at 12 °C, 85% RH. Light was obtained from Philips TLD 50W/84 lamps, and average photosynthetic active radiation (PAR) at soil level was 240 $\mu mol.m^{-2}.s^{-1}$. The soil was watered twice a week with a nutrient solution (Steiner, 1984). Plants density was 1 or 4 $plants.pot^{-1}$.

Fungal material. *A. caulina* was isolated from naturally infected *C. album* plants on different locations in Wageningen, 1990 or 1991. The isolates were axenically maintained

on oat meal agar. Three isolates were used in the experiments, (1) an isolate from a leaf of a plant on an arable field (isolate 90-1), (2) an isolate from a stem of a plant at a ruderal site (91-1), and (3) an isolate from a leaf of a plant from another arable field (91-2).
Pycnidiospores of *A. caulina*, to be used as inoculum in experiments, were produced on oat meal agar or on wheat bran medium (Chapter 2). Circa 4 h before application of spores to plants, cultures of *A. caulina* on the media were flooded with distilled water with 0.05% (v/v) Tween 80. After 3 h, supernatants with suspended spores were collected, filtered through cheese cloth, and diluted to desired densities.

Spore application and wetness period. When plants of a batch had reached a particular development stage, they were sprayed with a spore suspension till run off 4 h before the climate chambers went dark. A DeVilbiss atomizer was employed. Sprayed plants were placed in high-humidity chambers for a specified wetness period. Subsequently, they were returned to the climate chamber in which they were originally grown. Conditions in the high-humidity chambers were a near-saturated atmosphere (>95% RH) with a day-night regime of 14 h light (average PAR 210 $\mu mol.m^{-2}.s^{-1}$; Philips TLD 50W/84) at 18 °C and 10 h darkness at 12 °C. The surface of sprayed plants dried slowly but not completely during exposure to high humidity. When sprayed plants had been returned to the climate chamber at 75-85% RH, the surface of the plants dried within 30 minutes.

Disease assessment and data analysis. Plants were separated in leaves and stems. Per plant, the proportion of necrotic leaf area of each individual leaf was estimated by means of standard diagrams (Fig. 4.1a-4.1c). The area of each individual leaf was measured by means of a leaf area meter (LI-COR, USA; Model 3100). The necrotic area of each leaf was calculated by multiplying the leaf area with the estimated proportion of necrosis. The proportion of necrotic leaf area of a plant was calculated by dividing the summarized necrotic leaf area of a plant by the summarized leaf area of that plant. The proportion of necrotic stem area of a plant was directly estimated by means of standard diagrams (Fig.4.1d).
A monomolecular model (equation 1; *e.g.* Campbell and Madden, 1990) and a non-monotonic, critically damped model (equation 2; Gilligan, 1990) were used to describe courses of necrosis with time:

$$y = 1 - e^{\{-r*(t-a)\}} \qquad (1)$$

$$y = (b + c*(t-a))*e^{\{-r*(t-a)\}} \qquad (2)$$

In both models, y is the proportion of necrotic area per plant at time t, r is a rate parameter, t is time after spore application in days, and a is the incubation period (intercept of the curve with the x-axis). Parameters b and c in equation 2 are shape parameters. The models were fitted to the data by means of non-linear regression procedures of Genstat 5 (Payne *et al.*, 1987).
Treatment effects on angular-transformed proportions were determined by means of analysis of variance (ANOVA).

Experiment 1: Observations on spore germination and fungal development. Fungal development on inoculated leaves was assessed by means of the method of Bruzzese and Hasan (1983). *C. album* plants of population 'arable field 2' were grown at densities of 4 plants.pot^{-1}. When the plants had reached the 4-leaf stage, they were sprayed with a spore suspension of 10^6 spores.ml^{-1} of isolate 90-1. Sprayed plants were placed in a high-humidity chamber. Leaves were harvested from the plants at 3, 7, 11, 24, 48, 72 and 96 h after spore application, 8 leaves per harvest. The leaves were immersed in a clearing and staining solution for two days, transferred to a clearing solution for 1 day, and mounted on microscope slides for observations with a light microscope.

Experiment 2: Effect of source population and isolate on disease development. C. album plants of three populations (arable field 1, arable field 2, and ruderal site) were grown at densities of 4 plants.pot^{-1}. When the plants had reached the 6-leaf stage, they were sprayed with a suspension of 0 or 5.10^6 spores.ml^{-1}. Spore suspensions of three isolates (90-1, 91-1 or 91-2) were applied. Sprayed plants were placed in the high-humidity chamber for a wetness period of 24 h and subsequently returned to the climate chamber at 75-85% RH. The experiment consisted of 12 treatments (3 plant populations * 4 suspensions). A treatment consisted of 2 pots with 4 plants each. The pots were placed in a complete randomized design. Leaf necrosis on individual plants was assessed one week after inoculation.
At spraying time, one ml spore suspension of each isolate was plated on 1% (w/v) water agar in a Petri dish (∅ 9 cm). The Petri dishes were placed in the high-humidity chamber for 24 h. Percentages of spore germination were determined by observing 300 spores per isolate. A spore was considered germinated when the length of the germination tube exceeded the width of the spore (> 5µm).

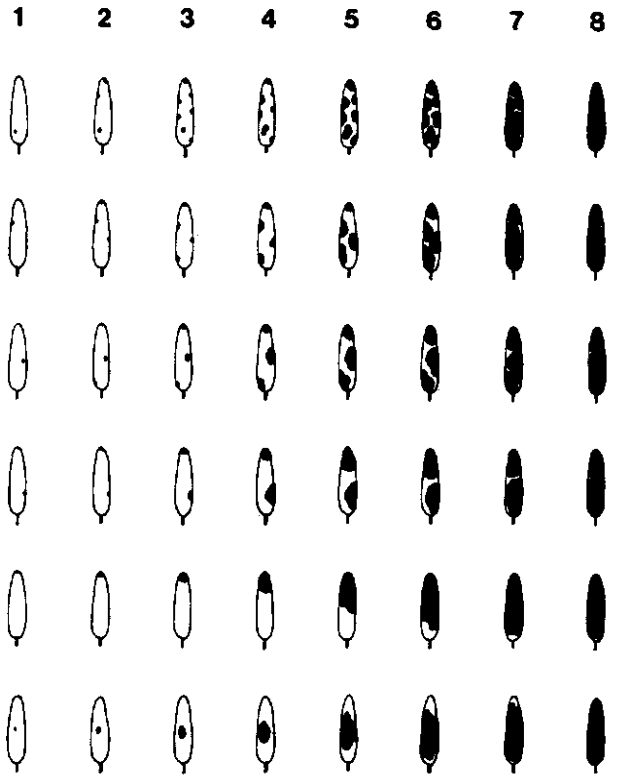

Fig. 4.1a, b, c, d Standard diagrams for the assessment of proportions of necrotic area on cotyledons (a), first two leaves (b), subsequent leaves (c), and stems (d) of young Chenopodium album plants. Numbers represent classes of necrosis and refer to proportions of necrotic area: 1 = 0.002, 2 = 0.015, 3 = 0.08, 4 = 0.24, 5 = 0.5, 6 = 0.76, 7 = 0.92 and 8 = 0.985.

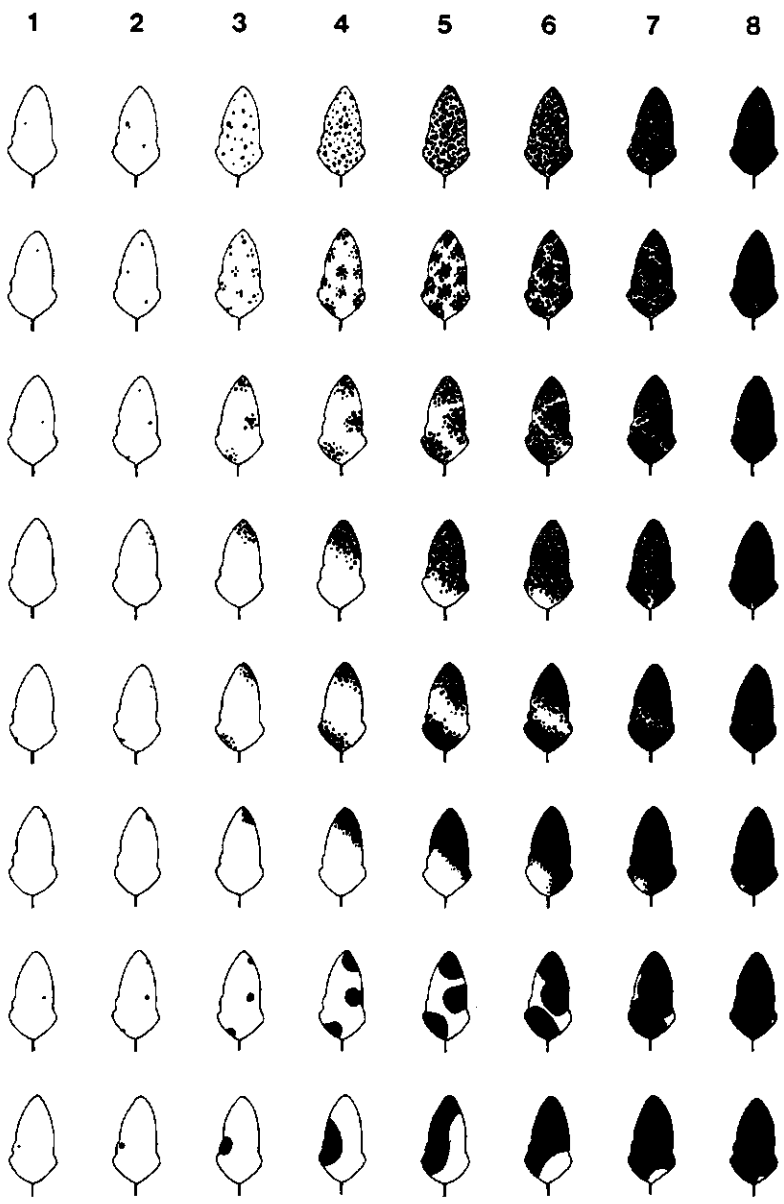

Fig. 4.1b

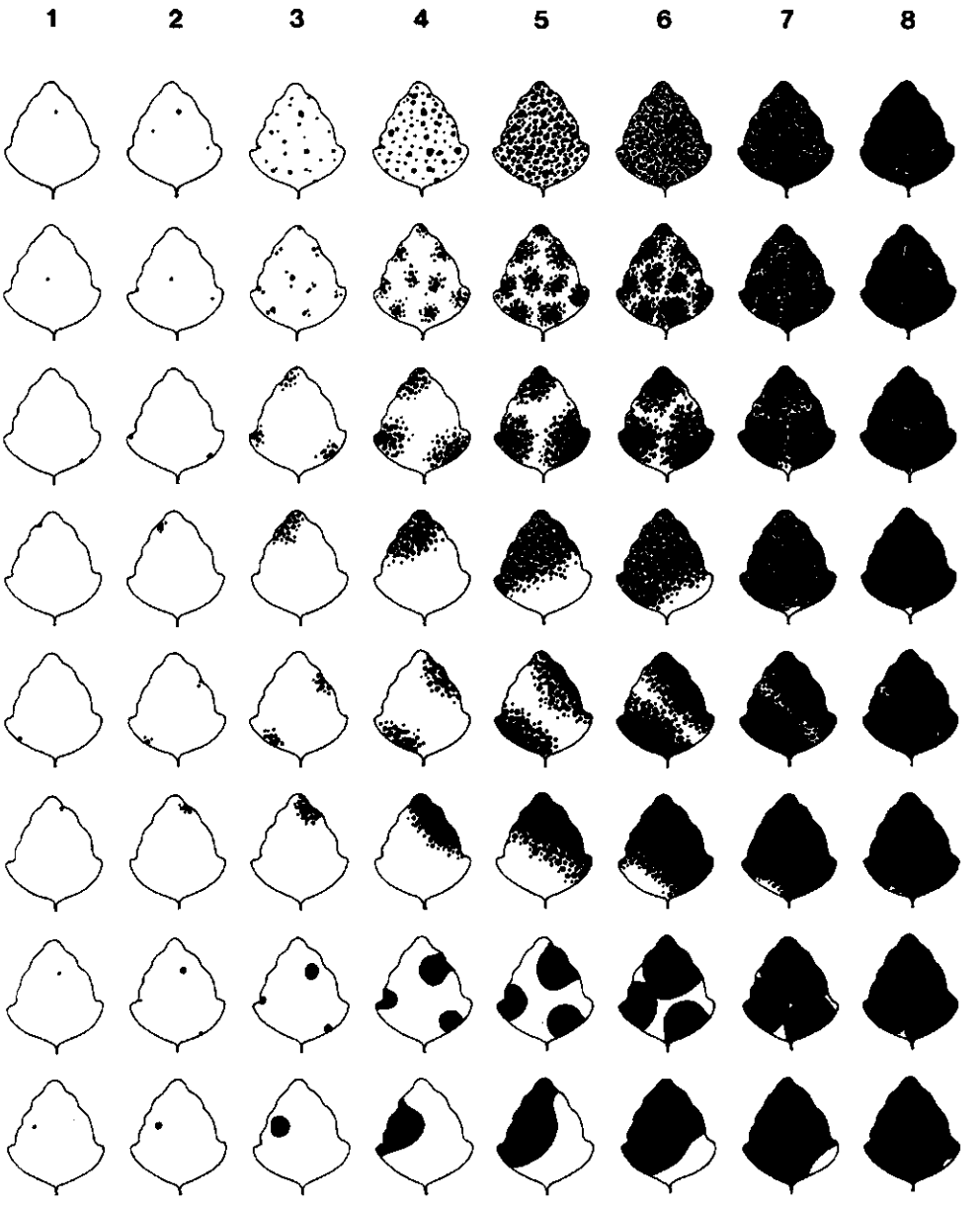

Fig. 4.1c

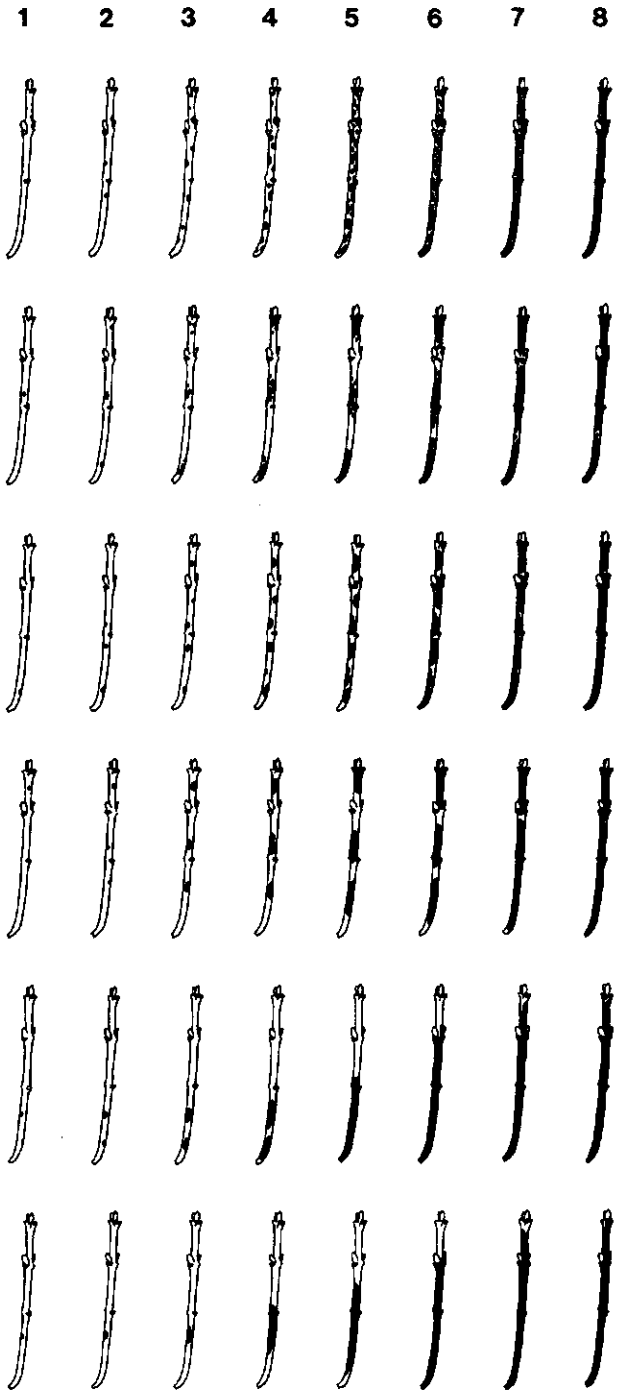

Fig. 4.1d

Experiment 3: Disease development with time. *C. album* plants of population 'arable field 2' were grown at densities of 1 plant.pot^{-1}. When the plants had reached the 4-leaf stage, they were sprayed with a suspension of 5.10^6 spores.ml^{-1} of isolate 90-1. Nutrients, 3.5 g.l^{-1} Czapek-Dox broth (DIFCO, USA) and 0.4 g.l^{-1} yeast extract (MERCK, Germany), were added to the spore suspension prior to spraying. Sprayed plants were placed in high-humidity chambers for wetness periods of 8, 16, 24 or 36 h and subsequently returned to the climate chamber at 75-85% RH. At nine dates after spore application, extended over a period of one month, 8 plants per treatment were taken from the climate chamber and assessed leaf and stem necrosis. The experiment consisted of 36 treatments (4 wetness periods*9 harvest dates). A treatment consisted of 8 pots with 1 plant each. The pots were placed in a randomized block design with 8 replicates.

Experiments 4a, 4b and 4c: Effect of plant development stage, spore density, and wetness period on disease development. Three experiments were conducted with three batches of *C. album* plants of population 'arable field 2'. Plant density was 4 plants.pot^{-1}. Plants of a batch were sprayed at a particular development stage: cotyledonous stage, 2-leaf stage or 6-leaf stage. The factor development stage at the time of spore application was confounded with the experiment number.

The three experiments were conducted in a similar way. When the plants of the particular batch had reached the specified development stage, they were sprayed with a suspension of 0, 10^5, 10^6 or 10^7 spores.ml^{-1} of isolate 90-1. Sprayed plants were placed in the high-humidity chamber for a wetness period of 0, 4, 8, 16, 28 or 44 h and subsequently returned to the climate chamber at 75-85% RH. Each experiment consisted of 24 treatments (6 wetness periods*4 spore densities). Each treatment was given to 8 plants divided over 8 pots. The pots were placed in a two-factor randomized split-plot block design. The factor wetness period was assigned to the main plot structure (blocks) and the factor spore density was assigned to the sub-plot structure (pots). Leaf necrosis on individual plants was assessed one week after spore application.

Experiment 5: Effect of temperature on disease development. Two batches of *C. album* plants of population 'arable field 2' were sown at a five days interval. Plant densities were 1 plant.pot^{-1}. The plants of the two batches were treated at the same time. They were sprayed with a suspension of 10^7 spores.ml^{-1} of isolate 90-1, when the plants of the two batches had reached the 4-leaf and 2-leaf stage, respectively. Sprayed plants were placed in one of five high-humidity chambers for wetness periods of 16 or 32 h. High-humidity chambers were set at temperatures of 6, 12, 18, 24 or 30 °C. The factor temperature was confounded with the humidity chambers. In each high-humidity chamber the pots were

placed in a two-factor (development stage and wetness period) randomized block design with 8 replicates. After the wetness period, the pots were returned to the climate chamber at 18/12 °C and 75-85% RH. Leaf necrosis on individual plants was assessed one week after spore application.
At spraying time, one ml spore suspension was plated on 1% (w/v) water agar in Petri dishes (Ø 9 cm). The Petri dishes were placed in the high-humidity chambers for periods of 16 or 32 h. The percentage of germinated spores at the five temperatures tested was determined by observing 200 spores per Petri dish.

Experiment 6: Host-specificity. Plants of 22 taxons, mostly *Chenopodiaceae*, were grown at densities of 4 plants.pot^{-1}. When the plants had reached the 4 to 6-leaf stage, they were sprayed with spore suspensions of 0 or 10^6 spores.ml^{-1} of isolate 90-1. Nutrients were added to the suspensions as described for experiment 3. Sprayed plants were placed in high-humidity chambers for a wetness period of 24 h and subsequently returned to the climate chamber at 75-85% RH. The experiment consisted of 44 treatments. A treatment consisted of 2 pots with 4 plants each. Leaf necrosis on the individual plants of a pot was assessed one week after spore application. The remaining plants were monitored for disease development for another two weeks.

RESULTS

Experiment 1: Observations on spore germination and fungal development. Fungal development on inoculated leaves of plants in the high-humidity chamber is described in chronological order. At 3 h a.i. (after inoculation), early signs of germination were observed. Some spores had developed a short (< 10 µm) germination tube. At 7 h a.i., more spores had germinated and germination tubes were longer. At 11 h a.i., some germination tubes had penetrated the leaves through stomata. At that time, appresoria-like structures at the end of some germination tubes were observed on top of stomatal cells as well as on epidermal cells. At 24 h a.i., mycelium was observed in the leaf tissue. At 48 h a.i., more mycelium was observed in the leaf tissue together with disintegrating host cells. At 72 h a.i., necrosis was observed macroscopically and early signs of development of pycnidia microscopically. At 96 h a.i., pycnidia were observed in the leaf tissue.

Experiment 2: Effect of source population and isolate on disease development. No significant differences were observed in the percentages of germinated spores of the three

isolates tested. On average, 75% of the spores had germinated on water agar after an incubation period of 24 h.

Plants sprayed with spore suspensions developed necrosis in the first week after inoculation while plants sprayed with the spore-free control fluid did not. Leaf necrosis at one week after spore application (Table 4.1) was influenced by isolates of *A. caulina* ($P<0.001$) but not by source populations of *C. album* ($P=0.064$).

Experiment 3: Disease development with time. Average leaf necrosis and average stem necrosis are shown in Fig. 4.2. Both leaf and stem necrosis showed an interaction ($P<0.001$) between wetness period and harvest date. Sprayed plants with a wetness period of 36 h showed a fast increase of necrosis, and complete necrosis of the aerial parts was reached circa one week after spore application. These plants died in the second week after spore application. Sprayed plants with a wetness period of 24 h or shorter also showed an increase of necrosis, but it was followed by a decline. The courses of necrosis with time could be described by the monomolecular model or the non-monotonic, critically damped model. The monomolecular model was used when complete necrosis occurred, and fitted well to the data. Percentages of variance explained by regressions were greater than 94. The critically damped model was used when increase of necrosis was followed by a decline. Regressions with this model could only be made when parameter a was set to a fixed value. Values for a (incubation period) were estimated from daily observations on disease development. The critically damped model underestimated the maximum necrosis levels. Percentages of variance explained by regressions were greater than 50. The parameter estimates are shown in Table 4.2.

Experiments 4a, 4b and 4c: Effect of development stage, spore density, and wetness period on disease development. Average leaf necrosis at one week after spore application is shown in Fig. 4.3. Plants sprayed with spore-free control liquid did not develop necrosis (not shown in Fig. 4.3). ANOVA of the combined data of the three experiments showed that leaf necrosis was significantly affected by the experiments ($P<0.001$). Experiments were confounded with development stages of the plants at the time of spore application. We ascribe the differences between the experiments to the effect of the development stage.

Each experiment showed an interaction ($P<0.001$) between spore density and wetness period. Leaf necrosis was near to zero, and not or hardly influenced by spore density when the sprayed plants had a short wetness period. With increasing wetness periods, leaf necrosis increased significantly and was significantly affected by spore density.

Table 4.1 Experiment 2. Average proportion of necrotic leaf area of young *Chenopodium album* plants one week after application of *Ascochyta caulina* spores as affected by source population of the weed and isolate of the fungus. Standard errors are in parentheses.

Population of	Isolate of *A. caulina*			
C. album	91-1	91-2	90-1	*mean*
arable field 1	0.02 (0.03) a	0.09 (0.06) b	0.37 (0.11) cd	*0.16 a*
arable field 2	0.03 (0.03) a	0.11 (0.08) b	0.37 (0.11) cd	*0.17 a*
ruderal site	0.03 (0.03) a	0.23 (0.15) bc	0.46 (0.14) d	*0.24 a*
mean	*0.03 a*	*0.15 b*	*0.40 c*	*0.20*

Treatment averages and means were separated with different letters according to LSD-tests of angular-transformed data ($P<0.05$).

Table 4.2 Experiment 3. Parameters of a monomolecular (mm) or a non-monotonic, critically damped (cd) model, fitted to data of leaf necrosis or stem necrosis of treated *Chenopodium album*. The monomolecular model was used when complete necrosis occurred, the critically damped model when an increase in necrosis was followed by a decline. Estimates of standard errors are in parentheses.

Plant part observed/ model fitted	Treatment[1)]	Parameter[2)]			
		b	*c*	*r*	*a*
Leaves/mm	36 h			0.40 (0.08)	0.9 (0.2)
Leaves/cd	24 h	0.01 (0.09)	0.30 (0.08)	0.25 (0.04)	1
Leaves/cd	16 h	0.02 (0.03)	0.20 (0.03)	0.32 (0.03)	1
Leaves/cd	8 h	0.01 (0.02)	0.06 (0.02)	0.28 (0.05)	1
Stem/mm	36 h			0.31 (0.03)	3.1 (0.1)
Stem/cd	24 h	-0.04 (0.11)	0.24 (0.09)	0.20 (0.04)	3.1
Stem/cd	16 h	-0.01 (0.09)	0.17 (0.09)	0.23 (0.07)	3.1
Stem/cd	8 h	-0.03 (0.05)	0.07 (0.04)	0.19 (0.06)	4

1) Wetness period after spore application.

2) Parameters are explained in Materials and methods.

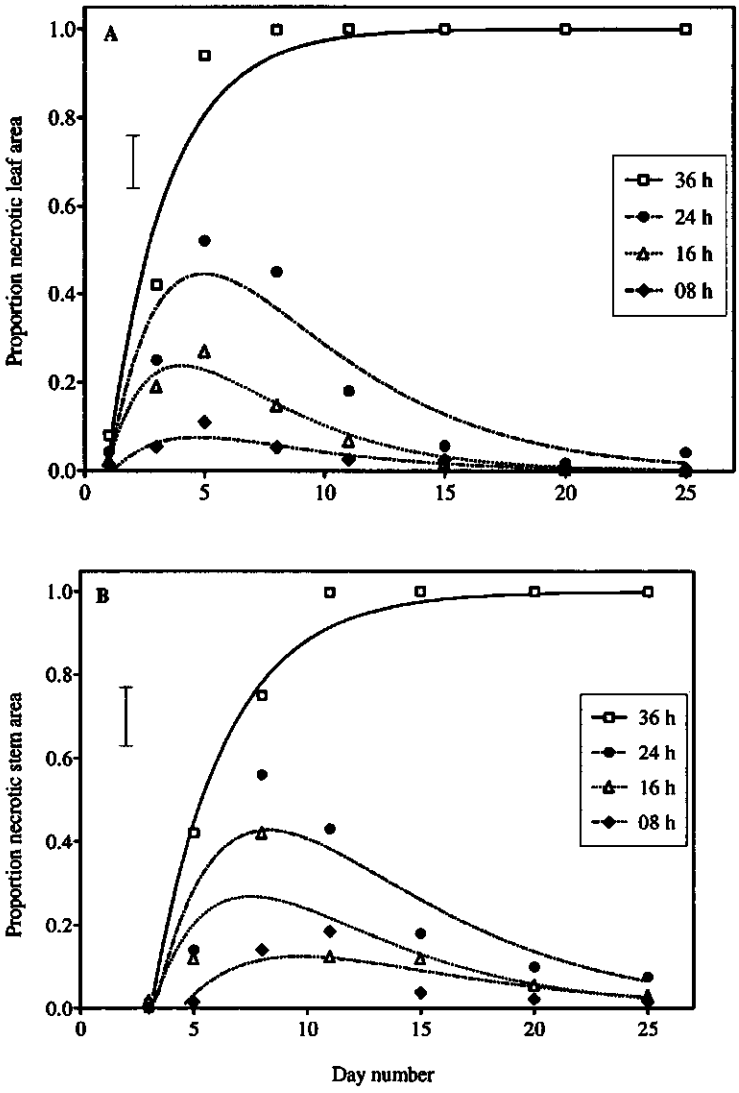

Fig. 4.2 Experiment 3. Dynamics of leaf necrosis (A) and stem necrosis (B) of *Chenopodium album* plants sprayed with a spore suspension of *Ascochyta caulina*. Sprayed plants were exposed to high humidity (>95% RH) for periods of 8, 16, 26 or 36 h and subsequently exposed to 75-85% RH. Entries are treatment averages. Curves show regressions of a monomolecular model (solid curves) or a non-monotonic, critically damped model (broken curves). Error bars indicate the angular-transformed LSD (P=0.05) divided by 100.

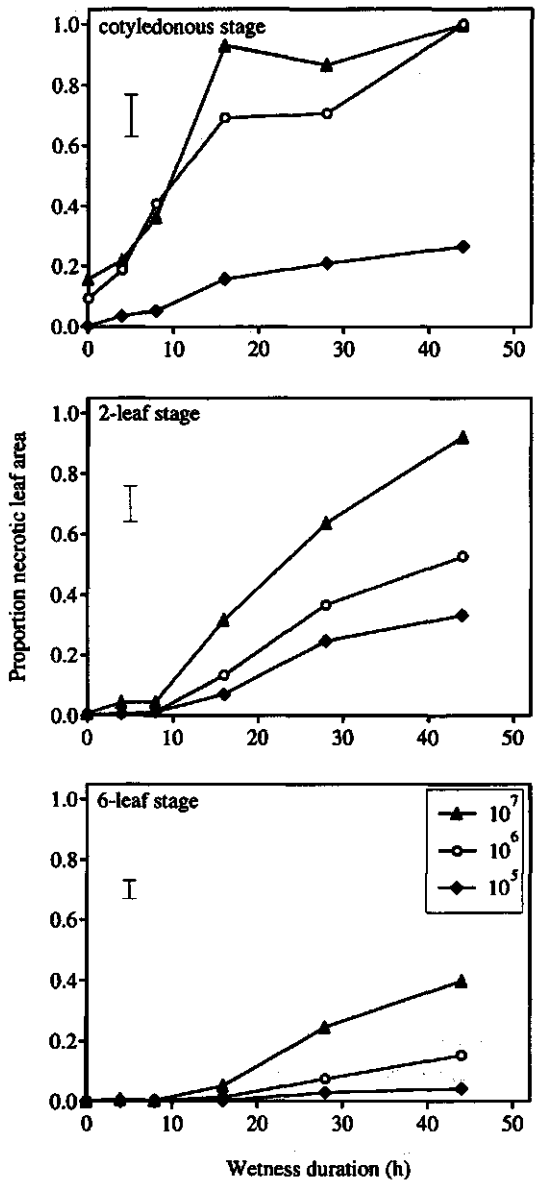

Fig. 4.3 Experiment 4. Effect of development stage (cotyledonous, 2-leaf or 6-leaf stage), spore density (10^5, 10^6 or 10^7 spores.ml^{-1}), and wetness period on leaf necrosis of *Chenopodium album* plants, assessed one week after application of *Ascochyta caulina* spores. Entries are treatment averages. Error bars indicate the angular-transformed LSD (P=0.05) divided by 100.

Experiment 5: Effect of temperature on disease development. Spore germination on agar was affected by temperature and incubation period (Table 4.3). Average leaf necrosis one week after spore application is shown in Fig. 4.4. Temperatures were confounded with high-humidity chambers. ANOVA of the whole data set showed a significant high-humidity chamber effect ($P<0.001$). We ascribe the differences between high-humidity chambers to a temperature effect. Leaf necrosis on plants exposed to 6 °C during the wetness period showed an interaction ($P<0.05$) between development stage and wetness period, while leaf necrosis on plants exposed to one of the other temperatures, were significantly ($P<0.05$) affected by both development stage and wetness period.

Experiment 6: Host-specificity. Disease symptoms were observed only on plants of the genera *Chenopodium*, *Atriplex* and *Spinacia* with significant differences between species. Plants sprayed with the spore-free contol fluid did not develop disease symptoms. Average leaf necrosis at one week after spore application is shown in Table 4.4. Plants that had not developed disease symptoms in the first week after spore application remained symptom free in the two consecutive weeks.

Table 4.3 Experiment 5. Effect of temperature on germination of *Ascochyta caulina* spores. Entries are percentages of germinated spores, assessed 16 h or 32 h after the spores were plated onto water agar in Petri dishes.

Incubation period	Temperature (°C)				
	6	12	18	24	36
16 h	5	46	72	85	79
32 h	35	85	77	82	80

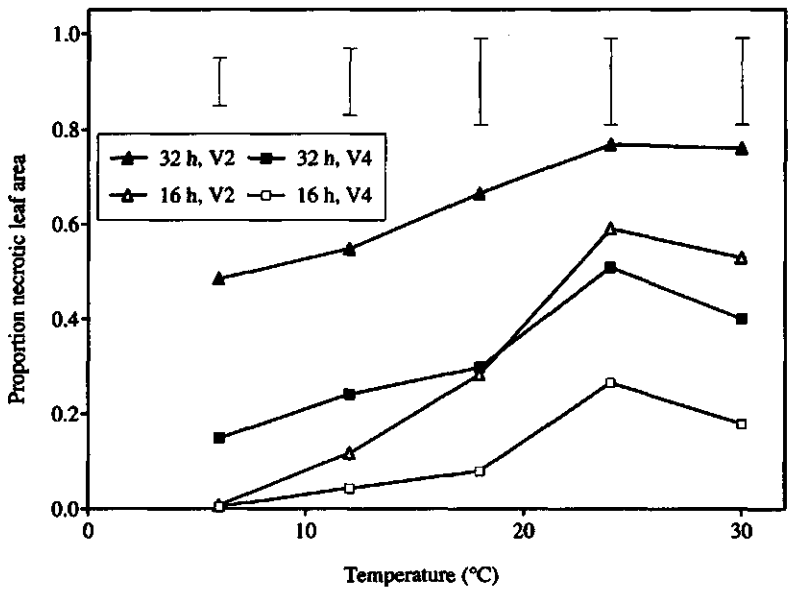

Fig. 4.4 Experiment 5. Effect of temperature, development stage (2-leaf or 4-leaf stage, V2 and V4 respectively), and wetness period (16 or 32 h) on leaf necrosis of *Chenopodium album* plants, assessed one week after application of *Ascochyta caulina* spores. Entries are treatment averages. Error bars indicate the angular-transformed LSD (P=0.05) divided by 100.

Table 4.4 Experiment 6. Average proportion of necrotic leaf area of juvenile plants of various plant taxons, assessed one week after application of *Ascochyta caulina* spores. Standard errors are in parentheses.

Plant taxon[1)]	Cultivar	Severity of leaf necrosis
Chenopodium album		0.30 (0.11)
Chenopodium ficifolium		0.35 (0.12)
Chenopodium quinoa	Elsevier	0.24 (0.10)
	Wild type	0.06 (0.05)
Chenopodium glaucum		0.11 (0.11)
Chenopodium polyspermum		0.02 (0.02) [2)]
Chenopodium rubrum		0 [2)]
Atriplex prostrata		0.35 (0.12)
Atriplex patula		0.27 (0.11)
Spinacia oleracea	Martine	0.02 (0.02)
	Amsterdams reuzenblad	0
Beta vulgaris subspecies *vulgaris*	Carla	0
	Lucy	0
	Univers	0
	Kyros	0
	Egyptische platte ronde	0
Corispernum marschallii		0
Zea Mays	Brazil	0
	Mandigo	0
Pisum sativum	Eminent	0
Triticum aestivum	Arminda	0
Brassica oleacerea ssp. *capitata*	Bartolo	0

1) The first 17 taxons are from genera of the plant family of Chenopodiaceae.

2) Chlorosis on leaves.

DISCUSSION

Disease development. Spray application of spores of *A. caulina* on *C. album* plants does not unconditionally result in disease development. A period of free moisture on inoculated plants or exposure of inoculated plants to high humidity was required for germination, infection, and development of necrosis. The necrosis development that was observed after spore application is part of a monocyclic process. *A caulina* infects the host tissue resulting in necrosis, but mature pycnidia and new spores are not formed at low RH and secondary spread does not occur. The course of necrosis with time could not be described by one model because of a dual response, necrosis either increased and subsequently decreased with time or necrosis increased with time up to complete necrosis. The former could be described by a non-monotonic, critically damped model, but the maximum necrosis levels were underestimated. The latter could be described by a monomolecular model. The meaning of the parameters of the non monotonic, critically damped model are poorly understood, unfortunately. Parameter *c* was the only parameter that was affected by treatments (Table 4.2). Parameter *c*, the rate of increase of necrosis when the first symptoms appear, is the tangent of the curve at the intercept with the *x*-axis. Gilligan (1990), who used this model to describe dynamics of fungi in soil, also observed that parameter *c* was most sensitive to changes in a system.

Disease development was affected by interactions between wetness period, spore density, plant development stage, and temperature. Disease was favoured by a long wetness period after spore application, a high number of spores applied, a young development stage at the time of spore application, and a temperature that allows fast spore germination (20-25 °C). Eggers and Thun (1988) reported 25 °C as the optimum temperature for germination of *A. caulina* spores. Variation in pathogenicity between the *A. caulina* isolates tested was large. We observed only small differences in resistance between source populations of *C. album*. However, this cannot be a generalized because of the small number of populations tested.

Wetness period appeared to be the crucial factor for disease development. A minimum wetness period of roughly 8 h was required for penetration of leaves by germination tubes and development of necrosis, under the given temperatures. However, there was one exception. Plants sprayed with spore suspensions at the cotyledonous stage (experiment 4a) developed necrosis, even when they were not exposed to high humidity. This result might suggest involvement of another, minor factor in disease development. Capasso *et al.* (1991) demonstrated that water-soluble toxins can be harvested from cultures of *Ascochyta* species. The necrosis on these young plants could have been caused by the action of a toxin in the suspension. If so, young *C. album* plants are more sensitive to the toxin than older plants.

Perspectives for biological control. Charudattan (1989) proposed two major criteria to assess the perspectives of plant pathogenic fungi as mycoherbicides: host specificity and efficacy of control. Charudattan's criteria were used to evaluate perspectives of *A. caulina*. Disease development by *A. caulina* was limited to plant species of three genera, *Chenopodium*, *Atriplex* and *Spinacia*. Among the susceptible species, there were two species with some economic importance, *S. oleracea* (a vegetable crop) and *C. quinoa* (an arable crop). We do not consider the susceptibility of these two crops a serious constraint to a further development of *A. caulina* into a mycoherbicide. However, more crop species have to be tested on host specificity before a final judgements of the agricultural risks involved with the use of *A. caulina* as a mycoherbicide.

Charudattan (1989) differentiated efficacy of control in amount, speed and ease of weed control. Amount and speed of control required will vary with each weed problem. At the present stage of knowledge, *A. caulina* will meet the demands for amount and speed of control when conditions are favourable for infection. Complete control can be attained within 14 days after spore application. Ease of control was defined by Charudattan as the possibility to produce, store and apply inoculum, and by the independence of disease development on environmental conditions. Evidently, disease development strongly depended on environmental conditions. This dependence might be a limitation to the use of *A. caulina* in practice because long periods of high humidity or free moisture on the target plants are infrequent under field conditions.

We hypothesize that incomplete control of *C. album* (less than 100% plant mortality) could be acceptable under certain conditions. Plants with a severity of leaf necrosis greater than 0.8 at one week after spore application all died in the second week after spore application. Plants with a severity of leaf necrosis of 0.6 to 0.8 at one week after spore application, either died in the second week after spore application, or were severely retarded in growth. Plants with a severity of leaf necrosis smaller than 0.2 at one week after spore application were hardly affected in their growth. Considering growth of sublethally infected plants, we expect that *C. album* plants with a severity of leaf necrosis greater than 0.6 will hardly cause competition damage to crops.

ACKNOWLEDGEMENTS

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PHOTOSYNTHETIC RESPONSE OF *CHENOPODIUM ALBUM* LEAVES TO LEAF NECROSIS CAUSED BY *ASCOCHYTA CAULINA*

C. Kempenaar, P.J.F.M. Horsten and P.C. Scheepens

ABSTRACT

The effect of leaf necrosis caused by *A. caulina* on photosynthesis of leaves of *C album* was studied in two climate chamber experiments. Leaves of young *C. album* plants were sprayed with a suspension of *A. caulina* spores or a spore-free control, and exposed to high relative humidity for 6, 18 or 24 h to cause different levels of necrosis. Individual leaves were assessed for proportion of necrotic leaf area and net photosynthetic rate 3, 4 and 7 days after treatment. The net photosynthetic rate of leaves decreased with increasing proportion of necrotic leaf area. The relationship between the two was linear in 5 of 6 regression analyses carried out. Linearity indicated that the necrotic leaf tissue hardly affected the surrounding leaf tissue during the early stage of necrosis development. The relationship between proportion of necrotic leaf area and net photosynthetic rate was constant during the early stage of necrosis development. Proportions of necrotic leaf area that caused a net photosynthetic rate of zero ranged from 0.2 to 0.5.

INTRODUCTION

Ascochyta caulina (P. Karst.) v.d. Aa & v. Kest. is one of the causal organisms of a local lesion disease of plants belonging to genera of *Chenopodium* L. and *Atriplex* L. (Van der Aa and Van Kesteren, 1979). The fungus may have potential as a mycoherbicide against *Chenopodium album* L., an important weed in many arable crops. In climate chamber experiments, we have demonstrated that the application of pycnidiospores of *A. caulina* to young *C. album* plants can have a large impact on these plants (Chapter 4). Infected plants showed retarded growth or died depending on the amount of necrosis developed. Plants that had developed a proportion of necrotic leaf area of 0.6 and more one week after application of pycnidiospores hardly showed any further growth. How necrosis affects growth in this pathosystem is poorly understood. In other pathosystems, it has been demonstrated that plant growth reduction due to disease depends on interactions between the plant, the pathogen and the environment, the plant development stage at infection and the subsequent duration and severity of the attack (Madeira *et al.*, 1994). Disease may affect plant growth in several ways. Changes in photosynthesis, respiration, transpiration and translocation of carbohydrates due to infection by fungi have been reported (Ayres, 1984). Probably, some of the physiological changes mentioned also occur in *C. album* leaves infected by *A. caulina*.

Objectives of the study presented here were to find a relationship between leaf necrosis caused by *A. caulina* and photosynthesis of leaves of young *C. album* plants, and to estimate the level of leaf necrosis at which the net photosynthetic rate becomes zero.

MATERIALS AND METHODS

Plant and fungal material. Two experiments were carried out with two batches of *C. album* plants. *C. album* seeds, harvested from a plant in a sugar beet crop at Wageningen, The Netherlands, in 1992, were stored at 5 °C in the dark until use. Seeds were planted in a peat soil in 600 ml pots in 1993 (experiment 1) and 1994 (experiment 2). The peat soil consisted of a mixture of 10 volumes of Triomf no. 17 peat (Trio B.V., The Netherlands) and 1 volume of coarse sand. The pots were placed in a climate chamber with a day-night regime of 14 h light at 18 °C and 75% RH and 10 h darkness at 12 °C and 85% RH. Light was obtained from Philips TLD 50W/84 lamps, and photosynthetic active radiation (PAR) at soil level ranged from 190 to 230 $\mu mol.m^{-2}.s^{-1}$. Plant density was 2 plants per pot. The soil was watered two times per week with a nutrient solution (Steiner, 1984).

An isolate of *Ascochyta caulina* (code 90-1), obtained from a naturally infected *C. album* plant at Wageningen, 1990, was used. Pycnidiospores of *A. caulina* were grown on wheat bran medium, and stored at 5 °C until use (Chapter 2). Spores were harvested from the wheat bran medium circa 3 h before their application to leaves. Cultures on the medium were flooded with distilled water with 0.05% (v/v) Tween 80, and 2 h later supernatants with suspended spores were collected. The suspensions were filtered over cheese cloth, diluted to 5.10^6 $spores.ml^{-1}$ and used as inoculum. A spore-free solution, consisting of distilled water with 0.05% (v/v) Tween 80, was used as a control.

Inoculation. When the plants of a batch had reached the 8-leaf stage, the inoculum or the spore-free control solution was applied to leaves of the second leaf pair of the plants. The leaves were sprayed till run-off using a DeVilbiss atomizer. At the day of application, the leaves of the second leaf pair were fully expanded, their average specific leaf dry matter weight was circa 30 $g.m^{-2}$. Time of application was 4 h before the lights went off in the climate chamber. Per experiment, leaves of at least 30 plants were sprayed with inoculum and leaves of 10 plants were sprayed with the spore-free control. Plants sprayed with inoculum were placed in a high humidity chamber (RH >95%, average PAR 200 $\mu mol.m^{-2}.s^{-1}$ from Philips TLD 50W/84 lamps, 18/12 °C) for 6, 18 or 24 h and

subsequently returned to the climate chamber in which they were originally grown. Plants sprayed with the spore-free solution were placed in the high humidity chamber for 24 h, and subsequently returned to the climate chamber. Conditions in the high humidity chamber and the climate chamber were synchronized.

Photosynthesis measurements and necrosis assessments. In both experiments, net photosynthetic rates of leaves were measured using equipment (*e.g.* Bastiaans and Roumen, 1993; Meurs and Kreuzer, 1995) placed inside the climate chamber. The equipment consisted of a leaf chamber, a CO_2 analyzer, an air-supply device and a data logger (Analytical Development Co., UK). Measurements were made 3, 4 and 7 days after inoculation, between 4 and 6 h after the lights had went on in the climate chamber. At each observation date, the net photosynthetic rate (P, in $\mu g\ CO_2.m^{-2}.s^{-1}$) of at least 24 inoculated leaves and 8 control leaves was assessed. Proportions of necrotic leaf area of inoculated leaves were visually assessed by means of standard diagrams (Chapter 4) at the time of measurement. Average PAR measured inside the leaf chamber differed between the two experiments, it was 160 or 210 $\mu mol.m^{-2}.s^{-1}$ for experiments 1 and 2, respectively.

Data analysis. Two equations were used to analyse the relationship between proportion of necrotic leaf area and net photosynthetic rate:

$$P_x = P_a + (P_0 - P_a) * (1 - x)^b \qquad (1)$$

$$P_x = P_0 - (P_0 - P_a) / (1 + e^{(-b*(x-m))}) \qquad (2)$$

where P_x is the net photosynthetic rate ($\mu g\ CO_2.m^{-2}.s^{-1}$) of a leaf with a proportion of necrosis of x, P_a is the net photosynthetic rate of a completely necrotic leaf, P_0 is the net photosynthetic rate of an uninfected leaf, and b and m are constants. Equation 1 was originally described by Van der Werf *et al.* (1990), it has a linear ($b = 1$) and a non-linear ($b \neq 1$) version. Equation 2 was derived from a simple logistic model. The models were fitted to data of each observation date using non-linear regression procedures of Genstat 5 (Payne *et al.* 1987). Treatment effects were tested by means of a t-test ($P<0.05$).

RESULTS AND DISCUSSION

The first necrotic spots on inoculated leaves were observed two days after application of pycnidiospores in both experiments. During the next 5 days, more necrosis developed (Fig 5.1). Levels of necrosis were affected by exposure time of inoculated leaves to high RH (>95%) and by day of observation, in correspondence with earlier observations (Chapter 4). In the second week after inoculation, leaves with severe necrosis (proportion of necrotic leaf area larger than 0.75) began to shrivel and dropped off. Therefore, the experiments were ended one week after inoculation. In experiment 1, levels of leaf necrosis were higher than in experiment 2.

Average net photosynthetic rate of uninfected leaves (P_0) differed significantly between the two experiments, but was not affected by the date of measurement. P_0 was 174 (± 41) $\mu g\ CO_2.m^{-2}.s^{-1}$ for experiment 1 and 306 (± 63) $\mu g\ CO_2.m^{-2}.s^{-1}$ for experiments 2, averaged out of the 3 times 8 assessments per experiment. The difference in average P_0 between the two experiments is explained by the difference in average PAR in the leaf chamber during the photosynthesis measurements.

Net photosynthetic rates of inoculated leaves are shown in Fig 1. Net photosynthetic rates decreased with increasing leaf necrosis. The level of leaf necrosis was confounded with exposure time to high RH. In preliminary experiments, we observed that net photosynthetic rates of leaves sprayed with water with 0.05% (v/v) Tween 80 and exposed to high RH for 24 h did not significantly differ from photosynthesis of untreated leaves under the given conditions. We ascribe the decrease of net photosynthetic rate directly to the increase of leaf necrosis and not to the increase of high RH.

The relationships between proportion of necrotic leaf area and net photosynthetic rate could be described by both models, but overall the model of Van der Werf gave better regressions than the logistic model. In experiment 1 the two models fitted equally well, but not in experiment 2 where the model of Van der Werf fitted much better than the logistic model. We decided to present only regressions with the model of Van der Werf. It appeared that the estimated values of parameter *b* did not differ significantly from 1 in 5 of the 6 data sets analysed indicating linear relationships between leaf necrosis and leaf photosynthesis. When parameter *b* did not differ significantly from 1, regressions with the linear version of the model are presented, otherwise the non-linear version (Table 5.1). The linearity of the relationships between leaf necrosis and leaf photosynthesis indicates that the necrotic leaf tissue hardly affected the surrounding visually healthy leaf tissue during the early stage of necrosis development. At a later stage of necrosis development (experiment 1, 7 days after inoculation) parameter *b* was significantly greater than 1. So, it is to be expected that the value of parameter *b* is not constant over time.

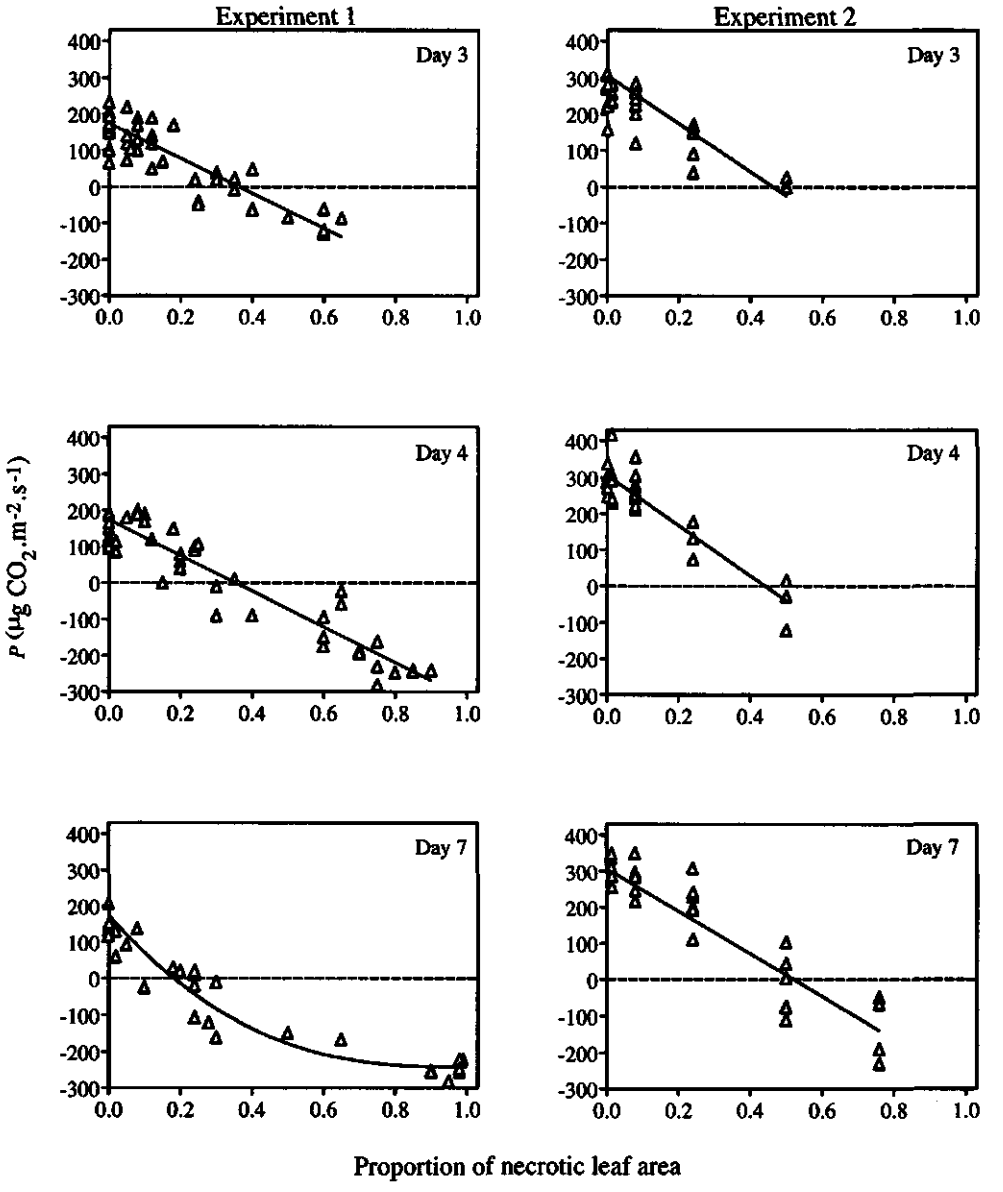

Fig 5.1 Relationships between proportion of necrotic leaf area and net photosynthetic rate (P_x) of *Chenopodium album* leaves infected by *Ascochyta caulina*, in two experiments. Net photosynthetic rates were measured 3, 4 and 7 days after application of pycnidiospores. Triangles are rates of individual leaves.

Table 5.1 Parameter values of a model[1)] describing a relationship between proportion of necrotic leaf area (x) and net photosynthetic rate (P_x, in $\mu g\ CO_2.m^{-2}.s^{-1}$) in two experiments and at three dates. Estimates of standard errors are in parentheses. R^2_{adj} is percentage of variance explained by regression. $X_{P=0}$ is the proportion of necrotic leaf area at which $P_x = 0$.

Days after	Experiment 1				Experiment 2			
inoculation	P_a	b	R^2_{adj}	$X_{P=0}$	P_a	b	R^2_{adj}	$X_{P=0}$
3	-306 (27)	1 [2)]	78	0.36	-360 (59)	1	61	0.46
4	-318 (19)	1	87	0.35	-388 (49)	1	83	0.44
7	-242 (16)	2.7 (0.3)	91	0.19	-280 (32)	1	84	0.52

1) $P_x = P_a+(P_0-P_a)*(1-x)^b$; parameters are explained in Material and methods.

2) when $b = 1$, the linear version of the model was fitted.

Estimates of $X_{P=0}$ of experiment 1 were smaller than the ones of experiment 2. In experiment 1, $X_{P=0}$ was affected by the date of observation, but not in experiment 2. The estimates of $X_{P=0}$ matched observations in other pathosystems. For instance, data on photosynthesis of rice leaves infected by *Pyricularia oryzae* Cavara, measured under field conditions, indicate that the net photosynthetic rate of leaves becomes less than zero when the proportion of necrotic leaf area becomes larger than 0.4 (Bastiaans, 1991). For *Septoria nodorum* (Berk.) Berk. and wheat, $X_{P=0}$ was estimated at 0.5 (Rooney, 1989).
We can use the estimates of $X_{P=0}$ for comparison of regression lines, for in our model $X_{P=0}$ determines the slope of the regression line (the absolute value of the slope is calculated by dividing P_0 by $X_{P=0}$). Regression lines are similar when both the intercepts and the slopes of the lines are similar. Since the intercepts (P_0) were set to a constant, similarity of regression lines can be determined on basis of the slopes of the regression lines. In experiment 1, the slopes of regression lines of days 3 and 4 after inoculation did not differ significantly, but they differed from the one at 7 days after inoculation. In experiment 2, the slopes were not affected by the date of observation. We can conclude that the relationship between leaf necrosis and leaf photosynthesis was constant during the early stage of necrosis development.

ACKNOWLEDGEMENTS

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CHAPTER 6

GROWTH AND COMPETITIVENESS OF *CHENOPODIUM ALBUM* AFTER FOLIAR APPLICATION OF *ASCOCHYTA CAULINA* AS A MYCOHERBICIDE

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ABSTRACT

To study perspectives of *A. caulina* as a post-emergence mycoherbicide against *C. album*, we conducted three field experiments in maize or sugar beet crops. *C. album* was transplanted into the crops. Suspensions of *A. caulina* pycnidiospores were applied shortly after *C. album* plants had emerged. Wetness durations after application of spores were varied to reach different levels of control. Disease development, plant mortality, dry matter weight and seed production of the weed, and dry matter weight of the crops were assessed throughout the vegetation season. Application of *A. caulina* resulted in necrosis of *C. album* plants. Average proportions of necrotic leaf area one week after application of spores ranged from 0.35 to 0.75. In the second and third week after application of spores, some plants died from infection. Plant mortality reached its maximum in the third week after application of spores. Average proportions of dead plants ranged from 0.05 to 0.65. Necrosis and plant mortality were affected by wetness duration in one of the three experiments. *C. album* plants that survived infection were reduced in size. Increase of dry matter weight of *C. album* plants with time could be described by a Gompertz model. Maximum dry matter weight of *C. album* plants was affected by the crop and by the amount of necrosis developed. Numbers of fruits per *C. album* plant showed a positive, almost linear relationship with plant dry matter weight. Seed weight was less affected by necrosis than number of fruits per plant. Competitiveness of *C. album* was reduced after infection by *A. caulina*. In maize, yield reduction by competition was prevented by application of *A. caulina*. In sugar beet, the effect of infection on growth of *C. album* was to small to prevent competition damage. A negative relationship between amount of control of *C. album* and yield reduction of sugar beet was demonstrated.

INTRODUCTION

Chenopodium album L. is an annual dicotyl plant species, considered world-wide as an important weed in many arable crops (*e.g.* Holm *et al.*, 1977). In Europe, *C. album* was ranked the most important weed in 10 major crops (Schroeder *et al.*, 1993), because of abundance, competitiveness, fertility, longevity of seeds in soil, and resistance developed to some herbicides. Presence of *C. album* plants in crops may cause severe crop yield reduction by competition; up to 100% yield losses have been reported (Zimdahl, 1980).

Crop yield reduction by competition of *C. album* depends on crop and weed characteristics, densities and spatial arrangements of plants, relative emergence dates, and environmental conditions. Seed production of *C. album* plants in crops also depends on the factors mentioned. Numbers of seeds produced may vary from 10 to 100,000 seeds per plant (Holm *et al.*, 1977).

Weed control in arable crops mainly consists of combinations of broad spectrum, chemical and mechanical methods. Selective weed control methods become of interest when broad spectrum methods fail or when the weeds on the field are dominated by one species. In this paper we describe a study on selective, biological control of *C. album* by a plant pathogenic fungus *Ascochyta caulina* (P. Karst.) v.d. Aa & v. Kest. Under natural conditions, *A. caulina* causes necrotic lesions on leaves and stems of plants of *Chenopodium* L. and *Atriplex* L. species. The fungus is endemic in Europe and Siberia, related to *Ascochyta hyalospora* (Cooke & Ellis) Boerema *et al.* (Van der Aa and Van Kesteren, 1979) which occurs on *Chenopodium* species in Northern America. Suspensions of pycnidiospores of *A. caulina* can be applied to plants in an inundative strategy after the mycoherbicide concept described by Daniel *et al.* (1973). We observed in climate chamber experiments that growth of *C. album* was reduced after infection by *A. caulina* according to the amount of necrosis developed (Chapter 4). Necrosis development of *C. album* plants depended on many factors among which number of spores applied, wetness duration after spore application, and plant development stage at the time of spore application were the most important ones. Considering the relationship between necrosis development and plant growth, we expect that competitiveness and seed production of *C. album* will be reduced after infection by *A. caulina* according to the amount of necrosis developed.

The objective of the study presented here was to investigate the effect of incomplete control of *C. album* on competition between crops and *C. album*, and seed production of the weed. Experiments with uniform densities and arrangements of *C. album* plants were conducted in maize and sugar beet crops. Spore suspensions of *A. caulina* were applied, and wetness durations were varied to reach different levels of control. Growth of *C. album* and crops plants was assessed throughout the vegetation seasons.

MATERIALS AND METHODS

Experimental design. Experimental fields were laid out in maize crops (*Zea mays* L. cv. Brazil), 1992 and 1993, and in a sugar beet crop (*Beta vulgaris* subspecies *vulgaris* cv. Univers), 1993, on an experimental farm near Wageningen, The Netherlands.

Experiments were encoded '92M, '93M and '93B, respectively. Soil type was a loamy fine sand with pH-KCl 5-6 and 2.5-3.5% organic material. Crop management was according to standard practice in the region, weed control excepted. Weeds were removed from experimental plots by hand. The experimental fields had dimensions of circa 80 by 30 m, and consisted of plots with a mono-culture of the crop or a bi-culture of *C. album* and the crop. Length of plots was 6 m. Width of plots was 4.5 m in maize or 3 m in sugar beet. The long axes of the plots were in the direction of the crop rows. Shortly after the crops were sown, germinated *C. album* seeds were transplanted into the crop rows of plots that were assigned a bi-culture after the method described in Chapter 3. Transplants were grown from seeds harvested from plants on arable fields near Wageningen in the years preceeding the experiments, and stored at 5 °C until use. Germinated seeds, together with small pieces of water agar, were transplanted onto positions in the crop rows, one transplant between every two crop plants in experiment '92C, or two transplants between every two crop plants in experiments '93M and '93B. Experimental data are given in Table 6.1. All established *C. album* plants were marked with labels.

A. caulina was isolated from a *C. album* plant at Wageningen in 1990, and maintained axenically on oat meal agar slants in test tubes at 5 °C. Pycnidiospores of the isolate were produced for application in the field. In 1992, they were produced on oat meal agar medium, in 1993 on a wheat bran medium (Chapter 2). At the day of application, cultures of *A. caulina* on the media were flooded with water with 0.05% (v/v) Tween 80. After 3 h, supernatants with suspended spores were collected and filtered through cheese cloth. Spore density was determined by means of a haemocytometer under a light microscope. The suspensions were diluted to 10^7 spores.ml^{-1}. Nutrients (3.5 g Czapek Dox broth l^{-1} and 0.4 g yeast extract l^{-1}) were added to the suspensions 2 h prior to application. Controls were treated by a spore-free solution of water, Tween 80 and nutrients.
The spore suspensions and the spore-free solutions were applied to plots by means of hand-automated, air pressure sprayers when *C. album* plants had 2 to 4 true leaves. Spray volume was such that adaxial sides of *C. album* leaves were completely wetted by the spray and run-off occurred. Spore density on *C. album* after spore application was circa 200 spores.mm^{-2}, assessed after the method of Bruzesse and Hasan (1983). Weather conditions were determined by the temperate sea climate of western Europe. Temperature, relative humidity and leaf wetness durations were measured in the experimental fields. Dates of spore application, and weather conditions after spore application are given in Table 6.2.

Experiment '92M consisted of 5 treatments, a mono-culture of maize (weed free control treatment; WF), bi-cultures of maize and *C. album* that were sprayed with a spore-free solution or a spore suspension (treatments S0 and S1, respectively), and bi-cultures of maize and *C. album* that were sprayed with a spore-free solution or a spore suspension followed by an artificial extension of the plant wetness duration (treatments S0** and S1**, respectively). Wetness duration was artificially extended by placing white plastic jars over sprayed *C. album* plants for 24 h. The jars had a content of 1100 cm^2 (Ø 10 cm, height 15 cm). Experiment '92M had a randomized block design with 4 replicates.
Experiment '93B consisted of 6 treatments, a mono-culture of sugar beet (WF), bi-cultures of sugar beet and *C. album* that were sprayed with a spore-free solution followed by no or an artificial extension of the wetness duration of 24 h (treatments S0 and S0**, respectively), and bi-cultures of sugar beet and *C. album* that were sprayed with a spore suspension followed by no or an artificial extension of the wetness duration of 16 or 24 h (treatments S1, S1* and S1**, respectively). Wetness durations were artificially extended by spraying the plots with 0.02 l water.m^{-2} every 20 minutes, from shortly after spore application (19.15) till after sun set (22.00) and during the next day from sun rise (05.30) till 11.00 or 19.00. Experiment '93B had a randomized block design with 6 replicates.
Experiment '93M was a replicate of experiment '93B, the crop excepted. Due to a whole day of rain after spore application, wetness durations were not artificially extended in this experiment.

Plants were harvested from the experimental plots at 5 to 7 dates during the vegetation season. *C. album* and maize plants were cut off at ground level. Sugar beet plants were carefully pulled out of the soil. Harvested area per plot were 0.3 m^2 at the first harvest date, 1.5 m^2 per plot at the last harvest date, and 0.5 to 1.2 m^2 at the intermediate harvest dates. Harvested plants were separated in leaves, stems, and, if present, generative parts and beet roots.

Assessments.

1. Leaf necrosis. Proportions of necrotic leaf area per plant were determined one week after spore application. At first, proportions of necrotic leaf area of individual leaves were estimated by means of standard diagrams (Chapter 4). Area of the individual leaves were measured by means of a leaf area meter (LI-COR, USA; Model 3100). Necrotic area of the individual leaves were calculated by multiplying the leaf area by the corresponding proportion of necrotic area. Proportions of necrotic leaf area of plants were calculated by dividing the summarized the necrotic leaf area of a plant by

the summarized leaf area of that plant. Average proportions of necrotic leaf area per plot were calculated.

2. Plant mortality. Proportions of dead *C. album* plants were determined when plant mortality had reached its maximum by observing 60 planting positions per plot.
3. Plant dry matter weight. Harvested plants were dried at 70 °C for two days. Average dry matter weights per plot were determined.
4. Number of fruits per *C. album* plant. At harvest dates 224, 242 and 247 for experiments '93B, '93M and '92M, respectively, numbers of fruits per plant were determined and averaged per plot. Both fruits with immature and with mature seeds were counted.
5. Seed weight. At the last harvest dates, 242, 247 and 263 for experiments '93M, '92M and '93B, respectively, circa 1000 dry *C. album* seeds were taken from the seeds harvested from a plot and weighted.

Data analysis. Treatment effects on data of a particular harvest date were tested by means of analysis of variance (ANOVA) using Genstat 5 software (Payne *et al.*, 1987). In some analyses, treatments were grouped and contrasts between groups were tested. Variances were balanced, if required, with angular or natural log transformations.
Dry matter production of *C. album* plants in the experimental plots was described with a Gompertz model (*e.g.* Campbell and Madden, 1990):

$$y = c * \exp(-\exp(-b * (t - m)))$$

where y is average dry matter per plant (gram) at time t (day number of the year), c is maximum dry matter per plant (gram), b is a shape parameter (day number^{-1}), t is harvest date (day number), and m is a location parameter (day number). The model was fitted to data of each plot by means of non-linear regression procedures of Genstat 5. Numbers of plants harvested per plot at a particular harvest date were used as a weight factor in the regressions. Treatment effects on parameter estimates were tested by means of ANOVA.

Table 6.1 Dates of action or events, and plant arrangements in three experiments in 1992 and 1993; M indicates maize, B sugar beet. Dates are expressed as day number of the year.

	Experiment		
	'92M	'93M	'93B
Crop			
- date of sowing	128	118	103
- date of emergence	139	129	115
- density (plants.m^{-2})	10	11	11
- row distance (m)	0.75	0.75	0.50
Chenopodium album			
- date of transplanting	139	134	119
- date of emergence	142	138	123
- density (plants.m^{-2})	9	22	22

Table 6.2 Weather conditions after spore application in experiments '92M, '93M and '93B.

	Experiment		
	'92M	'93M	'93B
Date of spore application	156	147	132
Time of spore application (h)	16.00	16.00	19.00
First 24 h after spore application			
- weather type	Cloudy, showers	Cloudy, showers	Cloudy, a thunder storm at 22.00
- leaf wetness duration (h)	18	23	12
- average temperature (°C)	14	11	15
- min. - max. temperature (°C)	12 - 18	10 - 14	12 - 24
- average relative humidity	87	91	79
- min. - max. relative humidity	59 - 100	84 - 100	43 - 100
First week after spore application			
- weather type	Comparable to the first 24 h, hardly any dew	Little warmer, a few short showers, some dew	Little colder, some showers, hardly any dew

RESULTS AND DISCUSSION

Disease development was observed only on *C. album* plants that were sprayed with a spore suspension of *A. caulina*. Onset of necrosis was 2 to 3 days after spore application. Average proportions of necrotic leaf area one week after spore application are shown in Table 6.3. Extension of wetness duration resulted in significantly different levels of leaf necrosis only in experiment '93B.

In the second and third week after spore application, *C. album* plants died from infection according to the amount of necrosis developed. Maximum levels of plant mortality were reached in the third week after spore application (Table 6.3). Extension of wetness duration resulted in significantly different levels of plant mortality only in experiment '93B.

C. album plants that survived infection dropped their infected leaves, and formed new leaves with hardly any secondary infections. As a result, proportions of necrotic leaf area of these plants decreased after the maximum was reached in the third week after spore application. The time course of leaf necrosis (an increase of necrosis followed by a decrease) of the survivors was as observed for sublethally infected *C. album* plants in climate chamber experiments (Chapter 4).

Table 6.3 Effect of application of spore suspensions of *Ascochyta caulina* to *Chenopodium album* plants on leaf necrosis and plant mortality. Standard errors of the mean are given in parentheses.

Experiment	Treatment	Average proportion of necrotic leaf area after 1 week		Average proportion of dead plants after > 2 weeks	
'92 Maize	S1	0.39	(0.08) a[1]	0.30	(0.11) a
	S1**	0.58	(0.09) a	0.50	(0.17) a
'93 Maize	S1	0.74	(0.03) a	0.62	(0.06) a
	S1*	0.61	(0.04) a	0.51	(0.06) a
	S1**	0.70	(0.03) a	0.65	(0.09) a
'93 Beet	S1	0.35	(0.03) a	0.05	(0.01) a
	S1*	0.65	(0.02) b	0.26	(0.03) b
	S1**	0.75	(0.06) b	0.43	(0.06) c

1 Treatment averages per experiment were separated with different letters according to LSD-tests of angular-transformed data ($P<0.05$).

C. album plants that survived infection were retarded in their growth according to the amount of necrosis developed. Relationships between harvest date and average dry matter of *C. album* plants (expressed as gram.plant^{-1}) in a plot could be described by the Gompertz model for experiments '92M and '93M, and for experiment '93B when the data of the last harvest date were excluded from the regressions. The reason for exclusion of these data was that the *C. album* plants had lost a large number of their senescent leaves at the last observation date, resulting in an apparent decrease of dry matter. Treatment averages of the estimated parameters are shown in Table 6.4. Parameter *c* (maximum dry matter per plant) was significantly affected by the spore application treatments in all experiments and by artificially extension of wetness duration in experiment '93B.

Table 6.4 Effect of treatments on parameters of a Gompertz model for sigmoidal growth fitted to data of dry matter weight of *Chenopodium album* plants. Treatment averages and, in parentheses, standard errors of the mean are presented.

Experiment	Treatment	Parameters		
		c (gram.plant^{-1})	*b* (day number^{-1})	*m* (day number)
'92 Maize	S0	24.6 (1.0) a[1]	0.11 (0.07) a	195 (5) a
	S0**	19.6 (1.4) a	0.05 (0.01) a	196 (4) a
	S1	7.2 (4.3) b	0.18 (0.08) a	189 (5) a
	S1**	2.4 (3.5) b	0.17 (0.08) a	198 (17) a
'93 Maize	S0	12.8 (1.0) a	0.16 (0.07) a	184 (1) a
	S1	4.7 (1.4) b	0.15 (0.06) a	192 (4) ab
	S1*	5.4 (1.3) b	0.10 (0.06) a	196 (3) b
	S1**	4.6 (0.6) b	0.18 (0.05) a	191 (2) ab
'93 Beet	S0	110 (10) a	0.09 (0.02) a	190 (3) a
	S1	72 (11) b	0.08 (0.04) a	190 (3) a
	S1*	46 (7) c	0.15 (0.03) a	193 (5) a
	S1**	46 (5) c	0.12 (0.02) a	194 (4) a

1 Treatment averages per experiment were separated with different letters according to LSD-tests ($P<0.05$).

We observed that the average time of onset of flowering of *C. album* plants that survived infection did not differ from uninfected control plants. In the second half of August, the flowering plants had formed most of their fruits. Average numbers of fruits per plant at day numbers 224, 242 and 247 for experiments '93B, '93M and '92M, respectively, are given in Table 6.5. At the observation dates, 30-60% of the fruits carried a mature seed. Average number of fruits per plant was significantly affected by the spore application treatments in all experiments, and by extension of wetness duration in experiments '92M and '93B. It showed a positive relationship with average plant dry matter weight. Seed weight, assessed at the last harvest dates, was less affected by treatments than numbers of fruits per plant (Table 6.5).

Table 6.5 Effect of treatments on average number of fruits per plant, and seed weight. Treatment averages were assessed at times the numbers of fruits per plant and the seed weights were maximum. Standard errors of the mean are in parentheses.

Experiment	Treatment	Number of fruits per plant (*10^3)	Seed weight (mg)
'92 Maize	S0	11.5 (1.6) a[1]	0.58 (0.02) a
	S0**	9.3 (2.0) a	0.56 (0.01) a
	S1	2.4 (0.4) b	0.57 (0.01) a
	S1**	0.6 (0.4) c	0.46 (0.04) b
'93 Maize	S0	9.3 (0.7) a	0.53 (0.03) a
	S1	2.7 (0.8) b	0.44 (0.03) ab
	S1*	2.3 (0.6) b	0.35 (0.03) b
	S1**	2.6 (0.6) b	0.44 (0.04) ab
'93 Beet	S0	71 (6) a	0.66 (0.02) a
	S1	43 (6) b	0.63 (0.02) a
	S1*	29 (4) b	0.68 (0.01) a
	S1**	30 (4) b	0.64 (0.02) a

1 Treatment averages per experiment were separated with different letters according to LSD-tests ($P<0.05$).

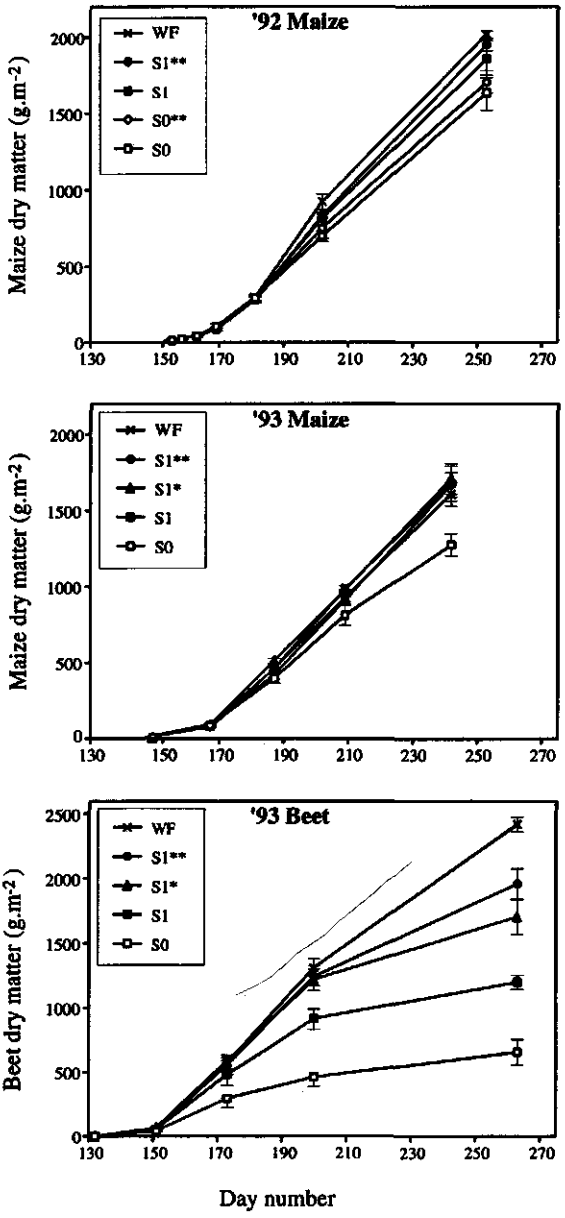

Fig. 6.1 Effect of presence or absence of *Chenopodium album*, and control of *C. album* by *Ascochyta caulina* on maize and sugar beet dry matter weight in experiments in 1992 and 1993. Entries are treatment averages, error bars standard errors of the mean. The dry matter consisted of aerial parts for maize and both aerial parts and beet roots for sugar beet.

Dry matter weight (DM; expressed as gram.m^{-2}) of maize was less affected by competition with *C. album* than DM weight of sugar beet. DM showed a positive relationship with the level of *C. album* control (Fig. 6.1). In experiments '92M and '93M, DM weight of maize of treatment S0 (no control) differed significantly from that of treatment WF (weed free) after day 200 (P<0.05). At the last harvest dates of these experiments, S0 were circa 20% smaller than those of WF. All spore application treatments did not differ from WF at any observation date (P>0.05).
In experiment '93B, DM weight of sugar beet of treatment S0 differed significantly from that of WF beginning at day 173. At the last harvest date of this experiment, S0 was 80% smaller than WF. S1 differed significantly (P<0.05) from WF after day 200. S1* and S1** differed significantly from WF at the last harvest date only (P<0.05). In sugar beet, specific leaf area (average leaf area per plant divided by average leaf dry matter weight) and shoot-root ratio of S0 were significantly greater than those of the other treatments (P<0.05), indicating that S0 was more affected by competition for light than the other treatments.

The data presented show that application of pycnidiospores of *A. caulina* to young *C. album* plants in a crop can have a large impact on the weed. Necrosis of *C. album* plants can be observed within several days after application of the fungus, plant mortality within two to three weeks. Necrosis development and plant mortality were affected by wetness duration after spore application. *C. album* plants that survived infection by *A. caulina* produced less dry matter, less fruits (≈ seeds) and were less competitive compared to uninfected plants. Average number of fruits per *C. album* plant showed a positive relationship with plant dry matter weight. An evaluation of propagation of *C. album* at incomplete levels goes beyond this study as acceptability depends on population dynamics of *C. album* seeds that are already present in the soil, population dynamics of the seeds that are added to the seed bank, and availability of control methods in forthcoming years (discussed in Chapter 9). In maize, but not in sugar beet, yield reduction by competition of *C. album* was prevented at incomplete levels of control, indicating that incomplete levels of control of *C. album* can be accepted more easily in a tall crop.

ACKNOWLEDGEMENTS

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CHAPTER 7

INFECTION AND CONTROL OF *CHENOPODIUM ALBUM* BY APPLICATION OF *ASCOCHYTA CAULINA* TO THE SOIL

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ABSTRACT

The effect of application of pycnidiospores of *A. caulina* to the soil on *C. album* and five cultivated plant species was investigated under controlled conditions. Spores of *A. caulina* were applied to the soil either by mixing spore suspensions through the top layer of the soil or by spraying spore suspensions on the soil. The presence of spores of *A. caulina* in the soil resulted in disease development on *C. album* and to a lesser degree on *Spinacia oleracea* seedlings, but not on *Beta vulgaris* subspecies *vulgaris*, *Zea mays*, *Triticum aestivum* and *Pisum sativum*. Affected *C. album* seedlings had an abnormal olive-green colour or carried necrotic spots on cotyledons and hypocotyls. They were retarded in growth or died due to infection. Affected *S. oleracea* seedlings were pale or carried necrotic spots on the cotyledons. Time courses of disease incidence and of mortality of *C. album* could be described by a monomolecular model. Six factors that may limit disease development, and thus efficacy of the mycoherbicide, were studied. These factors were spore density in the soil, way of spore application, sowing depth, soil moisture content, soil type, and survival during a host-free period. Disease incidence and mortality were influenced by spore density, soil moisture and soil type, not or hardly by way of spore application or sowing depth. Spores maintained their effectiveness in soil for a period of at least one week. We estimated that 10^9 to 10^{10} spores.m^{-2} were required for 50% mortality of emerged *C. album*.

INTRODUCTION

The fungus *Ascochyta caulina* (P. Karst.) v.d. Aa & v. Kest. (Van der Aa and Van Kesteren, 1979) causes necrotic spots on leaves and stems of *Chenopodium* L. and *Atriplex* L. species. It was studied for use as a mycoherbicide against *Chenopodium album* L. Mycoherbicides are environmentally benign biological control agents developed from indigenous fungi that normally remain at endemic levels (Templeton, 1992). Natural levels are increased by applying the fungi in an inundative strategy. *C. album* is an annual herbaceous plant which can be found on arable fields and ruderal sites in almost all inhabited areas of the world. *C. album* seeds can survive in soil for many years and *C. album* plants have a relatively strong competitive ability. These characteristics rank *C. album* in the top of the list of noxious weeds (Holm *et al.*, 1977; Schroeder *et al.*, 1993).

Application of pycnidiospores of *A. caulina* to young *C. album* plants showed promising control of the weed (Chapters 4 and 6). The level of control largely depended on availability of water for germination and infection. In search for possibilities to lower the dependency of water we studied the effect of application of *A. caulina* to the soil on control of *C. album*. The objectives were twofold: first to investigate infection of *C. album* and five cultivated plant species by pycnidiospores of *A. caulina* applied to the soil, and second to investigate factors that may limit disease development (Table 1.1). Six factors, spore density, way of spore application, depth from which plants emerge, soil moisture content, soil type and survival of spores in soil, were studied in greenhouse experiments.

MATERIALS AND METHODS

Inoculum production. A caulina was isolated from a naturally infected *C. album* plant in an arable crop at Wageningen, The Netherlands, 1990. The isolate was stored on oat meal agar slants in test tubes in the dark at 5 °C until use. Oat meal agar was made by suspending 60 g oat meal in 1 l demineralized hot water. The suspension was boiled and stirred for several minutes. Finally agar was added (2% w/w) to the suspension. The oat meal agar was sterilized at 120 °C for 30 min.
Pycnidiospores of *A. caulina* were produced on oat meal agar plates in Petri dishes (⌀ 9 cm). The plates were inoculated with spores of *A. caulina* suspended in demineralized water (circa 10^6 spores per plate) and these were placed in an incubation chamber under continuous light (75 $\mu mol.m^{-2}.s^{-1}$; Philips TL 13W/83) at 20 °C. After 9-16 days, cultures on the plates were flooded with 10 ml sterile demineralized water. Supernatants with suspended spores were collected after 3 h. Suspensions were filtered through cheese cloth and adjusted with demineralized water to desired densities. Spore densities were determined by means of a haemocytometer under a light microscope (Tuite, 1969).

Plant production. Seeds of *C. album*, collected from plants on arable fields at Wageningen, The Netherlands, in 1992 were stored in jars in the dark at 5 °C until use. Seeds of *Beta vulgaris* subspecies *vulgaris* cv. Carla (sugar beet), *Spinacia oleracea* cv. Martine (spinach), *Zea mays* cv. Mandigo (maize), *Triticum aestivum* cv. Arminda (wheat) and *Pisum sativum* cv. Eminent (pea) were taken from commercial seed batches. If coatings were present on seeds, they were removed by several washings in water.
Germination of seeds was induced prior to sowing. The seeds were placed on water agar (1% w/w) in Petri dishes (⌀ 9 cm) and these were placed in an incubation chamber with a

day-night regime of 14 h light (20 $\mu mol.m^{-2}.s^{-1}$; Philips TL 8W/33) at 25 °C and 10 h darkness at 15 °C for two days. Germinated seeds (maximum root tip length was 2 mm) were sown in soils in plastic pots (soil volume 600 ml and soil surface 72 cm^2). The seeds were sown in a square pattern, 25 seeds per pot for *C. album* and 16 seeds per pot for the other plant species tested. The pots were placed in a greenhouse. Growth conditions were 12-22 °C, 65-90% relative humidity and a 15-17 h day light period. Daily incoming photosynthetic active radiation was circa 20 to 30 $mol.m^{-2}.day^{-1}$. A sterilized sandy soil (Sand_1), a mixture of coarse sand and silver sand (1:1 w/w), was used in all experiments. Sand_1 contained very small amounts of nutrients and organic material. In one experiment three other soils were also used, a sandy soil from an arable field at Wageningen (Sand_2), a loamy clay soil from an arable field at Lelystad, The Netherlands and a commercial peat soil. The water content of the soil was kept between pre-set levels. Every two to three days the weight of the pots was determined. If the weight had dropped below the pre-set level, the pot was placed in a tray with water. When the soil had absorbed the desired amount of water, the pot was placed back in the greenhouse.

Spore application. Two application methods were used. With method 1, spores were applied prior to sowing. Pots were partly filled with 500 ml soil and topped with a layer of 100 ml soil (layer thickness 1 cm) through which a spore suspension was mixed. Germinated seeds were sown in the soil at specific depths, *C. album* at a depth of 0.5 cm and the other plant species at a depth of 1.5 cm. With method 2, spores were applied after sowing. Germinated seeds were sown in pots with 600 ml of soil. Subsequently, 5 ml spore suspension were sprayed on the soil of a pot with a DeVilbiss sprayer. Spore density in/on the soil was calculated from the number of spores applied.

Disease assessment and data analysis. The number of emerged, diseased and dead plants per pot was regularly counted over a period of about one month. The numbers of diseased and dead plants per pot were summarized which resulted in the number of affected plants per pot. Numbers were converted into proportions. Proportions of emerged plants per pot were related to the number of seeds per pot and proportions of affected and dead plants per pot were related to the number of emerged plants per pot.
A monomolecular regression model (equation 1; Campbell and Madden, 1990) was used to describe disease incidence with time and mortality with time. In equation 1, y is a proportion of plants at time t, r is the rate parameter (effective contacts per plant per day), t is time after sowing and a is the intercept of the regression line with the x-axis.

$$y = 1 - e^{\{-r*(t-a)\}} \qquad (1)$$

A logit-log regression model (equation 2; Zadoks and Schein, 1979) was used to estimate spore densities that caused disease symptoms on 50% of the plants (ED_{50}) and mortality of 50% of the plants (LD_{50}). In equation 2, y is a proportion of plants, a is the intercept of the regression line with the y-axis, x is the spore density and b is the slope of the regression line. ED_{50} and LD_{50} were calculated according to equations 3 and 4.

$$Logit(y) = a + b * Log(x) \tag{2}$$

$$ED_{50} = 10^{\{-a_{ED}/b_{ED}\}} \tag{3}$$

$$LD_{50} = 10^{\{-a_{LD}/b_{LD}\}} \tag{4}$$

Treatment effects on proportions were determined using a generalized linear regression model with a logistic link function (McCullagh and Nelder, 1989). Treatment effects on b_{ED}, b_{LD} and on log-transformed ED_{50}, LD_{50} and dry matter weight per plant were studied by analysis of variance. Regression analyses and analyses of variance were done by means of Genstat 5 software (Payne *et al.*, 1987).

Experiment 1: Host-specificity. Germinated seeds of *C. album* and cultivars of *B. vulgaris* subspecies *vulgaris*, *S. oleracea*, *Z. mays*, *T. aestivum*, and *P. sativum* were sown in sand through which spore suspensions were mixed. Spore densities were 0 or 10^6 spores.cm^{-2} soil surface area. Each treatment consisted of two pots with each 25 (*C. album*) or 16 (other species) seeds. The pots were placed in a complete randomized design. The soil moisture content was 15% at sowing and fluctuated between 12-18%. Disease development was monitored till the plants had four to six leaves.
Seventeen days after sowing some diseased plants were harvested to make re-isolations from diseased plant tissue. Small pieces of diseased tissue were surface-sterilized with sodium hypochlorite (3% v/v) for 30 s and placed on oat meal agar. Isolated colonies were compared with those of the original *A. caulina* isolate.

Experiments 2a and 2b: Effect of spore density and application method on disease incidence. In experiment 2a germinated seeds of *C. album* were sown in sand through which spore suspensions were mixed (application method 1). Spore density ranged from 0 to $1.5*10^6$ spores.cm^{-2} soil surface area. The experimental unit was a treated pot with 25 seeds. Each treatment consisted of five pots which were randomly assigned to five blocks.

The experiment had a one-factor (spore density) randomized block design with five replicates. Each replicate was a block with seven pots, one pot of each spore density.
In experiment 2b spore suspensions were sprayed over sand (application method 2) in which germinated seeds of *C. album* were already sown at a depth of 0.5 cm. In total there were five spore density treatments ranging from 0 to $1.6*10^6$ spores.cm^{-2} soil surface area. The experimental design was equal to that of experiment 2a. In both experiments the soil moisture content was 15% at sowing and fluctuated between 12%-18%.
Emergence, disease incidence and mortality were determined for both experiments every three to five days till 32 (expt 2a) or 30 (expt 2b) days after sowing. The proportions of emerged, affected and dead plants on the final day of observation were analysed statistically. The monomolecular model was fitted to data of each treatment. The logit-log model was fitted to data of each block of the final days of observation. The ED_{50} and LD_{50} were calculated.

Experiment 3: Effect of sowing depth on disease incidence. Germinated seeds of *C. album* were sown in sand at a depth of 0.1, 0.5 or 1.0 cm. Spore suspensions were sprayed on the soil surface. Spore densities were 0 or $1.6*10^6$ spores.cm^{-2} soil surface area. The soil moisture content was 15% at sowing and fluctuated between 12%-18%. The experiment had a two-factor (sowing depth and spore density) randomized block design with five replicates. The proportions of emerged, affected and dead plants 30 days after sowing were analysed statistically.

Experiment 4: Effect of soil moisture content on disease incidence. Germinated seeds of *C. album* were sown in sand through which spore suspensions were mixed. Spore density ranged from 0 to $1.4*10^6$ spores.cm^{-2} soil surface area. Soil moisture contents were either 10%, 15% or 18% at sowing and average (and min-max) soil moisture contents over a period of three weeks were 9.4 (8-10%), 14.4% (13-15%) or 17.1% (16-18%), respectively. The soil moisture range (circa pF 2.0 - pF 2.8) allowed plant growth to be undisturbed by water stress. The experiment had a two-factor (soil moisture content and spore density) randomized block design with five replicates. The logit-log model was fitted to data of each block of the final day of observation (28 days after sowing). The proportion of emerged plants, ED_{50}, b_{ED}, LD_{50} and b_{LD} on this day were analysed statistically.

Experiment 5: Effect of soil type on disease incidence and plant growth. Germinated seeds of *C. album* were sown in soils through which spore suspensions were mixed. Spore density ranged from 0 to $2.8*10^6$ spores.cm^{-2} soil surface area. The soil types were

sand_1, sand_2, clay and peat with a soil moisture content of 15%, 15%, 35% or 70% at sowing, respectively. The soil moisture contents fluctuated between 12-18%, 14-21%, 34-42% and 60-75%, respectively. The highest soil moisture levels were close to the maximum water capacities of the respective soils. The experiment had a two-factor (soil type and spore density) randomized block design with four replicates. The logit-log model was fitted to data of each block of the final day of observation (22 days after sowing). The proportion of emerged plants, ED_{50}, b_{ED}, LD_{50} and b_{LD} on this day were analysed statistically. On the final day of observation plants that had not died were uprooted, washed and dried at 105°C for two days. Plant dry matter weight was determined and analysed statistically.

Experiment 6: Effect of time between sowing and inoculation on disease incidence. Spore suspensions were mixed through sand and the mixture was stored in the greenhouse until use. During storage the soil moisture content was 15%. Germinated seeds of *C. album* were sown in the soil on the day of inoculation (day 0), or 7 or 14 days later. Spore densities were 0 or $3.9*10^5$ spores.cm^{-2} soil surface. The soil moisture content fluctuated between 12-18% after sowing. The experiment had a two-factor (relative time of sowing and spore density) randomized block design with five replicates. The proportions of emerged, affected and dead plants 32 days after sowing were analysed statistically.

RESULTS

Experiment 1: Host-specificity. Disease symptoms developed on seedlings of *C. album* and *S. oleracea* that emerged from inoculated soil, but symptoms were far more severe on *C. album* than on *S. oleracea*. The fungus that was isolated from diseased *C. album* and *S. oleracea* tissue on oat meal agar matched the characteristics of the original *A. caulina* isolate. Seedlings of *B. vulgaris* subspecies *vulgaris*, *Z. mays*, *T. aestivum* and *P. sativum* that emerged from inoculated soil showed neither disease symptoms nor growth reduction.

On *C. album* the first symptoms were observed a few days after emergence. Symptoms consisted of an abnormal olive-green shoot colour or necrotic spots on cotyledons and hypocotyls. Olive-green plants often wilted and died within a week after emergence. Necrotic spots on cotyledons of *C. album* were round to irregularly shaped and had a yellow-brown colour. Cotyledons of affected plants often curled downward and shrivelled. Necrotic spots on hypocotyls were round to oval shaped and had a grey-brown colour. Hypocotyl spots appeared most frequently near the soil surface. Spots that girdled

the hypocotyl resulted in toppling and dying plantlets. Pycnidia were observed on dead plant tissue that had dropped on the soil surface.
On *S. oleracea* the first symptoms were observed in the second week after emergence. Affected plants were pale or carried irregularly shaped red-brown spots on cotyledons. They were retarded in growth but did not die.

Experiments 2a and 2b: Effect of spore density on disease incidence. Most plants emerged within seven days after sowing. The proportion of seeds that emerged, 0.76 (expt 2a) and 0.59 (expt 2b), was not influenced by the spore density in the soil ($P>0.05$). The proportion of affected plants increased with time, at least when the soil contained spores, and increased faster at higher spore densities (Figs 7.1A and 7.2A). The proportion of affected plants on the final day of examination was influenced by spore density ($P<0.001$). The proportion of dead plants increased with time, at least when the soil contained spores, and increased faster at higher spore densities (Figs 7.1B and 7.2B). The proportion of dead plants on the final day of examination was influenced by spore density in the soil ($P<0.001$). The monomolecular regression model showed significant relationships between disease incidence and time, and mortality and time ($P<0.05$). Parameter a was hardly influenced by the treatments. We decided to set parameter a to a fixed value, 6 for disease incidence and 8 for mortality. Without this symplification, some regressions could not be made. Estimated values of parameter r and $R^2_{adj.}$ are given in Table 7.1. The ED_{50} and the LD_{50} on the final days of observation are given in Tables 7.2 and 7.3.

Table 7.1 Rate parameters r_d and r_m of a monomolecular regression model fitted to data of disease incidence and mortality of experiments 2a and 2b. S.e. are estimates of standard errors. $R^2_{adj.}$ are percentages of variance explained by regressions.

Experiment	Spore density (spores.cm^{-2})	Disease incidence			Mortality		
		r_d	s.e.	R^2_{adj}	r_m	s.e.	R^2_{adj}
2a	$1.5*10^6$	0.124	0.013	82	0.055	0.006	60
	$1.3*10^5$	0.119	0.013	86	0.027	0.004	31
	$1.0*10^4$	0.038	0.011	72	0.0061	0.0014	18
	$1.9*10^3$	0.0055	0.0006	54	0.0015	0.0005	13
2b	$1.6*10^6$	0.545	0.024	54	0.283	0.012	86
	$1.7*10^5$	0.121	0.012	71	0.012	0.0009	67
	$1.7*10^4$	0.0465	0.0039	55	0.0042	0.0009	14
	$1.7*10^3$	0.0073	0.0006	46	0.0003	0.0001	7

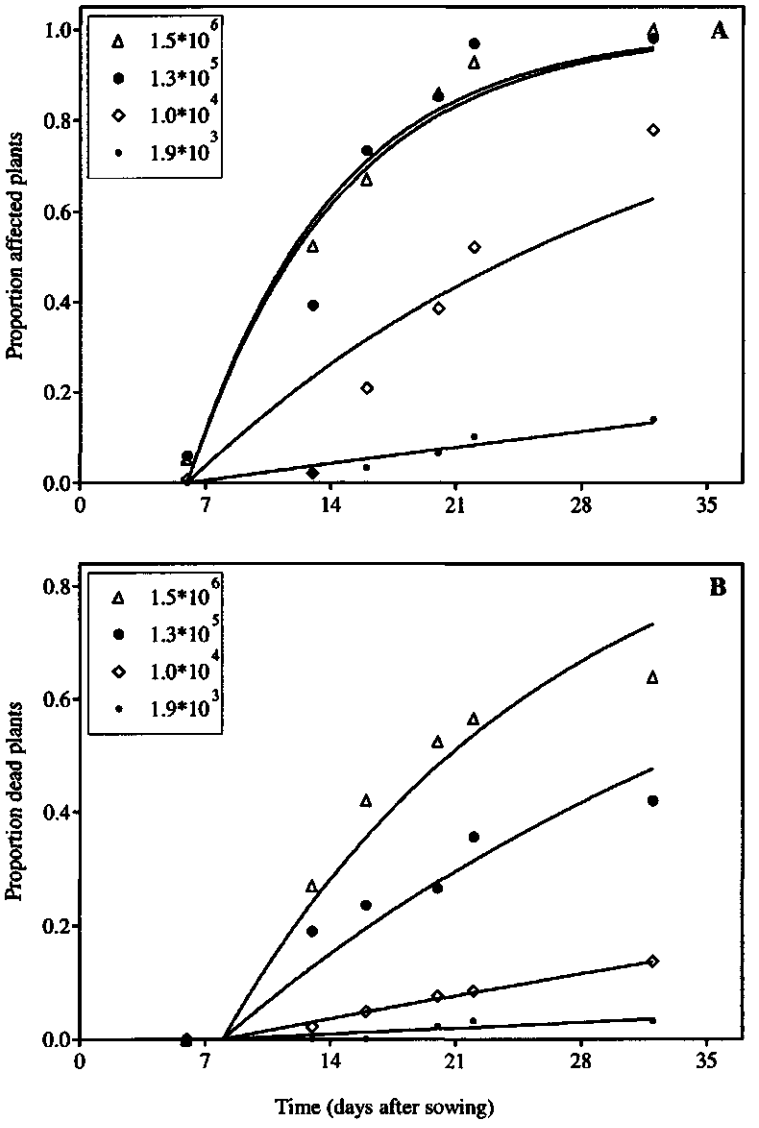

Fig. 7.1 Monomolecular curves describing disease incidence on (A) and mortality of (B) *Chenopodium album* plants for treatments of expt 2a. The plants emerged from soil through which spore suspensions of *Ascochyta caulina* were mixed. Parameter values of the curves are given in Table 7.1. Entries are treatment averages. Densities are expressed as spores.cm^{-2}.

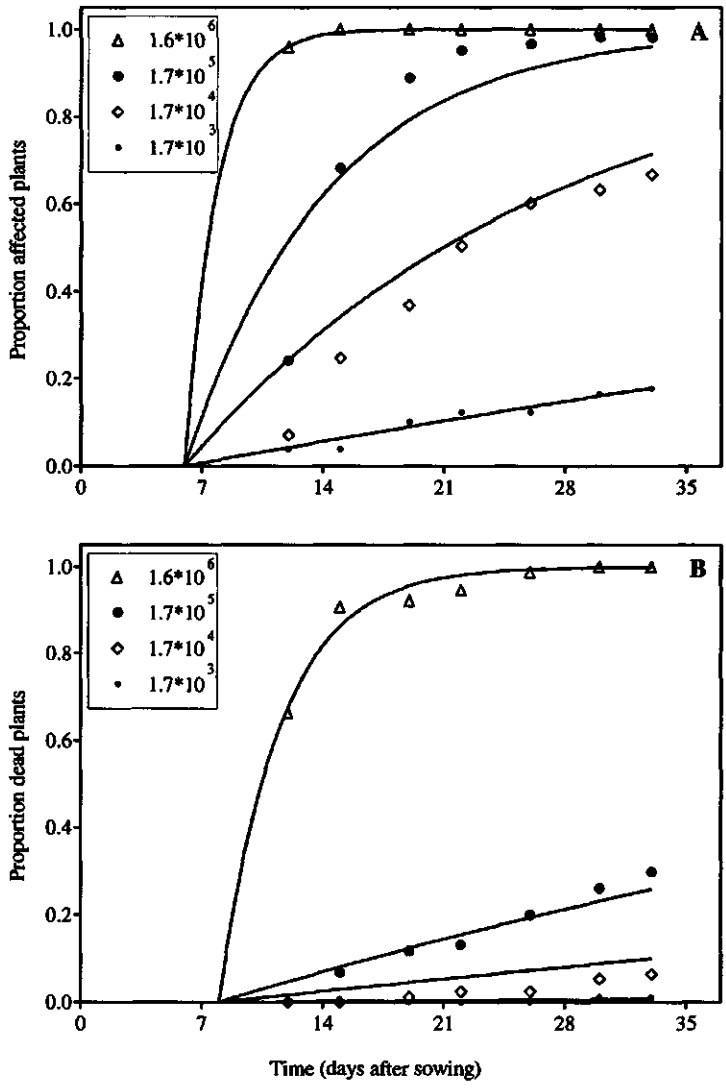

Fig. 7.2 Monomolecular curves describing disease incidence on (A) and mortality of (B) *Chenopodium album* plants for treatments of expt 2b. The plants emerged from soil on which spore suspensions of *Ascochyta caulina* were sprayed. Parameter values are given in Table 7.1. Entries are treatment averages. Densities are expressed as spores.cm^{-2}.

Table 7.2 Densities (ED_{50}) of pycnidiospores of *Ascochyta caulina* in/on soil that caused disease symptoms on 50% of the *Chenopodium album* plants on the final day of observation (days after sowing). ED_{50} values were determined by means of a logit-log regression model. Standard errors of the mean are in parentheses.

Experiment	Treatment	Time of observation	*a*	*b*	ED_{50} (*10^3 spores.cm^{-2})
2a. Spore density	-	32	-6.9 (0.6)	1.9 (0.1)	3.8 (0.9)
2b. Spore density	-	30	-7.6 (0.7)	2.4 0.2)	1.9 (0.2)
4. Soil moisture	10%	28	-10.5 (0.5)	2.3 (0.1)	42.4 (4.8)
content	15%	28	-8.3 (0.6)	2.0 (0.1)	18.2 (5.1)
	18%	28	-10.0 (1.1)	2.6 (0.3)	6.5 (0.8)
5. Soil type	Sand_1	22	-8.4 (1.3)	2.4 (0.4)	3.5 (1.4)
	Sand_2	22	-6.0 (0.5)	1.6 (0.1)	5.8 (1.2)
	Clay	22	-6.5 (0.4)	1.5 (0.1)	23.8 (3.0)
	Peat	22	-8.3 (0.9)	2.0 (0.3)	14.8 (3.0)

Table 7.3 Densities (LD_{50}) of pycnidiospores of *Ascochyta caulina* in/on soil that caused mortality of 50% of the *Chenopodium album* plants on the final day of observation (days after sowing). LD_{50} values were determined by means of a logit-log regression model. Standard errors of the mean are in parentheses.

Experiment	Treatment	Time of observation	*a*	*b*	ED_{50} (*10^3 spores.cm^{-2})
2a. Spore density	-	32	-7.6 (0.9)	1.4 (0.2)	4.5 (1.2)
2b. Spore density	-	30	-14.0 (0.6)	3.1 (0.1)	0.4 (0.1)
4. Soil moisture	10%	28	-19.9 (2.1)	3.3 (0.4)	14.0 (2.7)
content	15%	28	-24.0 (2.4)	4.1 (0.5)	5.8 (1.3)
	18%	28	-26.4 (2.3)	4.7 (0.4)	3.8 (0.3)
5. Soil type	Sand_1	22	-9.2 (0.7)	1.8 (0.1)	1.4 (0.1)
	Sand_2	22	-11.3 (1.3)	1.8 (0.3)	43.6 (23.8)
	Clay	22	*[1]	*[1]	*[1]
	Peat	22	-14.9 (0.6)	2.4 (0.1)	25.5 (5.5)

[1] No regression analyses done because mortality did not exceed 50%. The percentage dead plants at the highest spore density tested (2.8*10^8 spores.cm^{-2}) was 17%.

Experiment 3: Effect of sowing depth on disease incidence. The proportion of seeds that emerged (0.60) was not influenced by sowing depth or spore density ($P>0.05$). The proportions of affected plants and dead plants 30 days after sowing (Table 7.4) were influenced by spore density ($P<0.001$), not by sowing depth ($P>0.05$).

Experiment 4: Effect of soil moisture content on disease incidence. The proportion of seeds that emerged (0.78) was not influenced by soil moisture content ($P>0.05$) or spore density ($P>0.05$). The ED_{50} and the LD_{50} (Tables 7.2 and 7.3) 28 days after sowing were influenced by soil moisture content ($P<0.001$). Unlike the b_{ED}, the b_{LD} was influenced by soil moisture content ($P>0.05$ and $P<0.01$, respectively).

Table 7.4 Experiment 3. Effect of sowing depth of *Chenopodium album* seeds and pycnidiospores of *Ascochyta caulina*, sprayed on the soil surface, on disease incidence 30 days after sowing. Standard errors of the mean are in parentheses.

Sowing depth	Spore density ($*10^6$ .cm^{-2})	Proportion affected plants	Proportion dead plants
0.1	0	0.00 (0.00)	0.00 (0.00)
0.5	0	0.01 (0.01)	0.00 (0.00)
1.0	0	0.00 (0.00)	0.00 (0.00)
0.1	1.6	1.00 (0.00)	0.98 (0.02)
0.5	1.6	1.00 (0.00)	1.00 (0.00)
1.0	1.6	1.00 (0.00)	0.92 (0.05)

Table 7.5 Experiment 6. Effect of time of inoculation of the soil with pycnidiospores of *Ascochyta caulina*, relative to time of sowing of *Chenopodium album* seeds, on disease incidence 30 days after sowing. The spores were mixed through the top soil. Standard errors of the mean are in parentheses.

Relative time of sowing	Spore density ($*10^5$ .cm^{-2})	Proportion affected plants	Proportion dead plants
0	0	0.00 (0.00)	0.00 (0.00)
7	0	0.01 (0.01)	0.00 (0.00)
14	0	0.00 (0.00)	0.00 (0.00)
0	3.9	1.00 (0.00)	0.99 (0.01)
7	3.9	1.00 (0.00)	0.99 (0.01)
14	3.9	0.95 (0.01)	0.69 (0.03)

Experiment 5: Effect of soil type on disease incidence and plant growth. The proportion of seeds that emerged (0.78) was not influenced by soil type ($P>0.05$) or spore density ($P>0.05$). The ED_{50} and the LD_{50} (Tables 7.2 and 7.3) 22 days after sowing were both influenced by soil type ($P<0.001$). The b_{ED} and the b_{LD} were not influenced by soil type ($P>0.05$). The experiment was terminated relatively early, because size-mediated competition between plants was to be expected in certain treatments. Plant dry matter weight 22 days after sowing (Fig. 7.3) showed an interaction between soil type and spore density ($P<0.001$). Dry matter weight of plants grown on sand_1, sand_2 and clay was significantly influenced by spore density over the whole range of spore densities, while dry matter weight of plants grown on peat showed a significant weight reduction only at the highest spore density tested.

Experiment 6: Effect of time between sowing and inoculation on disease incidence. The proportion of seeds that emerged (0.86) was not influenced by relative time of sowing or by spore density ($P>0.05$). The proportion of affected plants (Table 7.5) 30 days after sowing was influenced by spore density ($P<0.001$) but not by relative time of sowing ($P>0.05$). The proportion of dead plants (Table 7.5) 30 days after sowing was influenced by both spore density and relative time of sowing ($P<0.001$).

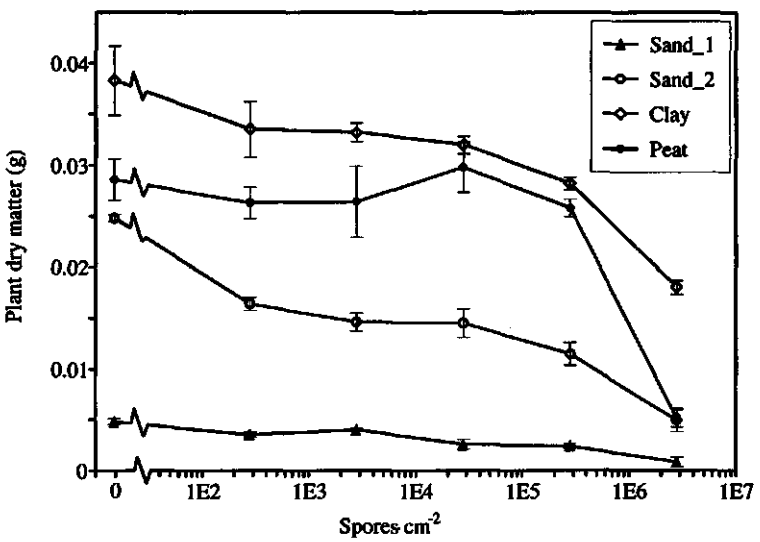

Fig. 7.3 Effect of soil type and spore density of *Ascochyta caulina* in the soil (spores.cm^{-2}) on average plant dry matter weight of *Chenopodium album* 22 days after sowing. Error bars represent standard errors of the mean.

DISCUSSION

Disease development. The life cyle of *A. caulina* is known only in part. Pycnidiospores of the fungus were associated with infection of aerial parts of plants of several *Chenopodium* and *Atriplex* species (Van der Aa and Van Kesteren, 1979; Boerema *et al.*, 1985). This study showed that the presence of pycnidiospores of *A. caulina* in soil can result in disease on seedlings of *C. album*. *A. caulina* could be re-isolated from diseased *C. album* seedling tissue and, therefore, pycnidiospores of *A. caulina* can infect *C. album* plants when present in or on soil. Susceptibility was not limited to *C. album* since one of the five other tested plant species, *S. oleracea*, also developed disease symptoms. Severity of symptoms differed between the two susceptible species, *S. oleracea* was far less affected than *C. album*. A limited host-range with some susceptible species belonging to a few genera within the *Chenopodiaceae* is expected on basis of this study. A host-specificity test with application of *A. caulina* to young plants showed a similar host-range (Chapter 4).
The symptoms observed on *C. album* seedlings partly resembled the symptoms on mature *C. album* plants. Necrosis on seedlings and mature plants was similar. The necrotic spots on cotyledons and hypocotyls probably resulted from infections during seedling emergence when plant tissue was exposed to spores. Cotyledon tissue covered by the seed coat during emergence never showed necrotic spots. The abnormal olive-green colour of seedlings was a new phenomenon for this pathosystem. This symptom might be caused by a toxin. Capasso *et al.*, (1991) identified water-soluble toxins for many *Ascochyta* species.
The time course of disease incidence and of mortality could be described by a monomolecular model. The model was selected because it is convenient for description of time courses of disease incidence of soil-borne diseases (Zadoks and Schein, 1979). It can be argued that a sigmoidal model would fit better in some regressions. Overall we obtained the best regressions with the monomolecular model. The regression analyses provided us rate parameters with an epidemiological meaning. Parameter values of r greater than 0.1 can be considered high, and were estimated for the higher spore density treatments.

Limitations to disease development. Spore density in the soil had a large effect on both disease incidence and on mortality of *C. album*. The infection rates were higher when more spores were applied to the soil indicating that disease incidence and mortality can be manipulated. Way of spore application, mixing through the top layer or spraying on the soil, had only little effect on disease incidence and mortality. Sowing depth did not significantly effect disease incidence and mortality in the range 0.1-1 cm tested. Van den Brand (1985) reported that *C. album* mainly emerges from depths between 0-0.5 cm under field conditions. Soil moisture content had a significant effect on disease incidence and

mortality. Both increased with increasing soil moisture. However, at the lowest soil moisture level tested, effectivity of inoculum was still considerable. Soil type had a significant effect on disease incidence and mortality. The effect of soil type should be assessed carefully, because many soil properties are involved such as water and nutrient content, and physical and biological characteristics. Inoculum was effective in both sterilized and unsterilized, natural soils. Survival of pycnidiospores was not tested long enough to observe substantial loss of infectivity. Inoculum maintained its effectiveness in moist sand for at least one week.

Perspectives for biological control. The two major considerations in the selection of plant pathogenic fungi for weed control are host specificity and efficacy (Charudattan, 1989).
Host specificity of *A. caulina* was not limited to the target weed *C. album* only. The cultivated plant species *S. oleracea* was also affected by the fungus. If this crop is considered in a rotation, a detailed risk analysis has to be conducted to evaluate soil application of *A. caulina*. This analysis requires more information on survival of the fungus in soil. If *S. oleracea* is not considered in the rotation, there are no limitations imposed by lack of host specificity. However, more species have to be tested for a sound evaluation of host specificity.
Efficacy of *A. caulina* against *C. album* was demonstrated under a broad range of controlled conditions. The investigations did not show major constraints on disease development. Approximately 10^9 to 10^{10} spores.m^{-2} were required for 50% mortality of emerged *C. album*. These densities are high but not unrealistic. The plants that survived infection were considerably retarded in growth and will be less competitive than healthy plants.
Obviously, there are perspectives for the use of *A. caulina* as a soil-applied mycoherbicide. In the field, the environmental conditions will be different from the experimental conditions in the greenhouse which may influence disease development. Resistance to *A. caulina* and variation in emergence of *C. album* will be encountered in the field. Timing of application of the fungus in relation to field emergence of the weed, optimal infection and/or survival structures of the fungus, and prevention of build up of resistance will be the next important research topics in the development of *A. caulina* into a soil-applied mycoherbicide against *C. album*.

ACKNOWLEDGEMENTS

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CHAPTER 8

APPLICATION OF *ASCOCHYTA CAULINA* TO FLOWERING *CHENOPODIUM ALBUM* PLANTS AND ITS EFFECT ON PROPAGATION OF THE WEED

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ABSTRACT

Effect of application of *A. caulina* to flowering *C. album* plants was studied in field experiments with maize and sugar beet crops, in 1993 and 1994. At three or four dates, suspensions of *A. caulina* pycnidiospores or spore-free controls were applied to the plants. At the end of each vegetation season, seeds of the *C. album* plants were harvested. Numbers of seeds per plant and seed weights were determined. In one experiment, the presence of *A. caulina* in and on seeds was determined as well as disease incidence on plants grown from infected seeds. We observed that flowers of *C. album* could become necrotic after application of *A. caulina*. Necrosis development was affected by year of experimentation and time of application of spores, but not by crop. It was favoured by rain. Number of seeds per plant and seed weight were also affected by year of experimentation and time of application of spores, and not by crop. Severe necrosis of flowers early in the season resulted in abortion of all flowers, while light necrosis of flowers at the end of the season had no effect on seed production. Seeds of *C. album* plants treated with *A. caulina* may be infected by the fungus, internally and externally, as demonstrated by a blotting paper test. Infected seeds resulted in a lower number of emerged plants and in necrosis of some of the emerged plants when the seeds were sown in a peat soil in a greenhouse.

INTRODUCTION

Chenopodium album L. is an important weed in almost all spring sown, arable crops (*e.g.* Holm *et al.*, 1977). Time of emergence of this annual plant species is adapted to climatic conditions and cropping systems. In The Netherlands, emergence of *C. album* plants on arable fields may occur from March till October and is most frequent in May. Emergence is stimulated by soil tillage (Van den Brand, 1985). When *C. album* plants emerge late relative to the crop, yield reduction of the crop is not likely because the *C. album* plants will not reach a competitive size. However, some farmers try to control such *C. album* plants, not because of expected crop yield reduction but because of propagation of the weed. Control of late emerged *C. album* plants is difficult because the size of the crop does not permit chemical or mechanical methods.

Ascochyta caulina (P. Karst.) v.d. Aa & v. Kest is a plant pathogenic fungus that causes necrotic spots on leaves and stems of plants belonging to *Chenopodium* L. and *Atriplex* L.

species (Van der Aa and Van Kesteren, 1979). The fungus may have potential as a mycoherbicide against *C. album*. We have already demonstrated in field experiments that the application of pycnidiospores of *A. caulina* to young *C. album* plants results in severe necrosis of the plants when conditions are favourable for infection, and that growth and competitive ability of infected *C. album* plants is reduced according to the amount of necrosis developed (Chapter 6).

It seems likely that *A. caulina* can also infect flowers and seeds of *C. album*. This assumption was based on observations of related *Ascochyta* species (*e.g.* Maden *et al.*, 1975; Boerema *et al.*, 1977) which can infect flowers and seeds of their hosts. If *A. caulina* can indeed infect flowers and seeds of *C. album*, application of the fungus to flowering plants could be a way to prevent propagation of the weed. Objectives of the present study were twofold, to quantify the effect of application of *A. caulina* to flowering *C. album* plants on propagation of the weed, and we wanted to study a part of the life cycle of the fungus.

MATERIALS AND METHODS

Experimental design. Three experimental fields were laid out in arable crops at an experimental farm near Wageningen, The Netherlands, two in maize (*Zea mays*) in 1993 and 1994, one in a sugar beet (*Beta vulgaris* subspecies *vulgaris*), 1994. Experiments were encoded 93_M, 94_M and 94_B, respectively. Maize cultivars were Brazil in 1993 and Mandigo in 1994, the sugar beet cultivar was Univers. Soil type was a loamy fine sand with pH-KCl 5 and 2-3% organic material. Experimental data are given in Table 8.1. Crop management was according to standard practice in the region, except for weed control. Weeds in maize were controlled by hand, in sugar beet by both a post-emergence herbicide treatment (600 g phenmedipham.ha^{-1} on day 136) and by hand.

The experimental fields consisted of plots of 6 by 4 m with the long axis in the direction of the crop rows. *C. album* was transplanted into the plots as described in Chapter 6. Transplants were grown from seeds harvested from plants on arable fields near Wageningen in the years preceding the experiments. Germinated seeds, together with a small piece of water agar, were transplanted into the centre row of each plot, 10 transplants per plot in positions half-way the crop plants. Minimum distance between transplants in a row was 18 cm. After emergence, *C. album* plants were thinned to 4 plants per plot. Intra-specific competition during the vegetation season was not expected because of the low density of *C. album* and the late emergence of *C. album* relative to the crop.

A. caulina was obtained from a naturally infected *C. album* plant at Wageningen in 1990, and maintained on oat meal agar slants in test tubes at 5 °C in the dark. Three months prior to application in the field, pycnidiospores of *A. caulina* were axenically grown on wheat bran medium (Chapter 2), and stored on the medium in jars at 5 °C until use. At the day of application, medium with the fungal culture was taken from cold storage and flooded with water with 0.05% (v/v) Tween 80. After 3 h, supernatants with suspended spores were collected. The suspensions were filtered through cheese cloth. Spore densities in the suspensions were determined and diluted to 10^7 spores.ml^{-1}. Nutrients (3.5 g Czapek Dox broth l^{-1} and 0.4 g yeast extract l^{-1}) were added to the suspensions 2 h before application. Controls were treated with the same amount of a spore-free solution of water, Tween 80 and nutrients.
Spore suspensions (S1) or spore-free solutions (S0) were applied to *C. album* plants at 4 different dates using a hand-automated, air pressure sprayer. The plants were sprayed till run-off. Dates of application (T1 through T4; Table 8.1) were selected according to plant development stages and weather conditions. At T1 the *C. album* plants had formed a few flowers, and some of which were open. At the later dates more flowers were formed, and flowering and seed development had progressed. At T4 more than 50% of the flowers had formed a mature seed. Weather conditions were determined by the temperate sea climate of western Europe. When no rain was expected, plants were treated shortly before sunset; otherwise they were treated at 16.00 h. Temperature, relative humidity, leaf wetness duration and precipitation were measured in the experimental fields. Weather conditions at the dates of application are indicated in Table 8.2. Rains were more than average during the treatment period of 1993, but not in 1994 (Fig. 8.1).

Experiment 93_M consisted of 8 treatments, 4 dates of application (T1, T2, T3 or T4) and 2 spore densities applied (S0 or S1). All treatments had 4 replicates (plots). The experiment had a complete randomized design. Experiment 94_M consisted of the same treatments as 93_M, but had a randomized block design with 6 replicates. Experiment 94_B was a replicate of experiment 94_M, the crop excepted. In 94_B, treatments at T1 were not carried out.

Table 8.1 Some data on experiments 93_M, 94_M and 94_B (M indicates maize, B sugar beet). Dates are expressed as day number of the year.

	Experiment		
	93_M	94_M	94_B
Crop			
- date of sowing	118	122	115
- date of emergence	129	131	125
- density (plants.m^{-2})	11	11	11
- row distance (m)	0.75	0.75	0.50
Chenopodium album			
- date of transplanting	139	150	150
- date of emergence	143	153	153
Dates of application of *A. caulina*			
T1	186	201	not carried out
T2	195	209	209
T3	208	217	217
T4	232	223	223

Table 8.2 Weather conditions during the first 24 h after plants were treated in 1993 (experiment 93_M) and 1994 (experiments 94_M and 94_B). Average leaf wetness duration, average day temperature and average relative humidity are indicated in parentheses.

	1993	1994
T1	Very cloudy, showers (15 h, 14 °C, 85% RH)	No clouds, little dew (4 h, 21 °C, 72% RH)
T2	Very cloudy, showers (19 h,14 °C, 92% RH)	No clouds, little dew (5 h, 21 °C, 76% RH)
T3	Cloudy, showers (15 h, 15 °C, 88% RH)	Cloudy, some dew (10 h, 20 °C, 78% RH)
T4	Cloudy, little dew (5 h, 17 °C, 83% RH)	Cloudy, showers (15 h, 16 °C, 93% RH)

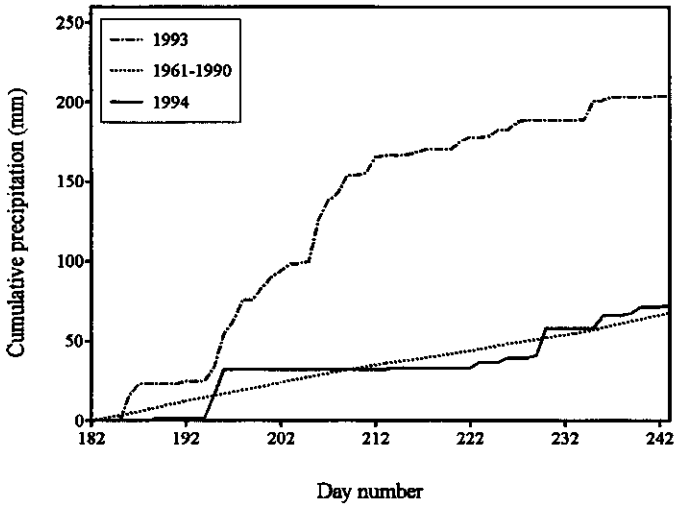

Fig. 8.1 Cumulative precipitation (mm) on the experimental fields in July and August of 1993 and 1994 and average precipitation in The Netherlands in the period 1961 through 1990 (KNMI, 1992).

Observations and assessments.

1. Disease development. Disease development of plants was assessed in the field by regular observations.
2. Seed production. Aerial parts of the treated *C. album* plants were harvested from the plots at day 238 (1993) or 248 (1994). The plants were cut off near the soil level and placed in a greenhouse (60% RH, 18 °C) to dry. After one month, seeds were separated from the other aerial plant parts and stored at 5 °C in the dark. The number of seeds per plot was determined. Four hundred seeds per plot were dried at 105 °C for 3 days and subsequently weighted to determine the average seed weight.
3. Seed infection. Presence of *A. caulina* on seeds of treatments T3S0, T3S1, T4S0 and T4S1 of experiment 93_M was tested by a blotting paper test carried out one month after the seeds were placed in cold storage. Eighty seeds per plot were assessed. Forty of them were surface-sterilized by submerging the seeds in water with 1.5% (v/v) sodium hypochlorite for 2 to 3 minutes. The other 40 seeds per plot were submerged in water. After these treatments, the seeds were placed on wet blotting paper in Petri dishes (Ø 9 cm), 20 seeds per Petri dish. The Petri dishes were placed in an incubation chamber with a day-night regime of 14 h light (20 $\mu mol.m^{-2}.s^{-1}$) at 25 °C and 10 h darkness at 15 °C. After 1 week, the number of seeds per plot with one or more pycnidia of *A. caulina* was determined using a stereo microscope.

4. Disease incidence of plants grown from infected seeds. One hundred seeds per plot of treatments T3S0, T3S1, T4S0 and T4S1 of experiment 93_M were sown in a peat soil in trays of 30 by 30 by 5 cm (length x width x height) 5 months after the seeds were placed in cold storage. The trays were placed in a greenhouse (12-22 °C and 65-90% RH). The peat soil was watered twice a week. The number of emerged plants per plot and the number of diseased plants per plot was determined one month after sowing.

Data analysis. Treatment effects were tested by means of analysis of variance (ANOVA) using Genstat 5 software (Payne *et al.*, 1987). Variances were balanced, if required, by angular or natural log transformations.

RESULTS AND DISCUSSION

Observations on disease development. Disease development was observed only on *C. album* plants that were sprayed with a spore suspension of *A. caulina*. Amount and rate of disease development was affected by year of experimentation and time of application of spores, not by crop.

In 1993, application of spores at dates T1, T2 and T3 resulted rapidly in severe necrosis of flowers, leaves and stems of treated *C. album* plants. Three days after spore application, early necrotic spots were observed on flowers and leaves. During the next days the amount of necrosis increased rapidly. Treatment T1S1 resulted in complete necrosis of flowers and mortality of all plants about 3 weeks after spore application. Treatment T2S1 had nearly the same effect as T1S1, but some plants survived in 2 of the 4 plots. Treatment T3S1 resulted in severe necrosis of flowers, but plant mortality did not occur. Treatment T4S1 resulted in light necrosis of flowers.

In 1994, disease development in the two experiments (94_M and 94_B) was similar. Until day 230, application of spores resulted in light necrosis of flowers, leaves and stems of the treated plants. We estimated that at day 230 less than 5% of the flowers of these plants carried necrotic spots or had become completely necrotic. After day 230, an increase in the amount of necrosis was observed. At day 230, most flowers had already formed seeds. At harvest (day 248), nearly all flowers of plants treated with *A. caulina* spores had necrotic spots. The increase of necrosis coincided with several rain showers in the second half of August. In 1994, necrosis development was hardly affected by time of application of spores.

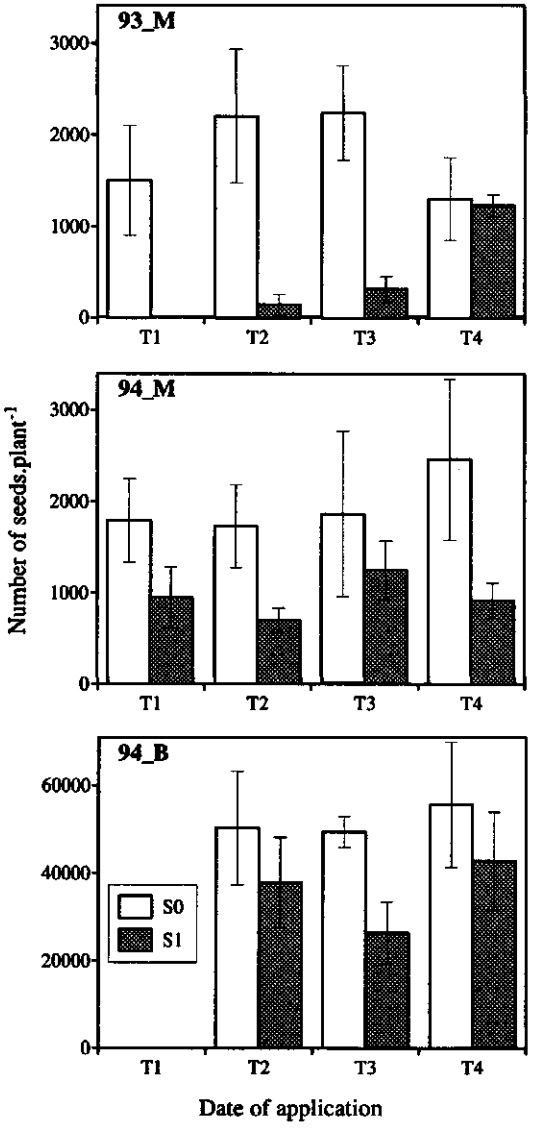

Fig. 8.2 Average number of seeds of *Chenopodium album* plants treated with a suspension of spores of *Ascochyta caulina* (S1, shaded columns) or a spore-free control solution (S0, white columns) at different dates in three experiments. Error bars indicate standard errors of the mean. T1 through T4 are application dates, explained in Material and methods.

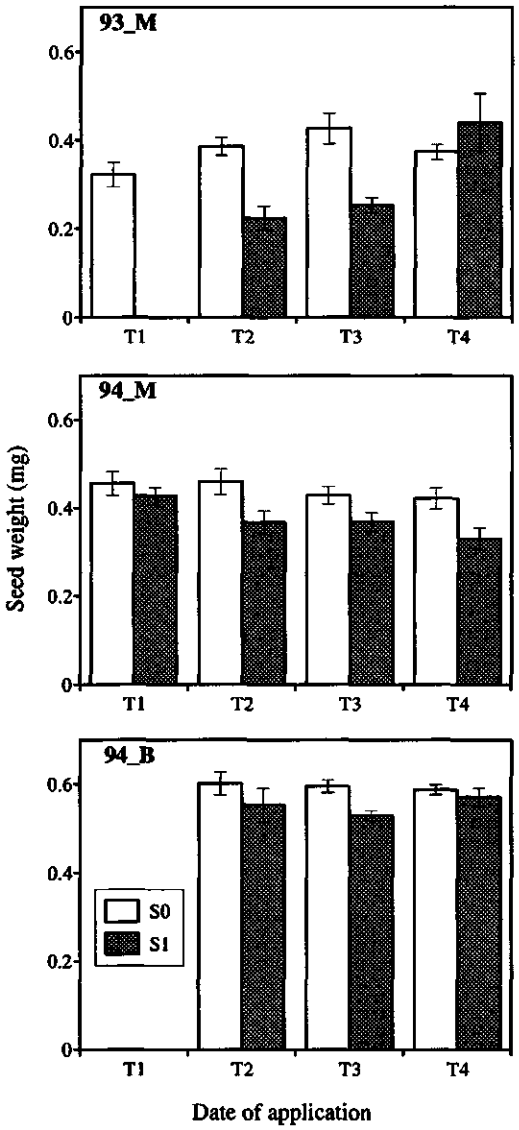

Fig. 8.3 Average weight of *Chenopodium album* seeds harvested from plants treated with a suspension of spore of *Ascochyta caulina* (S1, shaded columns) or a spore-free control solution (S0, white columns) at different dates in three experiments. Error bars indicate standard errors of the mean. T1 through T4 are application dates, explained in Material and methods.

Seed production. Fig. 8.2 shows average numbers of seeds per plant at harvest. Experiment 93_M showed an interaction between application of spores and date of application ($P<0.001$). Application of spores had a significant effect on number of seeds per plant in experiments 94_M ($P<0.01$) and 94_B ($P<0.05$).
Fig. 8.3 shows average seed weights at harvest. Experiment 93_M showed an interaction between application of spores and date of application ($P<0.01$). Application of spores had a significant effect on seed weight in experiments 94_M ($P<0.001$) and 94_B ($P<0.1$).

Seed infection. Presence of *A. caulina* on and in seeds of *C. album* plants was demonstrated by the blotting paper test (Table 8.3). Surface sterilization had a significant effect ($P<0.001$) on presence of pycnidia of *A. caulina* on seeds. Less seeds with pycnidia were counted when seeds were surface-sterilized. Obviously, the fungus can be present outside as well as inside seeds. ANOVA also showed a significant interaction ($P<0.001$) between application of spores, yes or no, and date of application.

Disease incidence on plants grown from infected seeds. Plants emerged from 4 to 20 days after sowing of the seeds. One month after sowing, proportions of emerged plants (relative to number of seeds) were significantly ($P<0.05$) smaller when the parent plants of the seeds were treated with *A. caulina* at T3 but not T4 (Table 8.4).
Some of the plants grown from seeds of plants, to which spores were applied, developed disease symptoms. A few days after emergence some plants had small necrotic spots on their hypocotyls or on their cotyledons. Later on, more plants developed necrotic spots and some diseased plants died from infection. *A. caulina* was isolated from infected plant tissue. Proportions of diseased plants one month after sowing are shown in Table 8.4. ANOVA showed a weak but significant interaction ($P<0.05$) between application of *A. caulina* to the parent plants and the time of application.

The results show that the application of *A. caulina* to flowering *C. album* plants can have a large effect on the propagation of this weed. When the plants were treated before the flowers had formed mature seeds, and when conditions were favourable for infection, propagation of the weed was reduced to a low level. When treated later in the season, or when conditions were less favourable for infection, propagation of the weed was only slightly reduced. It is to be expected that the progeny of *C. album* plants treated by *A. caulina* during flowering will have less vigour because seed weight is likely to be reduced and seeds may be infected or infested by *A. caulina.*
The study also clarified a part of the life cycle of *A. caulina.* The fungus can infect flowers and seeds of *C. album*, it can survive a plant-free period on infected seeds, and necrosis of

C. album seedlings can originate from seed-borne inoculum. It is likely that survival of *A. caulina* on seeds is not the only survival strategy of the fungus during winter. We expect that *A. caulina* can also survive on plant debris in the soil, but this survival strategy has not yet been demonstrated experimentally.

Table 8.3 Experiment 93_M. Average proportions of *Chenopodium album* seeds with one or more pycnidia of *Ascochyta caulina* after one week on wet blotting paper, as affected by application of the fungus to flowering plants (S0 or S1), dates of application (T3 or T4) and surface sterilization of the seeds. Standard errors of the means are in parentheses.

Treatment	Not surface-sterilized		Surface-sterilized	
T3S0	0.019	(0.006) ab[1]	0.004	(0.004) a
T3S1	0.36	(0.02) e	0.26	(0.02) d
T4S0	0.019	(0.011) ab	0.000	(0.000) a
T4S1	0.13	(0.04) c	0.04	(0.01) b

[1] Treatment averages were separated by different letters according to an LSD-test of the angular-transformed data ($P<0.05$).

Table 8.4 Experiment 93_M. Average proportions of emerged plants and average proportions of diseased plants one month after seeds were sown in a peat soil. The seeds were harvested from *Chenopodium album* plants treated with *Ascochyta caulina* or not (S1 and S0) at different dates (T3 and T4). Standard errors of the means are in parentheses.

Treatment	Proportion of emerged plants		Proportion of diseased plants	
T3S0	0.28	(0.01) a[1]	0.00	(0.00) a
T3S1	0.14	(0.04) b	0.40	(0.02) c
T4S0	0.28	(0.01) a	0.01	(0.00) a
T4S1	0.23	(0.04) ab	0.11	(0.01) b

[1] Treatment averages per column were separated by different letters according to LSD-tests of the angular-transformed data ($P<0.05$).

GENERAL DISCUSSION

Several scientific, technical, economical and practical factors must be considered in the development and commercialization of a mycoherbicide. Some of these considerations are discussed here, directed to the use of *A. caulina* as a mycoherbicide. Present knowledge about the *A. caulina* - *C. album* pathosystem is reviewed in the first part of the discussion. In the second part biological control of *C. album* by *A. caulina* is discussed. A view on the future is given in the last part of the discussion.

The pathosystem

Knowledge about the *A. caulina* - *C. album* pathosystem was limited at the start of the research reported in this thesis (Kempenaar and Horsten, 1994). Two useful publications were found. Van der Aa and Van Kesteren (1979) associated *A. caulina* with necrosis of leaves and stems of mature plants belonging to genera of *Chenopodium* and *Atriplex*. Eggers and Thun (1988) published a study on infection of leaves of vegetative *C. album* plants after application of pycnidiospores of *A. caulina* to the leaves. They observed that penetration was only through stomata, and that the frequency of penetration was lower when leaves were older. The latter points to an age-dependent resistance. Senescing plants probably loose this mature-plant resistance, as observations show that senescing plants can have a higher incidence of necrosis caused by *A. caulina* than fast-growing, vegetative plants.

The present thesis shows that *A. caulina* can infect *C. album* plants at all plant stages (Chapters 4, 7 and 8). These observations imply that control of *C. album* may be achieved not only by application of the fungus to young plants, as was expected at the beginning, but also by application of the fungus to the soil and to flowering plants. All plant organs are susceptible, but one may expect differences in susceptibility as the surfaces of the respective plant organs differ greatly (*e.g.* variation in stomatal density, presence of a cuticle or a seed coat).

Significant differences in pathogenicity between isolates of *A. caulina* were observed (Table 4.1). Present knowledge is too limited to verify whether differences in virulence or aggressiveness between the isolates occur. Pathogenicity of the isolate used in all experiments (isolate code 90-1) was limited to species within *Chenopodium*, *Atriplex* and *Spinacia*, with differences between the species (Chapters 4 and 7). As only a small number of isolates were tested, further selection of isolates on pathogenicity is advisable if *A. caulina* is considered for use as a mycoherbicide. Variation in resistance of *C. album*

was small (Table 4.1), but this observation cannot be generalized. One may expect that more variation in resistance will be found when an extensive survey will be carried out.
Pathogenesis was not studied in detail, but some remarks can be made. Observations of infected leaves showed that host cells near hyphae of *A. caulina* were often necrotic (Chapter 4). Involvement of a fungal toxin in the pathogenesis was suggested in several Chapters of this thesis. Vurro (personal communications, for methodology see Capasso *et al.*, 1991) recently showed that necrosis of *C. album* leaves could be induced by application of metabolites extracted from cultures of *A. caulina*, an observation indicating that *A. caulina* produces one or several toxins indeed. The isolate of *A. caulina* tested by Vurro was identical to the one used in the experiments described in this thesis.
The harmful effect of necrosis on *C. album* plants was organ-specific and growth stage-specific, comparable to harmful effects of other diseases (*e.g.* Zadoks and Schein, 1979). Organ specificity was indicated in Chapter 7; seedlings with a necrotic spot on the hypocotyl had a higher probability to die than seedlings with a necrotic spot on a cotyledon. Growth stage specificity was indicated in Chapters 4 and 7; plants being less affected by necrosis when they were older. Necrosis affected the firmness of plant tissue (Chapter 7, damping-off of infected seedlings) and photosynthesis (Chapter 5). It is to be expected that more physiological plant processes are affected by necrosis (*e.g.* Ayres, 1984). For one, transpiration was affected by necrosis. In the experiments of Chapter 5 necrosis reduced transpiration as well.

Control of *Chenopodium album* by *Ascochyta caulina*

Understanding of the tri-partite system studied can be obtained in various ways. One way is to describe the relationship between time and the population sizes of both *C. album* and the crop as affected by treatments. Such descriptions are presented in Chapters 6 through 8. Another approach could have been a description of the relationship between level of disease shortly after treatment and level of control, analogous to the relationship between severity of disease and yield loss in a crop (*e.g.* Zadoks and Schein, 1979). Such an analysis was not presented in Chapters 6 through 8, partly because the experiments were not designed for that purpose and partly because the predictive value was small. Still, some extra information (general trends) can be indicated by such an analysis, and is presented hereafter.
Severity of necrosis one week after application of *A. caulina* to *C. album* plants can be used as an independent variable in the relationship between level of disease and level of control. Control of *C. album* can be expressed as percentage of plant kill, biomass reduction or reduction of seed production. When severity of necrosis one week after

application of *A. caulina* to young *C. album* plants (data are in Table 6.3) is plotted versus percentage of plant kill, biomass reduction or reduction of seed production (data are in Tables 6.3, 6.4 and 6.5), the relationship between severity of necrosis and percentage of control can be described by a power law equation ($y = 100*x^b$ with $b > 1$). The predictive value of the relationship was, unfortunately, small as the value of parameter b was affected by experiment, indicating that level of control is affected by more factors than severity of necrosis.

The relationship between level of control and crop yield can be used to assess whether some weed infestation in crops is acceptable. Percentage of plant kill three weeks after application of *A. caulina* to *C. album* plants can be used as an independent variable in such a relationship. Crop yield can be expressed as g biomass.m^{-2}. When percentage of plant kill (Table 6.3) is plotted versus crop yield at the end of the season (Fig. 6.1), the relationship between the two variables can be described by a logistic equation. The parameters (explained in *e.g.* Chapter 2) of the equation were affected by crop. In maize, but not in sugar beet, yield reduction was prevented at incomplete levels of control, indicating that some weed infestation can be accepted in a tall crop.

The potential of a mycoherbicide is partly determined by efficacy of control. Relationships as indicated in the former two paragraphs can be used in the evaluation of efficacy of control. Charudattan (1989) defined efficacy of control as the ability to provide a satisfactory amount, speed, and ease of weed control. In Chapters 4 and 7, efficacy of control by *A. caulina* was discussed briefly. Here it is discussed in more detail.

The amount or level of weed control considered satisfactory varies with the weed species targeted and the crop. Two criteria may be applied for satisfactory control of *C. album*: the population has to be reduced to a size at which (1) damage to the crop by competition does not occur or (2) propagation of the weed does not result in unacceptable levels of weed infestation in the following years. The former can be achieved more easily than the latter. Application of *A. caulina* to the soil and to young *C. album* plants may give nearly 100% plant kill (Chapters 4 and 7). So, the level of control that can be achieved by *A. caulina* will be satisfactory for use in practice. Relationships between percentage of plant kill and crop yield (Chapter 6 and former paragraph) show that a certain level of weed infestation can be accepted, if prevention of competition damage is the only objective. Some seed production by *C. album* plants in crops is acceptable if control methods will be available during the following years. If not, acceptability of seed production by *C. album* plants in crops is close to zero because of the low mortality rate of seeds in the soil, which varies between 2% and 5% per year (Lewis, 1973; Roberts and Feast, 1973; Van den Brand, 1985).

Speed of control considers how quickly control can be achieved. The term speed suggests that there is a rate involved, but this is not the case here as it refers to a period needed for weed control. The period available for weed control also varies with the weed species targeted and the crop. During a cropping season, there are a limited number of periods available for weed control. For *C. album*, control must be achieved before crop-weed competition occurs. The time at which competition begins, depends on many factors (explained in Chapters 1, 3 and 6). In spring-sown crops such as maize and sugar beet, the beginning of competition varies from 30 to 50 days after emergence of the crop (De Groot and Groeneveld, 1986; Hall *et al.*, 1992). Application of *A. caulina* to the soil and to young *C. album* plants may produce control within a short period. A high level of disease can be achieved within a week after treatment and mortality within two to three weeks (Chapters 4, 6 and 7). So, the time needed to reach high levels of control by *A. caulina* is available in spring-sown crops.

Ease of control considers biological, technical and economical aspects such as production of the mycoherbicide, application technology, ability to use the mycoherbicide over a broad range of conditions, and compatibility of the mycoherbicide with other culture measures. Using a solid-state fermentation process (Chapter 2), circa 10^8 pycnidiospores of *A. caulina* can be produced on 1 gram of wheat bran medium. In field experiments (Chapter 6), the application of 2.10^8 spores.m^{-2} resulted in high levels of control. With the two figures mentioned, the amount of medium needed for the treatment of an area of 1 m^2 is calculated to be 2 grams of wheat bran medium.

Among the factors determining ease of control, a negative point is that high levels of control by application of *A. caulina* to young *C. album* plants can only be achieved within a narrow range of environmental conditions. Post-emergence application of *A. caulina* was effective only when the environmental conditions were favourable for infection, i.e. high relative humidity and rain showers (Chapters 4, 6 and 8). Such conditions are not always present. Effectiveness of application of *A. caulina* to the soil seems to depend little on environmental conditions, but soil treatment has yet to be tested under field conditions. Several herbicides did not hamper germination of pycnidiospores of *A. caulina* (unpublished data). Additional and synergistic control effects were observed when *C. album* plants were sprayed with a suspension of pycnidiospores of *A. caulina* and a non-lethal dosage of a herbicide (unpublished data). These results indicate that probably the use of *A. caulina* as a mycoherbicide will not be hampered by current herbicide treatments. In fact, it may benefit from the use of herbicides. If the level of control of *C. album* achieved by application of *A. caulina* is not considered satisfactory, it may be supplemented by an additional stress factor such as a low dosage of a herbicide (Scheepens, 1987; Sharon *et al.*, 1992) or a secondary pathogen (Hallet *et al.*, 1990).

Incompatibility with fungicides may be a constraint to the use of *A. caulina* in some arable crops, as it is likely that fungicides will reduce effectivity of mycoherbicides. Control of *C. album* must be achieved relatively early in the growth season. Fungicides are usually applied after the time at which control of *C. album* must be achieved. Therefore, in most crops incompatibility with fungicides is not expected when *A. caulina* is used as a mycoherbicide early in the growth season. When applied later in the season, as with control of flowering plants, the effectivity may be hampered by fungicide treatments.

View on further development of *Ascochyta caulina* into a mycoherbicide

The data presented in this thesis indicate that there is promise in the use of *A. caulina* as a mycoherbicide against *C. album*. Application of *A. caulina* to the soil and to young plants seems to fit in current weed control strategies in arable crops. Application of *A. caulina* to flowering *C. album* plants may be an interesting long-term control strategy.

Three critical issues in the further development of *A. caulina* into a mycoherbicide are large-scale production and storage of pycnidiospores, optimization of application techniques and reduction of the variability of field performance. Optimization of production of pycnidiospores and application techniques may profit from the amount of work done in this field (Daigle and Connink, 1990; Boyette *et al.*, 1991). Feasibility is probably determined more by economical factors than by technical factors.

In decisions on further development of (myco)herbicides, agricultural and financial risks have to be evaluated. The risk of damage to non-target plants by application of *A. caulina* can be evaluated in part using the data already presented. Pathogenicity was limited to species within *Chenopodium*, *Atriplex* and *Spinacia* (Chapters 4 and 7). The only susceptible crops known so far are *Spinacia oleracea* and *Chenopodium quinoa*. The mycoherbicide probably cannot be used in these crops. Dispersal in space and in time to susceptible non-target plants are aspects that have to be considered too. The risk of dispersal in space (to another field) after application of *A. caulina* is not expected to be large as field experiments showed that secondary spread was limited (Chapter 6). The risk of dispersal in time (persistence in soil) cannot be assessed yet because data on survival of the fungus in the soil are not available. In the forthcoming years persistence of *A. caulina* in the soil has to be studied in relation to presence of a susceptible crop in the rotation to assess the risk of dispersal with time.

The financial risk involved in the development of *A. caulina* into a mycoherbicide against *C. album* is determined by the expected economic and technical life spans of the product. The economic life span is determined by a cost-benefit relationship and release of alternative, more profitable control methods. The technical life span is determined by

factors as persistence of *A. caulina* in the soil and development of resistance of *C. album* against *A. caulina*. Resistance development against mycoherbicides has not yet been reported. However, a lesson from the past is that every weed control strategy creates its own weed problems, and thus the risk of resistance development should not be underestimated. Development of resistance may be prevented by the use of mixtures of isolates of *A. caulina* or by the use of *A. caulina* in combination with other control methods

As mentioned, some investigations have still to be carried out for a sound evaluation of *A. caulina* as a mycoherbicide. However, at present the expectation is that the risks involved in the further development of *A. caulina* into a mycoherbicide will be acceptable.

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SUMMARY

Plant pathogenic fungi may be used as mycoherbicides to control weeds. The present thesis reports studies carried out to assess the perspectives of *Ascochyta caulina* (P. Karst) v.d. Aa & v. Kest. as a mycoherbicide against *Chenopodium album* L., world-wide a weed in arable crops. *A. caulina* is associated with necrosis of leaves and stems of plants belonging to genera of *Chenopodium* L. and *Atriplex* L. Three control strategies of *C. album* were studied: application of pycnidiospores of *A. caulina* to the soil, to young plants and to flowering plants. The studies were focused on recognition of factors that may limit disease development, and on relationships between disease development and level of control. Chapter 1 of this thesis is an introduction to the subject, Chapters 2 through 8 are descriptions of experiments carried out and Chapter 9 is a discussion on the use of *A. caulina* as a mycoherbicide.

Chapter 2. Pycnidiospores of *A. caulina* could be produced on various agar and grain seed media. Among these, an oat meal agar and a wheat bran medium were used to study the course of spore production with time, and germination capacity of spores. The course of spore production with time could be described by a Gompertz model. Under growth conditions of 20 °C and continuous light, maximum numbers of spores produced were reached within 14 days after inoculation of the media. A negative relationship was demonstrated between the age of pycnidiospores and their germination. Addition of nutrients to suspensions of pycnidiospores restored germination to high levels, even if the pycnidiospores were stored for longer than a year.

Chapter 3. A method to obtain fast and uniform emergence of *C. album* plants for experiments was developed. *C. album* seeds were placed on water agar in Petri dishes. The Petri dishes were placed in an incubator (light period 14 h, 25 °C; dark period 10 h, 15 °C) for three to four days. Germinated seeds with a small piece of water agar were taken from the Petri dishes and planted in soil. After emergence plants were thinned to desired densities. The method provided a faster and more uniform emergence of plants in comparison with a standard blotting paper method.

Chapter 4. Disease development after application of suspensions of *A. caulina* pycnidiospores to young *C. album* plants was studied in climate chamber experiments. Two courses of necrosis of *C. album* plants were observed, (1) an increase of necrosis followed by a decline, and (2) an increase of necrosis up to completion with subsequent

plant death. Courses of necrosis with time could be described by a non-monotonic, critically damped model when plants survived infection and by a monomolecular model when plants died from infection. Disease development was influenced by significant interactions between wetness period, density of the spore suspension applied, plant development stage at the time of spore application, and temperature. Disease was favoured by a long wetness period, a high number of spores applied, an early plant development stage at the time of spore application, and a temperature of circa 20 °C. A host-specificity test showed that disease development was limited to plant species of the genera *Chenopodium* L., *Atriplex* L., and to a lesser degree *Spinacia* L. Pathogenicity differed significantly between three *A. caulina* isolates tested. Variation in resistance between four source populations of *C. album* was small.

Chapter 5. The effect of leaf necrosis caused by *A. caulina* on photosynthesis of *C. album* leaves was studied in climate chamber experiments. Leaves of young *C. album* plants were sprayed with a suspension of pycnidiospores of *A. caulina* and subsequently exposed to high humidity for 6, 18 or 24 h to cause different levels of necrosis. Individual leaves were assessed for proportion of necrotic leaf area and net photosynthetic rate 3, 4 and 7 days after treatment. The net photosynthetic rate of leaves decreased with increasing proportion of necrotic leaf area. The relationship between the two was linear and constant during the early stage of necrosis development. Linearity indicates that the necrotic leaf tissue hardly affected the surrounding, visibly healthy leaf tissue. Levels of leaf necrosis that caused a net photosynthetic rate of zero ranged from 0.2 to 0.5 depending on light intensity and date after treatment.

Chapter 6. Control of *C. album* plants in crops by post-emergence application of *A. caulina* was studied in field experiments. Experimental fields were laid out in maize and sugar beet crops. Germinated *C. album* seeds were transplanted into the crop rows. Shortly after *C. album* plants had emerged, they were sprayed with a suspension of *A. caulina* pycnidiospores or a spore-free control. Beside prevailing conditions, treatments with extra water supply were carried out to reach different levels of control. Disease development, plant mortality, dry matter weight and seed production of the weed, and dry matter weight of the crops were assessed throughout the vegetation season. Application of *A. caulina* resulted in necrosis of *C. album* plants, but not of the crops. Average proportions of necrotic leaf area one week after application of *A. caulina* ranged from 0.35 to 0.75. In the second and third week after application of *A. caulina*, some plants died from infection. Plant mortality reached its maximum in the third week after application of *A. caulina*. Average proportions of dead plants ranged from 0.05 to 0.65. Necrosis

development and plant mortality were affected by wetness duration in one of the three experiments. *C. album* plants that survived infection were reduced in size. Increase of dry matter weight of *C. album* plants with time could be described by a Gompertz model. Maximum dry matter weight of *C. album* plants was affected by the crop and by the amount of necrosis developed. Numbers of fruits (≈ seeds) per *C. album* plant showed a positive, almost linear relationship with plant dry matter weight. Seed weight was less affected by necrosis than number of fruits per plant. Competitiveness of *C. album* was reduced by necrosis. Crop yield reduction by competition was affected by the amount of necrosis developed. In maize yield reduction by competition was prevented at incomplete levels of control, but not in maize.

Chapter 7. The effect of pre-emergence application of *A. caulina* to soil on *C. album* and five cultivated plant species was studied in greenhouse experiments. Suspensions of *A. caulina* pycnidiospores were applied to the soil either by mixing spore suspensions through the top layer of the soil or by spraying spore suspensions on the soil. The presence of spores of *A. caulina* in the soil resulted in disease development on *C. album* and to a lesser degree on *Spinacia oleracea* seedlings, but not on the other species tested. Affected *C. album* seedlings had an abnormal olive-green colour or carried necrotic spots on cotyledons and hypocotyls, were retarded in growth or died due to infection. Affected *S. oleracea* seedlings were pale or carried necrotic spots on the cotyledons. Courses of disease incidence and of mortality of *C. album* plants with time could be described by a monomolecular model. Disease incidence and mortality were influenced by spore density, soil moisture content and soil type, but not or hardly by spore application method and sowing depth. Spores maintained their effectivity in soil for a period of at least two weeks.

Chapter 8. The effect of application of *A. caulina* to flowering *C. album* plants was studied in field experiments. Experimental fields were laid out in maize and sugar beet as in Chapter 6. At three or four dates following flowering of *C. album* plants, suspensions of *A. caulina* pycnidiospores or spore-free controls were applied to the plants. Observations were made on disease development. At the end of each vegetation season, seeds of the *C. album* plants were harvested. Numbers of seeds per plant and seed weights were determined. Flowers of *C. album* developed necrosis after application of *A. caulina* depending on weather conditions and growth stage of *C. album*, but not on crop. Number of seeds per plant and seed weight were also affected by weather conditions, by growth stage of *C. album* and by crop. Severe necrosis of flowers early in the season resulted in abortion of all flowers, while light necrosis of flowers at the end of the season had no effect on seed production. Seeds of *C. album* plants treated with *A. caulina* could be

internally infected and externally infested by the fungus. Sowing of infected seeds resulted in a lower number of emerged plants and in necrosis of some of the emerged plants.

Chapter 9. Some scientific, technical, practical and economical factors were discussed, aimed at the use of *A. caulina* as a mycoherbicide against *C. album*. The research resulted in a better understanding of the *A. caulina* - *C. album* pathosystem. Besides leaves and stems, flowers and seeds of *C. album* plants could be infected too by *A. caulina*. Necrosis of *C. album* seedlings could originate from soil-borne and seed-borne inoculum. These observations imply that control of *C. album* may be achieved by application of *A. caulina* to the soil, to young plants and to flowering plants, which is more than originally expected. Efficacy of control was discussed on basis of the data presented in Chapters 4, 6, 7 and 8. It is concluded that the levels of control that can be achieved by application of *A. caulina* to the soil and to young plants, and the time needed to achieve high levels of control will be acceptable for satisfactory control of *C. album*. A negative point is the dependence of the effectivity on environmental conditions such as a high relative humidity, rain or dew. Approaches to reduce this dependency were discussed. Risks involved with the further development of *A. caulina* into a mycoherbicide were also discussed, and suggestions for further research were made. It was concluded that there is promise in the use of *A. caulina* as a mycoherbicide against *C. album*. Application of *A. caulina* to the soil and to young plants seems to fit in current weed control strategies in arable and vegetable crops. Application of *A. caulina* to flowering *C. album* plants may be an interesting long-term control strategy.

SAMENVATTING

Het gebruik van schimmels als mycoherbiciden heeft de afgelopen 20 jaar in toenemende mate aandacht gekregen van zowel het onderzoek als de praktijk. In dit proefschrift wordt een onderzoek beschreven naar de toepasbaarheid van de schimmel *Ascochyta caulina* (P. Karst) v.d. Aa & v. Kest. als mycoherbicide tegen *Chenopodium album* L. (melganzevoet), een wereldwijd onkruid in de akkerbouw. *A. caulina* is beschreven als een perthotrofe schimmel die necrotische vlekken kan veroorzaken op bladeren en stengels van planten behorende tot de geslachten *Chenopodium* L. and *Atriplex* L. Drie bestrijdingsstrategieën van melganzevoet werden bestudeerd: toediening van pycnidiosporen van *A. caulina* aan de bodem, aan jonge planten en aan bloeiende planten. Het onderzoek was gericht op het herkennen van factoren die aantasting negatief kunnen beïnvloeden en het vinden van relaties tussen aantasting en onkruidbeheersing. Het proefschrift bestaat uit een inleiding (Hoofdstuk 1), resultaten van experimenteel onderzoek (Hoofdstukkken 2 tot en met 8) en een afsluitende discussie (Hoofdstuk 9).

Hoofdstuk 2. Pycnidiosporen van *A. caulina* konden gekweekt worden op verscheidene voedingsbodems. Een havermoutagar- en een tarwezemelenmedium werden gebruikt in experimenten ter bestudering van het effect van leeftijd van cultures van *A. caulina* op produktie en kieming van pycnidiosporen. Het verloop van produktie van pycnidiosporen in de tijd kon beschreven worden met een Gompertz model. Het maximum aantal pycnidiosporen per eenheid medium werd onder omstandigheden van 20 °C en continue belichting binnen 14 dagen na inoculatie van het medium bereikt. De kiembaarheid van de pycnidiosporen nam af naarmate ze ouder waren. Toevoeging van voedingsstoffen aan suspensies van pycnidiosporen bracht de kiembaarheid terug op het oude niveau, zelfs indien de sporen meer dan een jaar bewaard waren.

Hoofdstuk 3. Een methode voor snelle en uniforme opkomst van melganzevoetplanten voor gebruik in experimenten werd ontwikkeld. Melganzevoetzaad uit voorraad werd op wateragar in Petrischalen gelegd. De Petrischalen werden in een kiemkast geplaatst gedurende 3 tot 4 dagen. De kast was afgesteld op afwisselend 14 uur licht en 25 °C, en 10 uur donker en 15 °C. Gekiemde zaden werden tezamen met een stukje wateragar uit de Petrischalen genomen en in de grond geplant. Na opkomst werden de planten gedund tot de gewenste dichtheid. De methode gaf betere opkomstresultaten en was handzamer dan een standaard filtreerpapiermethode.

Hoofdstuk 4. Aantasting van melganzevoet na toediening van pycnidiosporensuspensies van *A. caulina* aan jonge planten werd in klimaatkamerexperimenten bestudeerd. Het verloop van de hoeveelheid necrose van behandelde melganzevoetplanten was één van de twee mogelijkheden: of de hoeveelheid necrose nam toe en vervolgens weer af (1), of de hoeveelheid necrose nam alleen maar toe tot de plant geheel necrotisch was en alsgevolg doodging (2). Het verloop van de hoeveelheid necrose in de tijd kon beschreven worden met een niet-monotoon, gedempt model indien de planten de aantasting overleefden en met een monomoleculair model indien de planten de aantasting niet overleefden. Aantasting werd beïnvloed door significante interacties tussen de plantnatperiode van behandelde planten, de hoeveelheid toegediende sporen, het plantontwikkelingstadium op het moment van toediening en de temperatuur. Aantasting werd bevorderd door een lange plantnatperiode, een hoge hoeveelheid toegediende sporen, een jong plantstadium en een temperatuur van ongeveer 20 °C. Een waardspecificiteitstoets toonde aan dat planten uit de geslachten *Chenopodium* L., *Atriplex* L. en *Spinacia* L vatbaar waren. Pathogeniteit was soortspecifiek. Er bleek een significant verschil in pathogeniteit tussen isolaten van *A. caulina*. Verschil in resistentie tussen populaties van melganzevoet was gering.

Hoofdstuk 5. Het effect van bladnecrose op bladfotosynthese werd bestudeerd in klimaatkamerexperimenten. Bladeren van jonge melganzevoetplanten werden bespoten met pycnidiosporensuspensies van *A. caulina* en aansluitend blootgesteld aan hoge relatieve luchtvochtigheid (> 95%) gedurende 6, 18 of 24 uur om verschillende aantastingsniveau's te creëren. Drie, vier en zeven dagen na behandeling werden de bladeren beoordeeld op de fractie necrotisch bladoppervlak en werd de netto-fotosynthesesnelheid gemeten. De netto-fotosynthesesnelheid nam af met toenemende fractie necrtotisch bladoppervlak. De relatie tussen de fractie necrtotisch bladoppervlak en de netto fotosynthesesnelheid was lineair en constant tijdens het begin van necrose-ontwikkeling. De lineariteit geeft aan dat het necrotische bladweefsel nauwelijks effect had op het overige, op het oog gezonde, bladweefsel. Fracties necrotisch bladoppervlak die resulteerden in een netto-fotosynthesesnelheid gelijk aan nul lagen tussen 0,2 en 0,5, afhankelijk van het experiment en de waarnemeningsdatum.

Hoofdstuk 6. Bestrijding van melganzevoetplanten in gewassen door een na-opkomstbehandeling met *A. caulina* werd bestudeerd in veldexperimenten. Proefvelden werden aangelegd in maïs- en suikerbietengewassen. Gekiemde melganzevoetzaden werden geplant in de gewasrijen. Kort na opkomst werd een pycnidiosporensuspensie of een sporenvrije controlevloeistof over de planten verspoten. Naast de heersende omstandigheden werden behandelingen met extra toediening van water uitgevoerd om

meerdere aantastingsniveau's te krijgen. Aantasting, plantsterfte, drogestofgewicht en zaadproduktie van melganzevoet en drogestofgewicht van de gewassen werden bepaald op meerdere tijdstippen tijdens het groeiseizoen. Toediening van *A. caulina* resulteerde in aantasting van melganzevoet, maar niet van het gewas. Gemiddelde fracties necrotisch bladoppervlak één week na de behandeling varieerden van 0,35 tot 0,75. In de tweede en derde week na behandeling ging een aantal planten dood als gevolg van aantasting. Plantstserfte bereikte zijn maximum aan het einde van de derde week na behandeling. Gemiddelde fracties gedode planten varieerden van 0,05 tot 0,65. Aantasting en sterfte werden beïnvloed door toediening van extra vocht in één van de drie experimenten. Melganzevoetplanten die aantasting overleefden, waren geremd in groei. De toename van drogestofgewicht van deze planten in de tijd kon beschreven worden met een Gompertz model. Drogestofgewicht werd beïnvloed door het gewas en de mate van aantasting. Het aantal vruchtjes per melganzevoetplant toonde een positief, bijna lineair verband met het drogestofgewicht per plant. Het zaadgewicht werd minder beïnvloed door aantasting dan het aantal vruchtjes per plant. Het concurrentievermogen van melganzevoet werd verlaagd door aantasting. Opbrengstderving bij onvolledige bestrijding kwam voor bij suikerbiet, maar niet bij maïs.

Hoofdstuk 7. Het effect van vóóropkomsttoediening van *A. caulina* aan de bodem op melganzevoet en 5 andere plante-soorten werd bestudeerd in kasexperimenten. Pycnidiosporensuspensies van *A. caulina* werden gemengd door de bovenlaag van de grond of gespoten op het bodemoppervlak. Toediening van sporen aan de bodem resulteerde in aantasting van melganzevoetkiemplanten en, in mindere mate van spinaziekiemplanten. Suikerbiet, maïs, erwt en tarwe werden niet aangetast. Aangetaste melganzevoetkiemplanten hadden een afwijkende, olijfgroene kleur of hadden necrotische vlekken op de cotylen en het hypocotyl. Ze waren geremd in groei of gingen dood als gevolg van de aantasting. Aangetaste spinaziekiemplanten waren bleek of hadden necrotische vlekken op de cotylen. Het verloop van aantasting en sterfte van melganzevoetplanten kon beschreven worden met een monomoleculair model. Aantasting en sterfte werden beïnvloed door de hoeveelheid toegediende sporen, het bodemvochtgehalte en het bodemtype, en niet of nauwelijks door de manier van sporentoediening en de zaaidiepte. Sporen behielden hun activiteit in de bodem gedurende minimaal twee weken.

Hoofdstuk 8. Het effect van toediening van *A. caulina* aan bloeiende melganzevoetplanten werd bestudeerd in veldexperimenten. Proefvelden werden aangelegd in maïs- en suikerbietgewassen op dezelfde wijze als beschreven in hoofdstuk 6. Op drie of vier

tijdstippen nadat de melganzevoetplanten begonnen te bloeien, werd een sporensuspensie of een sporenvrije controlevloeistof over de planten verspoten. Aantasting werd beoordeeld in het veld. Aan het einde van het groeiseizoen werden de melganzevoetplanten geoogst. Het aantal zaden per plant en het zaadgewicht werden bepaald. Het bleek dat bloemen van melganzevoet aangetast werden na toediening van *A. caulina*. Aantasting werd beïnvloed door weersomstandigheden en groeistadium van de planten op het moment van toediening, maar niet door het gewas. Zaadproduktie werd beïnvloed door weersomstandigheden, groeistadium van de planten en het gewas. Necrose van bloempjes vroeg in het seizoen resulteerde in abortie, terwijl necrose laat in het seizoen geen effect had op zaadproduktie. Het bleek dat zaden van melganzevoetplanten zowel uitwendig besmet als inwendig geïnfecteerd konden zijn door *A. caulina*. Zaaien van besmette zaden resulteerde in een geringer aantal opgekomen kiemplanten en in necrose van enkele kiemplanten.

Hoofdstuk 9. In de afsluitende discussie werden enkele wetenschappelijke, technische, practische en economische factoren met betrekking tot het gebruik van *A. caulina* als mycoherbicide beschouwd. Het onderzoek resulteerde in een beter inzicht in het pathosysteem. Naast bladeren en stengels bleek ook bloemen en zaden van melganzevoet geïnfecteerd te kunnen worden door *A. caulina*. Necrose van melganzevoetkiemplanten kon ontstaan door inoculum van de schimmel in de bodem en door inoculum in en op het zaad. Dit houdt in dat bestrijding van melganzevoet bereikt kan worden door toediening van de schimmel aan de bodem, aan jonge planten en aan bloeiende planten, hetgeen meer is dan oorspronkelijk verwacht werd. Effectiviteit van bestrijding werd bediscussieerd op basis van gegevens die gepresenteerd zijn in de Hoofdstukken 4, 6, 7 en 8. De conclusie is dat de mate van bestrijding die bereikt kan worden en de tijdsduur die nodig is om dit te bereiken acceptabel zal zijn voor de praktijk. Een negatief punt is de afhankelijkheid van de effectiviteit van omgevingsfactoren zoals hoge luchtvochtigheid, regen of dauw. Mogelijkheden om de effectiviteit onder ongunstige omstandigheden te verhogen werden besproken. Risico's betreffende het gebruik van *A. caulina* als mycoherbicide werden ook besproken, en suggesties voor vervolgonderzoek werden gedaan. De algehele conclusie is dat er goede vooruitzichten zijn voor het gebruik van *A. caulina* als mycoherbicide tegen melganzevoet. Toediening van de schimmel aan de bodem en aan jonge planten lijkt te passen in huidige onkruidbestrijdingsstrategieën in landbouwgewassen. Toediening van de schimmel aan bloeiende planten lijkt een interessante, lange-termijn bestrijdingsstrategie.

CURRICULUM VITAE

Corné (Cornelis) Kempenaar werd geboren op 21 februari 1963 te Los Angeles (USA). In 1981 behaalde hij het Atheneum-B diploma op het Christelijk Lyceum in Alphen aan de Rijn. In datzelfde jaar begon hij de studie plantenziektenkunde aan de toenmalige Landbouwhogeschool (thans Landbouwuniversiteit) te Wageningen. Het kandidaat-diploma werd behaald in 1985, het doctoraaldiploma in 1988. Het vakkenpakket in de doctoraalfase bestond uit de hoofdvakken Fytopathologie, Virologie en Theoretische Produktie-ecologie. De praktijktijd werd doorgebracht aan de Department of Plant Pathology, University of California te Davis (USA).

Na het vervullen van de militaire dienst van mei 1988 tot mei 1989, trad hij in mei 1989 in dienst bij het Nederlands Graancentrum, vanwaar hij werd gedetacheerd bij het Proefstation voor de Akkerbouw en Groenteteelt in de Vollegrond. Tot januari 1991 werkte hij hier aan de optimalisering van de zaaizaadproduktie van erwten. Van januari 1991 tot mei 1991 werkte hij bij ditzelfde Graancentrum aan de optimalisering van de teelt van rogge. In mei 1991 trad hij voor 3 jaar en 11 maanden in dienst bij het voormalig Centrum voor Agrobiologisch Onderzoek (CABO-DLO), thans het DLO-Instituut voor Agrobiologisch en Bodemvruchtbaarheids onderzoek (AB-DLO), alwaar hij projectleider was van het onderzoek beschreven in dit proefschrift. Het onderzoek heeft geleid tot een 'testing agreement' tussen AB-DLO en Ciba Geigy te Bazel (Zwitserland) en een patent-aanvraag aangaande het gebruik van *Ascochyta caulina* als mycoherbicide tegen melganzevoet. Eén en ander heeft geleid tot een verlenging van zijn dienstverband bij AB-DLO tot december 1995.

NAWOORD

Nog zwaar onder de indruk van de geboorte van onze dochter Madelon op 9 april j.l. is het nu tijd om dit proefschrift af te ronden met een woord van dank voor een ieder die er een bijdrage aan geleverd heeft.

Toen ik op 22 januari 1991 op sollicitatiegesprek kwam bij het CABO (thans AB-DLO), had ik nog niet het idee dat ik in de opvolgende 4 jaar een promotie-onderzoek zou gaan uitvoeren. De functie waarop ik gesolliciteerd had betrof namelijk die van 'wetenschappelijk onderzoeker onkruidkunde/fytopathologie', welke additioneel gefinancieerd werd door de Directie Wetenschap en Kennisoverdracht van het Ministerie van Landbouw, Natuur en Visserij in het kader van het Meerjarenplan Gewasbescherming. Door de directie van het instituut werd echter vanaf het begin gestimuleerd dat ik een academisch proefschrift zou schrijven over het uit te voeren onderzoek. Na enige maanden bedenktijd heb ik besloten de geboden mogelijkheid aan te nemen. Graag wil ik de directie van het AB-DLO, met name de directeur van het instituut dr. ir. J.H.J. Spiertz, bedanken voor de mogelijkheid die mij geboden is om te promoveren.

In het najaar van 1991 heb ik prof. dr. J.C. Zadoks en dr. ir. P.C. Scheepens gevraagd of zij respectievelijk promotor en co-promotor van mijn proefschrift wilden zijn. Nadat zij mij dit hadden toegezegd, is de bal gaan rollen.
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de promovendi en de begeleider van groep 7 van de C.T. de Wit Onderzoekschool Produktie Ecologie,
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de Brainstormgroep, en Kamer 1.69.
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Wageningen, 25 april 1995

Corné Kempenaar