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THE POTENTIAL OF *STAGONOSPORA CONVOLVULI* AS A MYCOHERBICIDE FOR FIELD BINDWEED

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Hanspeter Andreas Pfirter

Dipl. sc. nat.

born June, 30th, 1968

citizien of Seegräben, ZH

accepted on the recommendation of

Prof. Dr. Geneviève Défago, examiner

Prof. Dr. Silvia Dorn, co-examiner

Prof. Dr. Heinz Müller-Schärer, co-examiner

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Zusammenfassung

Die tiefwurzelnde, mehrjährige Ackerwinde (*Convolvulus arvensis* L.), ein Windengewächs eurasiatischen Ursprungs, ist besonders im Getreidebau der gemässigten Klimazone als ertragsminderndes Unkraut weit verbreitet. Chemische wie auch mechanische Bekämpfung gestaltet sich schwierig. Mit der Entwicklung umweltverträglicherer Anbausysteme steigt das Interesse an einer biologischen Unkrautbekämpfung. Das Ziel der vorliegenden Arbeit war es, die Fähigkeit eines pathogenen Pilzes zur biologischen Kontrolle der Ackerwinde abzuschätzen und im Hinblick auf eine praktische Anwendung zu verbessern.

Stagonospora convolvuli LA39, ein Blattpathogen, wurde 1994 von einer Ackerwinde aus Long Ashton, England, isoliert. Alle untersuchten Wachstumsstadien der Ackerwinde waren anfällig. Krankheitssymptome wurden durch den Pathogen auch auf der Zaunwinde (*Calystegia sepium* [L.] R. Br.), ebenfalls einem bedeutenden Unkraut, sowie in weitaus geringerem Masse auf weiteren Spezies der Familie der Convolvulaceae hervorgerufen. Sämtliche getesteten Nutzpflanzen waren nicht anfällig. Bei der Anwendung des Pathogens in einem Feldversuch (1995) wurde eine starke Schädigung der Winde festgestellt, und die Zunahme der Bodenbedeckung durch das Unkraut konnte gestoppt werden.

Die Formulierung des Pathogens in einer 10% Öl-in-Wasser-Emulsion verbesserte seine Wirksamkeit signifikant. Die Verkürzung und zeitliche Verschiebung der Periode mit 100% relativer Luftfeuchtigkeit nach der Applikation hatte keinen Einfluss auf die Stärke der Krankheit. Eine Konzentration von 10^7 Sporen/ml in der Öl-Emulsion bewirkte selbst ohne eine Inkubation bei 100% relativer Luftfeuchtigkeit einen grossen nekrotisierten Blattflächenanteil. Durch Oberflächen-Fermentation auf Couscous (geschroteter Hartweizen) konnten bis zu $4x10^8$ Sporen/g Medium erzeugt werden. Diese waren ebenso pathogen wie die auf künstlichem Medium (V8-Medium) erzeugten Sporen. Auf Kaolin luftgetrocknete und bei 3°C gelagerte Sporen blieben während 140 Tagen lebensfähig und aggressiv. Die Massenproduktion der Sporen auf Couscous, die Lagerung auf Kaolin und die Formulation in einer Öl-Emulsion erscheint als geeignete Methode zur Entwicklung eines kommerziellen Produktes.

Für eine Sporenmischung von *S. convolvuli* LA39 und *Stagonospora* sp. LA30B konnte eine erhöhte Wirksamkeit im Vergleich zur Einzelapplikation festgestellt werden.

Verschiedene Ökotypen der Zaunwinde erwiesen sich als unterschiedlich anfällig gegenüber sechs verschiedenen *Stagonospora* sp. Isolaten. Nur *S. convolvuli* LA39 war gegenüber allen Ökotypen besonders aggressiv.

Die Entwicklung von genetischen Markern wird ermöglichen, den Kontroll-Organismus nach seiner Applikation im Feld zu verfolgen. Mit Hilfe zweier unterschiedlicher Methoden wurde die genetische Vielfalt von *Stagonospora* sp. Isolaten aus verschiedenen europäischen Ländern studiert. Die Analyse der ITS-Region mit PCR-RFLP zeigte eine geringe genetische Vielfalt auf; eine grössere wurde mit RAPD-PCR festgestellt. Die mit der Cluster-Analyse der RAPD-PCR-Daten identifizierten Gruppen stimmten mit denjenigen des RFLP-PCR überein. Als nächster Schritt sollte die Gültigkeit dieser Methoden, den Stamm *S. convolvuli* LA39 zu identifizieren und verfolgen zu können, im Feldversuch untersucht werden.

Die durchgeführten und beschriebenen Studien bilden wichtige Schritte zur Entwicklung eines Mykoherbizids. Das Potential von *S. convolvuli* Stamm LA39 als Kontrollorganismus wurde bestätigt. Zukünftige Arbeiten sollten sich auf die Anwendung des Mykoherbizids unter verschiedenen Umweltbedingungen und dessen Einbindung in den Integrierten Pflanzenschutz konzentrieren. Zusätzlich sollte eine Methode zur Reduktion des ausgedehnten Wurzelsystem der Ackerwinde gefunden werden, um dadurch neue Sprossungen zu unterdrücken.

Summary

Field bindweed (*Convolvulus arvensis* L.), a deep-rooted perennial of Eurasian origin, is most troublesome in cereals of the Temperate Zone. The control of the weed with chemical herbicides and mechanical practices is difficult. Further development of more ecological cropping systems intensified the interest in biological control of this weed. The aim of this thesis was to evaluate and improve the potential of a fungal pathogen as a biological control agent for field bindweed.

In 1994, the foliar pathogen *Stagonospora convolvuli* strain LA39 was isolated from a field bindweed plant collected in Long Ashton (UK). All growth stages of the weed tested were susceptible to the pathogen. Disease symptoms were not only induced on field bindweed, but also on hedge bindweed (*Calystegia sepium* [L.] R. Br.), another important weed, and, far less severely, on some other Convolvulaceae species. No crop tested was susceptible to the fungus. Applied in a field trial in 1995, severe disease symptoms were observed on bindweed, and the increase of ground coverage by the weed was stopped.

The formulation of the pathogen in a 10% oil-in-water emulsion significantly improved its efficacy. Shortening and delay of exposure to 100% relative humidity after inoculation did not affect disease severity, achieving high mean necrotic leaf area with 10^7 spores/ml in the oil-emulsion even in absence of exposure to 100% relative humidity. The solid state fermentation on cous-cous (cracked hard wheat) produced up to $4x10^8$ spores/g substrate, which were as pathogenic as those grown on artificial medium (V8-juice agar). The air-drying on kaolin followed by storage at 3°C kept spores viable and pathogenic for 140 days. The mass production of the pathogen on cous-cous, storage on kaolin and formulation in an oil-in-water emulsion seems to be an appropriate method for the development of a commercial product.

An additional improvement may be achieved by applying a mixture of two *Stagonospora* sp., as demonstrated for *S. convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B.

Distinct ecotypes of field bindweed reacted differently against six isolates of *Stagonospora* sp.. Only the biocontrol agent *S. convolvuli* strain LA39 was highly aggressive on all ecotypes.

The development of genetic markers will allow tracking of the biocontrol agent once released in the field. Therefore, the genetic variation among *Stagonospora* sp. isolates

collected in different European countries was studied using two different methods. Little variation was found with RFLP-analysis of the ITS-region, more with the RAPD-PCR assay. Groups determined with cluster analysis of the RAPD-PCR data and fragment pattern types of the RFLP-PCR were in agreement. As a next step, validity of these methods to identify and track *S. convolvuli* strain LA39 should be ensured in the field.

Several crucial steps have been taken towards the development of a mycoherbicide for field bindweed. The potential of *S. convolvuli* strain LA39 as a biological control agent has been demonstrated. Further research should be focused on field trials under different environmental conditions and the incorporation of the mycoherbicide into integrated pest management systems. In addition, a suitable method to control the weed's extensive root system and thereby stop regrowth is still needed.

Introduction

The most common definition of a weed is "a plant growing where it is not desired" (Buchholtz, 1967). It may also be defined as an "herbaceous plant not valued for use or beauty, growing wild and rank, and regarded as cumbering the ground or hindering the growth of superior vegetation" (Little *et al.*, 1974). It is obvious that a plant is characterised and classified as a weed not by nature but by man. Therefore, all types of plants can be regarded as undesirable – trees, broadleaf plants, grasses, sedges, aquatic plants and parasitic plants. In the species concept, a weed is defined as a plant species, which is adapted to man-made habitats and does interfere with human activities. The term weeds is used as the plural of weed (Müller-Schärer, 1999).

Weed control exists as long as man is growing plants as food crops. In the beginning weeds were removed by hand. Then man started to construct tools. As a next step animals were used to drag a hoe or plough, reducing human labour. Later, animals were replaced by tractors. Since the 1940's selective chemical herbicides have reduced labour and machine requirements and improved, together with other technology, crop production drastically. In Switzerland, in 1920, one farmer could feed 10 persons, that farmer could feed 14 persons in 1960, 31 persons in 1980 and 58 persons in 1996 (data from the Swiss Farmers' Union).

Although chemical herbicides have increased the efficiency of farming and have become safer and more effective, problems such as herbicide-resistant weed populations, residues of herbicides in soil and water, and effects on non-target organisms have arisen. Therefore interest in alternative weed management strategies, including biological control of undesired plants, is growing.

Biological weed control

Biological weed control can be defined as the use of living organisms "in maintaining another organism's population density at a lower average than would occur in their absence" (DeBach, 1964). Control agents are insects and fungi, but can also be plants with allelopathic effects, fishes, aquatic mammals or vertebrates. Organisms considered as biological control agents must first be sufficiently specific to suppress a target species, but not the nontarget species. Secondly, the agent must be able to persist in field situations under varying environmental conditions. Thirdly, it must affect the target organism at a critical point in its growth. Finally, its application must be practical, economical and compatible with other methods of weed control (Kennedy and Kremer, 1996). The aim of biological control is not necessarily the eradication of a weed, but a reduction below the so-called "economic injury level".

There are two major methodes known in the biological control of weeds: (i) the classical (or inoculative) approach and (ii) the inundative (or bioherbicide) approach. In addition, the integrated management approach has recently been proposed as a new strategy. It includes practices to conserve or enhance native enemies of weeds and thus reduce the competitive ability of the weed (Müller-Schärer and Frantzen, 1996).

The classical approach

The classical approach involves the release of one (or more) host-specific, exotic organisms into a region, where the target weed is introduced and where its natural enemies are absent. The exotic pest is combatted with an exotic antagonist, which, after release, is self-perpetuating, survives and establish itself. The control agent originates normally from the weed's native range and is well adapted to its host. In 1971, the rust *Puccinia chondrillina*, collected in the Mediterranean area, has been introduced into Australia and reduced the density of skeleton weed (*Chondrilla juncea*) dramatically (Cullen *et al.*, 1973). A weevil (*Rhinocyllus conicus* Froelich) from southern Germany was released in several states of the USA after 1969 to control musk thistle (*Carduus thoermeri* Weinmann) (Kok and Surles, 1975). In Missouri, infestation levels of flower heads ranged from 46% to 90% at the release site in the following year (Puttler *et al.*, 1978).

The inundative or bioherbicide approach

The inundative approach was first introduced by Daniel *et al.* (1973) as a massive, usually annual inoculation of a pathogen into specific weed-infested fields to infect and kill susceptible weeds. Most commonly the pathogen used is a fungus, so the term "bioherbicide" is often replaced by the term "mycoherbicide". The requirements for a successful agent have been summarised by Daniel *et al.* (1973) as follows: (i) it must be capable of producing abundant and durable inoculum in artificial culture, (ii) the pathogen must be genetically stable and specific to the target weed and (iii) must be infective and able to kill the target weed in a wide range of environments.

Commercial bioherbicides first appeared on the market in the USA in 1981 (DeVine®; Abbot Laboratories, North Chicago, IL, USA) and in 1982 (Collego®; Ecogen Inc., West Langhorn, PA, USA). DeVine consists of the fungus *Phytophthora palmivora* and is used against strangler-vine (*Morrenia odorata* [H&A] Lindl.), whereas Collego consists of the fungus *Colletotrichum gloeosporoides* f. sp. *aeschynomene* and is used against northern joint vetch (*Aeschynomene virginica* [L.] B.S.P.). But since then, not much was reported about new products. In 1992, the rust *Puccinia canaliculata* (Schw.) Lagerh. has been registered as Dr. Biosedge® for the control of yellow nutsedge (*Cyperus esculentus* L.) (Tifton Innovation Corp., Tifton, GA, USA). In the same year, BioMal® (comprised of spores of *Colletotrichum gloeosporioides* f. sp. *malvae*) was introduced by Philom Bios. Inc. (Saskatchewan, Canada) for the control of round-leaved mallow (*Malva pusilla* Sm.). However, both products as well as Collego are not available on the market anymore due to economic considerations. In China, *Colletotrichum gloeosporioides* f. sp. *cuscutae*, is used under the name Luboa II against dodders (*Cuscuta* spp.).

In the development of a mycoherbicide, there are crucial steps to take. Several of them are discussed below, starting by the collection and screening of mycoherbicides candidates up to their release and tracking in the field.

Collection and screening of pathogens

In the bioherbicide assay, every pathogen able to incite an endemic disease is a candidate for biological control. As a pathogen is co-adapted to its host, the best place to find them seems to be in the native range of a weed. In contrast, Hokkanen (1985) argued that new host-pathogen associations may produce more virulent and effective biological control agents by the lack of evolved homeostasis. After collection and isolation, screening of candidates is normally performed in a single plant, the weed-pathogen-system. However, their potential has to be tested under field conditions in combination with the crop. In addition, different ecotypes of the weed may react differently against one pathogen. For this reason, a collection of different isolates should be maintained to overcome the uncertainty of distinct ecotype susceptibility.

Host range

Host specificity, especially in the classical approach, is a major concern in biological weed control. It is essential to ensure that no damage on beneficial plants will occur after release of a control agent. Wapshere (1974) proposed the following strategy with two main components: exposing of the biological control agent (A) to the most closely related species, progressing to more and more distantly related plants until host range is determined and (B) as a safeguard, to cultivated plants, which should include (i) cultivated plants botanically related to the weed, (ii) cultivated plants with little entomological or mycological information, (iii) cultivated plants not previously exposed to the control agent, (iv) cultivated plants attacked by organism closely related to the control agent, and (v) any plant recorded as host of the control agent.

The determination of host range is a fundamental need. The impact of the control agent on the existing ecosystem has to be evaluated carefully. Recently, an increase in host range of an introduced biological control agent was reported by Louda *et al.* (1997). The weevil *Rhinocyllus conicus* Froeh., released in North America for the control of exotic thistles (*see above*), has been found attacking native thistle species as well. The seed production of native thistle flowerheads was significantly reduced. In addition, the density of native tephritid flies was significantly lower at high weevil density.

Formulation and application

Formulation of a bioherbicide is the blending of the active ingredient with a carrier or solvent. Other adjuvants (surfactants, stickers, anti-freezing compounds, humectants, etc.) are often added to produce a form which can be effectively delivered to the target weed (Boyette *et al.*, 1991). Fungal spores can be applied as liquid or as solid or granular formulations. One limitation to the use of fungal spores as mycoherbicides is the requirement for a sufficient dew period to enable efficient spore germination and to allow adequate infection of the target weed. An appropriate formulation of infective propagules which reduces or eliminates the dew requirement would greatly improve the potential of a mycoherbicide.

Liquid formulations include aqueous-, oil-, or polymer-based products (Boyette *et al.*, 1991). They are normally used as postemergence sprays to incite leaf and stem diseases on the target weed. Invert (water-in-oil) emulsions can provide a favourable micro-environment around the spores during the infection process, thereby reducing the time of dew needed as well as the amount of inoculum (Amsellem *et al.*, 1990; Boyette *et al.*, 1993; Connick *et al.*, 1991; Daigle *et al.*, 1990). Application of *Alternaria cassiae* Jurair & Khan on sicklepod (*Cassia obtusifolia* L.) seedlings in the invert emulsions without a dew period gave 88% mortality compared to 0% mortality when applied as conidial suspension (Quimby *et al.*, 1988). Unfortunately, due to their viscosity, invert emulsions are difficult to apply with standard equipment. More recently, the potential of oil-in-water emulsions has been reported (Potyka, 1996; Shabana, 1997). Improved efficacy is suggested either by retention of the water present in the emulsion or by inducing an exogenous supply of water, possibly from leaf tissue cells (Greaves *et al.*, 1998). Oil-in-water emulsions can be applied with standard equipment.

Granular formulation is an appropriate method for control agents applied on, or below, the soil surface. It is not well suited for foliar pathogens. Granules can protect the pathogen from environmental changes, provides a food source and is less likely washed away from the treated areas (Boyette *et al.*, 1991). Most attention has been given to alginate formulations. *Fusarium solani* (Mart.) Appel. & Wr. controlled 70% of the weed Texas gourd (*Cucurbita texana* [Scheele] Gray) when applied as alginate-kaolin granules in the field (Weidemann and Templeton, 1988). Other formulations based upon solid carriers consist of cornmeal, wheat kernels, peat moss, long-grain rice or vermiculite (Daigle and Connick, 1990).

Mass production and storage

The inoculum production for large-scale application is often a limiting factor in the development of a mycoherbicide, as it must be as inexpensive as possible while maintaining product quality. Generally, spore production by liquid fermentation is the most economic option. As an example, the commercial product COLLEGO® consists of a powder of dried spores produced in a liquid medium. However, alternative production methods are needed for pathogens that do not sporulate in liquid media. Solid-state fermentation, in which microbial growth occurs on the surface of a solid substrate, is a possible option for these control agents. Such processes are well established in the Orient for traditional food and enzyme production (Mudgett, 1986).

An adequate shelf life is needed for production of an inoculum in advance and will allow storage of the inoculum until environmental conditions are suitable for an application. However, this requirement may be bypassed through production for immediate use, but such production will be of negative impact for commercial utilisation. During conservation the control agent has to keep its pathogenicity and genetic stability.

Field application and tracking

Field inoculations should be performed during periods which will favour the control agent. An appropriate formulation (see above) may help to overcome environmental constraints. The ability to apply the agent with standard farm equipment will be advantageous to the agent's commercialisation. Total volume of application tends to become smaller to reduce the cost of carrying water and the time needed for application. In most cases, mycoherbicide candidates have been applied in the greenhouse at high volumes (up to 1000 l/ha). However, in a field experiment with *Colletotrichum orbiculare*, a mycoherbicide candidate for Bathurst burr (*Xanthium spinosum*), high volumes of 1000 l/ha were generally not superior to volumes of 500 and 250 l/ha (Klein and Auld, 1995).

Only little attention has been given so far to spray technology. But an effective infection can only be achieved when the inoculum is deposited on the target plant in an appropriate quantity and at the correct site. Small droplets provide a good coverage on

the target, but are highly susceptible to drift. Large droplets deposit well, but may result in insufficient coverage unless spray volumes are increased to high levels. Foliar-applied herbicides are often sprayed with droplets ranging between 100 and 400 µm to give good retention on the target surface (Knoche, 1994). In the case of *Stagonospora* sp., pathogenic on field bindweed, 20% of droplets up to 150 µm contained no conidia and are therefore wasted. Droplets of this size can make up to 50% of the applied spray volume with conventional flat-fan hydraulic nozzles. On the other hand, more than 60% of the larger droplets (290 µm) contained at least 20 conidia, which are probably more than required for a good infection (Lawrie *et al.*, 1997).

Once released in the field, tracking and identification of the control agent will help to study its dispersal, reproduction and survival. Classification of organisms with DNA-based methods has become more and more common. These methods will help to identify individual fungal isolates through genetic fingerprinting analysis and, in addition, to determine the genetic variability among various isolates from the same species.

The target weed *Convolvulus arvensis* L. (field bindweed) and potential biocontrol agents

Field bindweed (also known as small flowered or wild morning glory) is a deep-rooted perennial plant with numerous long twining stems found throughout the temperate regions of the world. It belongs to the family *Convolvulaceae* in which sweet potato (*Ipomea batatas* [L.] Lam.) is the best known crop. The botanic name is derived from the Latin words *convolvere* (=to roll or to entwine) and *arvenere* (=to plough or to cultivate), describing its climbing habit and its association with cultivated field. Field bindweed reproduces by seeds and by sending up new shoots from extensive root systems. Seeds can remain viable in the soil for 20 years or even more (Timmons, 1949). Root regeneration occurs mainly from vertical roots (Swan and Chancellor, 1976).

Field bindweed is a serious problem of cereals and horticulture (Holm *et al.*, 1977). The extensive root system makes field bindweed a successful competitor for nutrients and moisture. Under suitable conditions, especially where soil moisture is available throughout the summer and autumn, the weed can produce a bulk of vigorous growth that may entwine and choke desirable plants. It can cover bushes and small trees in fruit orchards, increasing the time taken for pruning and picking. In California, for example, 30.8% of the grape acreage was infested by field bindweed in 1980 (Rosenthal, 1983). The yields of winter wheat can be reduced by one-third, yields of summer crops even by three quarters (Philips, 1967).

The control of field bindweed with mechanical and chemical methods is poor due to the vigorous regeneration capacity from its roots. The repeated use of selective herbicides (2,4-D, dicamba, picloram, imazapyr) may reduce but not eradicate the weed (Derscheid *et al.*, 1970; Schoenhals *et al.*, 1990; Westra *et al.*1992). Therefore, interest in biological control of field bindweed is growing. Potential biological control agents and their status are presented in Table 1. Among these, the gall mite *Aceria malherbae* Nuzzaci and the fungi *Phomopsis convolvulus* Ormeno and *Phoma proboscis* Heiny have potential as bioherbicides.

Table 1. Potential biological control agents for field bindweed.

Agent and taxonomic reference	Status and degree of control
Exotic mites and insects[a]	
Aceria malherbae Nuzzaci (Acarina:Eriophyidae)	Canada. Ex Italy. Released in British Columbia and Alberta (1994), where the insect overwintered successfully, but establishment not confirmed. Not established in Manitoba or Saskatchewan.
	USA. Ex Greece. Released in Colorado, Maryland, Montana, New Jersey, Oklahoma, South Dakota, Texas and Washington (1989). Established in Montana, Texas[b] and Washington.
	South Africa. Ex Greece. Released in 1994[c].
Tyta luctuosa (Denis & Schiffermüller) (Lepidoptera:Noctuidae)	Canada. Ex Italy. Released in Alberta and Saskatchewan (1989). Not established.
	USA. Ex Italy. Released in Arkansas, Iowa, Missouri, Oklahoma and Texas (1987). Not established. Released in Maryland (1991) and Washington (1996). Establishment not confirmed.
Native mites and insects[a]	
Chelymorpha cassidea (Fabricius) (Coleoptera:Chrysomelidae)	Canada. Native organism collected in 1979 in Saskatchewan and released in Alberta in an attempt to extend its range. Not established.
Chirida guttata (Olivier) (Coleoptera:Chrysomelidae)	Canada. Native organism collected in 1979 in Saskatchewan and released in Alberta in an attempt to extend its range. Not established.
Metriona purpurata (Boheman) (Coleoptera:Chrysomelidae)	Canada. Native organism collected in 1979 in Saskatchewan and released in Alberta in an attempt to extend its range. Established.
Fungi	
Phomopsis convolvulus Ormeno[d] (Sphaeropsidales:Coelomycetes)	95% reduction in foliage biomass and up to 55% mortality on seedlings with 10^9 conidia/ml and 18h dew. Up to 100% biomass reduction when applied pre-emergence.
Phoma proboscis Heiny[e] (Sphaeropsidales:Coelomycetes)	Tested in the field during 1990-1993. Up to 90% of seedlings killed.

[a] Data from Julien and Griffiths (1998)

[b] Data from Boldt and Sobhian (1993)

[c] Data from Craemer (1995)

[d] Data from Morin *et al.* (1990) and from Vogelsang *et al.* (1998)

[e] Data from Heiny (1994)

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Objectives and outline

In 1994, a co-ordinated European research programme on "Biological control of weeds in crops" (COST action 816) was inaugurated. *Amaranthus* spp., *Convolvulus arvensis* L. / *Calystegia sepium* (L.) R. Br., *Chenopodium album* L., *Orobanche* spp. and *Senecio vulgaris* L. were chosen as target weeds. COST action 816 has facilitated establishment of well co-ordinated research procedures and an efficient project management and has greatly stimulated co-operation between the involved research groups (Müller-Schärer and Scheepens, 1997).

Within the framework of COST action 816, the aim of the present study was to investigate the possibility for biological control of *C. arvensis* through the inundative (or bioherbicide) approach by evaluating and improving the potential of a fungal pathogen.

In a first step, fungal pathogens from bindweeds (*C. arvensis* and *C. sepium*) were collected in different European countries and were screened for possible biocontrol activity. The most promising control agent, *Stagonospora convolvuli* strain LA39, was tested in a preliminary field experiment (Chapter 1). An ecological weed management system (i.e. maize with living mulch) is described and a possible integration of the fungal pathogen to control the escaping bindweed is discussed. In chapter 2, the potential of an oil-in-water formulation to improve the biocontrol agent was studied. Research was focused on spore concentration and exposure to 100% relative humidity (after inoculation) needed to induce severe disease symptoms. Host range was examined. As a next step, different solid media were evaluated for mass production of *S. convolvuli* strain LA39. Methods for prolonged storage of the fungal inoculum and for preservation of the spores at low temperatures were tested (chapter 3). In chapter 4, the degree of susceptibility of three ecotypes of field bindweeds against six isolates of *Stagonospora* sp. was analysed. The genetic variation among 38 *Stagonospora* sp.- and 10 *Septoria* sp.-isolates was determined using two molecular methods. This with regard to the possibility to track the control agent once applied in the field. In addition, the effect of a spore mixture of two pathogens on disease was studied. Finally, all the results are compiled and discussed in general.

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Müller-Schärer, H. and Scheepens, P.C. (1997). Biological control of weeds in crops: a coordinated European research programme (COST-816). *Integrated Pest Management Reviews* 2, 45-50.

Chapter 1:

Towards the management of field bindweed (*Convolvulus arvensis*) and hedge bindweed (*Calystegia sepium*) with fungal pathogens and cover crops

Abstract

The bindweeds *Calystegia sepium* and *Convolvulus arvensis* are difficult to control chemically. *Calystegia sepium* is often a problem in maize or in vineyards, while *C. arvensis* is an important weed of cereals. The biological control of these weeds with insects or fungal pathogens has been investigated since 1970. More than 600 fungi collected in countries throughout Europe have been isolated in our laboratories. The isolates with the highest and most stable pathogenicity against bindweed belong to the genus *Stagonospora*. In a field trial in maize in 1995, one of these *Stagonospora* isolates stopped the increase of ground coverage by the bindweeds. In response to public concern about environmental problems caused by modern agriculture, new cropping systems are being developed. Underseeding maize with a living green cover achieves good control of a large spectrum of the weed flora typical of conventional tillage systems. However, *C. sepium* and *C. arvensis* remain as problems. The research reported shows that *C. sepium* is partly suppressed by the green cover, but escapes control by climbing the stems of the maize plants. Therefore, the application of spores of *Stagonospora* sp. in a maize field underseeded with a living green cover may allow a large or a complete reduction of the herbicide input and promote a more sustainable agriculture.

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Introduction

Field bindweed (*Convolvulus arvensis* L.) and hedge bindweed (*Calystegia sepium* (L.) R. Br.) are perennial, noxious weeds in Europe and many agricultural areas of the world (Maillet, 1988; Weaver and Riley, 1982). Field bindweed has been described as the twelfth worst weed in the world (Holm *et al.*, 1977). Bindweeds are a serious problem in grapes, raspberries, beans and maize; the yields of winter wheat can be reduced by one-third and of summer-growing crops by three-quarters (Phillips, 1967). Bindweeds reduce the crop value through competition and by interfering with the harvest procedures. In addition, field bindweed provides a breeding site for insects attacking adjacent crops (Tamaki *et al.*, 1975) and serves as an alternative host of viruses which cause plant diseases (Feldman and Gracia, 1977; Holm *et al.*, 1977). The control of bindweed with mechanical and chemical methods is difficult because of its vigorous regeneration capacity. Some control but not eradication is obtained with chemical herbicides (Derscheid *et al.*, 1970; Westra *et al.*, 1992; Wiese and Rea, 1959). The selective herbicides in use are 2,4-D, dicamba, picloram (Westra *et al.*, 1992) and imazapyr (Schoenhals *et al.*, 1990); glyphosate, a non-selective herbicide, is also used (Westra *et al.*, 1992). Once a bindweed population is established it is very difficult to control. Repeated applications of herbicide may stop shoot growth and reduce the amount of root, but, even after applications for several years, some root growth, from which further shoots can develop, remains (Timmons, 1949).

Convolvulus arvensis, an important weed in cereal production (Holm *et al.*, 1977), has slender, twinning stems (1 to 3 m long) which spread over the soil surface and other plants. The funnel-shaped, white or pinkish flowers, 1.5 to 3 cm wide and long, with two bracts approximately 1.5 cm below the flower, are borne in leaf axils. The simple, alternate leaves are up to 6 cm long and 3 cm wide. The root system of *C. arvensis* may extend through a soil zone 6 m in diameter and up to 9 m deep (Holm *et al.*, 1977).

Calystegia sepium is a pest in maize and vineyards, but is also troublesome in cereals (see the section on new cropping systems). Its creeping and climbing stems can be up to 5 m long and bear longer (length $\geq$ 6 cm) leaves than *C. arvensis*. Flowers are white, 4 to 6 cm long and funnel shaped (Maillet, 1988).

Bindweeds produce numerous seeds (10^7 ha^{-1}) which survive for 20 to 30 years in the soil (Timmons, 1949). *Calystegia sepium* prefers moist soils whereas *C. arvensis* is more tolerant of dryness (Maillet, 1988).

The poor control of bindweeds by herbicides has stimulated studies on biological control since 1970. Insects and gall mites have been considered (Rosenthal and Carter, 1977; Rosenthal and Platts, 1990) and the gall mite *Aceria malherbae* Nuzzaci (Acari: Eriophyidae), imported from Greece, has been released as a potential biological control agent in some states of the USA (Boldt and Sobhian, 1993). The reports of biological control by fungi mainly concern *Phomopsis convolvulus* Ormeno used at the three- to five-leaf stage of *C. arvensis* (Morin *et al.*, 1989; Ormeno-Nuñez *et al.*, 1988) and *Phoma proboscis* Heiny (Heiny, 1990; Heiny and Templeton, 1991). Phytotoxic metabolites of *P. convolvulus* have been analysed (Tsantrizos *et al.*, 1992). An additional alternative to chemical control is the use of a living soil cover (Ammon, 1993; Ammon and Serafin, 1996). In this study we examine a possible combination of biological control of bindweed with fungal pathogens and crop management practices including the use of living soil cover.

Biological control of *C. arvensis* and *C. sepium* with fungal pathogens

Preliminary field survey

Since 1982, the Institute of Plant Sciences, Zürich, Switzerland, has been collecting diseased *C. arvensis* and *C. sepium* throughout Europe. In addition, specimens have been obtained from S. Hasan, CSIRO, Montpellier, France and E. E. Trujillo, University of Hawaii, USA. In total, 271 cultures representing 44 species in 30 genera were isolated and screened for pathogenicity (the data for the cultures from *Convolvulus arvensis* are presented in Table 1). The best candidates, all *Stagonospora* sp., were tested for host specificity and their potential evaluated in preliminary field trials in different locations conducted with 2 to 4 months-old bindweed plants of various ecotypes. Five fungi were successful against *C. sepium* and two against *C. arvensis*. For example, at Eschikon, Switzerland, isolate 214 Ca a reduced the ground cover by *C. sepium* in maize by 82% in 1990 and by 63% in 1991. In contrast, less control was achieved during 2 years of field trials at Montpellier, France (Hasan *et al.*, 1992).

Additional surveys of *C. arvensis* infestations at 15 sites across the south of England and 2 sites in the north of England by the group in Long Ashton have produced 268 pathogenic fungus isolates. These include *Phomopsis, Fusarium, Alternaria* and *Phoma* spp. Only *Phoma exigua* Desmazieres strains were found to be sufficiently effective to be considered as a potential mycoherbicide. Although *P. exigua* is normally a virulent pathogen of potato, the four strains isolated were not pathogenic to potato or to 16 other crops (M.P. Greaves and M.D. MacQueen, unpublished). The identity of the strains, which were all morphologically distinct, was confirmed by both the Commonwealth Mycological Institute (CMI), Kew, UK and the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands.

In laboratory experiments (M.P. Greaves and M.D. MacQueen, unpublished), each strain of *P. exigua* was shown to kill *C. arvensis* seedlings when applied to the foliage at the three- to five-leaf stage (10^6 conidia ml^{-1}) using an air brush sprayer and spraying to run-off. The sprayed plants were given 18 hours exposure to dew and then transferred to the glasshouse or were kept in humid propagation boxes without prior exposure to dew. In both cases, lesions were visible 2 days after spraying, the leaves were totally necrotic after 4 to 5 days and stems had died after 7 days. There was no regrowth from the roots. The treatment of mature plants with well-developed root systems resulted in stem death

in 7 to 10 days but this was followed some 10 to 14 days later by regrowth from the roots. After approximately 2 years subculture and storage on slopes all four isolates suddenly lost their virulence. Attempts to re-establish the virulence, by passage across the host, failed. Therefore, for convenience, subsequent research at Long Ashton concentrated on the *Stagonospora* sp. isolated by the Zürich group. All the *P. exigua* isolates were placed as confidential deposits in the CMI collection. The original sites of sampling were clearly documented. Subsequent visits have confirmed that the disease is still present.

The genus *Stagonospora*

Stagonospora, a genus of the Deuteromycota, class Coelomycetes, contains more than 350 species, which are mostly poorly described. The most important species is *Stagonospora nodorum* Berk. (= *Septoria nodorum* Berk.; telemorph: *Leptosphaeria nodorum* Müller). *Stagonospora* sp. produces brown lesions on the leaves of bindweeds.

Our *Stagonospora* strains grow well *in vitro* between 15 and 27°C. Spores are produced in mass on V8-juice agar and, to some extent, on several sterilised cereal grains (e.g. wheat, maize, barley and millet). None of the strains that are aggressive on *C. sepium* cause major disease symptoms on *C. arvensis* and *vice versa*. On bindweeds, they cause typical brown lesions on the leaves followed by defoliation, the reduction of plant growth and, occasionally, the death of young seedlings in the greenhouse. No attack was observed on stem or roots. The strains appear to produce one or more phytotoxins.

Table 1: Fungal strains isolated from *Convolvulus arvensis* L..

Classes	Species	Number
Agonomycetes	*Rhizoctonia* sp.	4
Ascomycetes	*Chaetomium* sp.	2
	Hypoxylon bipapillatum Berk. & Curtis	1
	fragiforme Kickx	2
	Pleospora herbarum Rabenhorst	5
	Sordaria fimicola Cesati & de Notaris	2
	macrospora Auerswald	1
	Sporormiella minima Ahmed & Cain	1
Basidiomycetes	*Aureobasidium pullulans* Arnaud	5
Coelomycetes	*Ascochyta* sp.	1
	Colletotrichum dematium Grove	4
	gloeosporioides Panzig & Saccardo	4
	Phoma cava Schulzer	2
	pomorum von Thümen	2
	Phoma sp.	12 (**3**)[a]
	Septoria sp.	17 (**9**)
	Stagonospora sp.	5 (**4**)
	Stagonospora /Ascochyta	2
Hyphomycetes	*Acremonium curvulum* Gams	2
	Acremonium sp.	1
	Alternaria alternata von Keissler	3
	tenuissima Wiltshire	5
	Alternaria sp.	8
	Bipolaris sp.	1
	Botrytis cinerea Persoon:Fries	1
	Cladosporium tenuissimum Cooke	1
	Epicoccum purpurascens Ehrenberg	4
	Fusarium arthrosporioides Sherbakoff	6
	lateritium Nees:Fries	3
	oxysporum Schlechtendahl:Fries	6
	solani Saccardo	7
	tricinctum Saccardo	2
	Fusarium sp.	1
	Geniculosporium serpens Chesters & Greenhalgh	1
	Goniotrichum sp.	1
Hyphomycetes	*Humicola grisea* Traaen	1
	Hyalodendron sp.	1
	Noedulisporium sp.	4
	Ovularia sp.	2
	Periconia igniaria Mason & Ellis	1
	Trichoderma sp.	1
Oomycetes	*Rhizopus stolonifer* Vuillemin	1
non determined		1
Mycelia sterila		17
	28 Genera	154 (**16**)

[a] The number in parentheses are the number of isolated strains pathogenic for *C. arvensis* and with a reduced host range. The strains were isolated from 57 specimens collected in Germany, France, Italy, Austria, Switzerland, Czechoslovakia and Yugoslavia.

Recent survey and field experiments

In summer and autumn 1994, diseased bindweed plants were collected in Romania and England. One isolate, a *Stagonospora* sp., was tested at Eschikon near Zürich in a maize field with an established bindweed population during the summer of 1995. Spores were applied at 3 x 10^6 spores ml^{-1} at 300 l ha^{-1} with a conventional backpack sprayer (motorised pump and 1.5 m spray boom with three Teejet 800067 nozzles operating at 2 bar). The control plots were repeatedly treated with Benomyl (fungicide) to prevent spread of the sprayed fungus. Untreated plots were sprayed with neither fungal spores nor Benomyl.

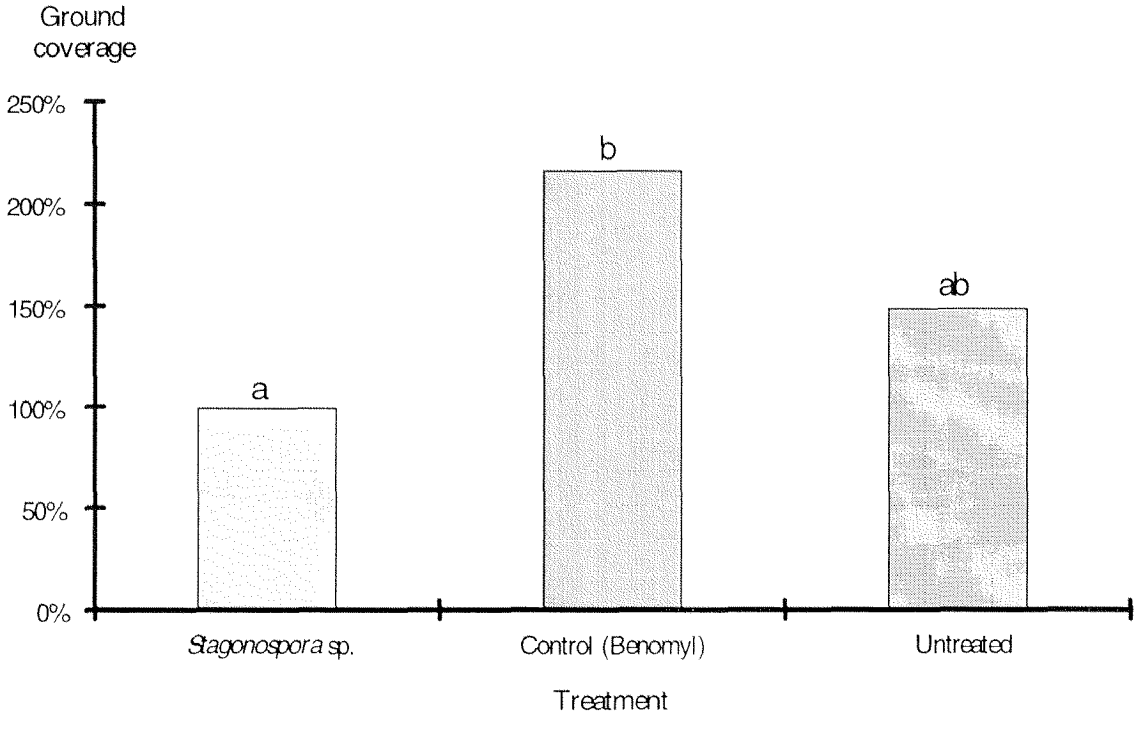

Figure 1: Development of percentage ground coverage with bindweed 3 weeks after the application of *Stagonospora* sp. (100% = ground coverage before the application; field trail 1995 at Eschikon, Switzerland). Columns with the same letter are not significantly different at the 5% level using the Student's *t*-test.

The percentage ground cover with bindweed in the *Stagonospora*-treated plots did not increase during the 3 weeks after treatment, while in the untreated plots it increased by 48% and in control plots by 115% (Figure 1). Leaf infection in the *Stagonospora*-treated plots reached 78% compared to only 38% in the untreated plots. In the *Stagonospora*-treated plots 45% of the leaves were dead, with 23% dead in the untreated plots (Table 2). The infections in the untreated plots might indicate that this isolate has the ability to spread or that *Stagonospora* was just generally present in the field. In the Benomyl control plots 14% of the leaves were infected and 6% were dead (Table 2).

Table 2: Percentage of leaves infected and dead three 3 after application of *Stagonospora* sp. (field trail 1995 at Eschikon, Switzerland).

Treatment	**% of leaves infected**[a]	**% of leaves dead**[a]
Stagonospora sp.	77.9 a	45.4 a
Control (Benomyl)	13.8 b	6.0 b
Untreated	37.8 b	23.3 ab

[a] Values followed by the same letter are not significantly different at the 5% level using the Student's *t*-test.

Control of bindweeds in new cropping systems

Why new cropping systems?

The development of European agriculture has produced many changes in recent decades. Notably, herbicides have replaced mechanical weed control. Most recently, new crops such as maize, soya and sunflower are sown late in spring rather than in autumn, as is usual with cereals. In addition, we now have less diversity in crop rotation, or even monocultures, in particular with maize and increasingly with wheat.

Without the adaptation of tillage and cropping techniques, these changes have given rise to environmental and weed problems. Nitrate losses are high during long fallow winter periods, one of main reasons for the increasing nitrate concentrations in the ground water. Further, the use of persistent, highly active herbicides increases soil erosion because the soils are weed free for longer. In addition, the repeated use of such herbicides results in resistant weeds or a shift of the weed flora. Effective weed control, thus, needed more herbicide and, as a result, herbicides in the ground and surface waters have increased. In addition, there is soil compacting during harvesting of the new, late harvested crops in fully weed-free soil.

An increased perception of and public concern about environmental problems in many countries in the 1990s produced demands for new concepts of land use and lower inputs of pesticides in agriculture and, in particular, in amenity and non-cropped areas. Three main improvements were adapted.

(1) Pesticide use was restricted by environmental legislation in agriculture, forestry, amenity and non-cropped areas. In some countries, herbicides were totally banned in forests and roadsides and their use severely restricted on railway tracks.
(2) Integrated pest management (IPM) was introduced in agriculture, coupled with subsidies in many countries, in addition to prescriptions of land use, crop rotation and tillage techniques.
(3) Organic farming production was increased to meet the demand of an increasing number of consumers. All synthetic pesticides are banned in these production systems.

Intensive research has been started to underpin these changes. New tillage or cropping techniques are being developed and land use systems adapted to the new crops and crop rotation systems. The research also includes the introduction of the new

cropping techniques into integrated crop production (ICP) systems and investigation of biological pest, disease and weed control, as potential partners in integrated control programmes.

Integrated production system for maize

Long periods of uncovered soil are regarded as the main reason for ecological problems in maize. These include soil erosion and the movement of adsorbed pesticide, in particular atrazine, into adjacent water bodies. In recent years, it has been shown that maize is only affected by weeds during the critical period lasting from approximately the three- to four to the six- to eight-leaf stage (Koch and Kemmer, 1980; Zink and Hurle, 1990). There is, thus, no need to maintain the field weed free during the whole growing season to permit full yields. A green soil cover produced by underseeding with *Trifolium* species after mechanical weed control is tolerated (Ammon and Scherrer, 1995). Mulch seeding techniques, using the dead plant residues of the preceding crop or a frost- or herbicide-killed catch crop are common practice in many countries. Recent studies have shown that the catch crop need not be ploughed in or removed with herbicides before seeding the maize. Direct seeding of the crop into the living plant cover is possible with appropriate machinery and in an adapted system, the plants may be kept as living mulch in maize. The timing and the methods of regulating this soil cover mechanically or with herbicides and the influence of the catch crop species on the yield, are described in Ammon *et al.* (1995) and Hartwig (1983). In new trials the influence of the green cover on pests, diseases and other soil parameters has been studied. Here we report the influence on the weed flora, in particular on *C. sepium*.

Materials and methods

Maize cropping with a living mulch

The conventional maize tillage system, ploughing in autumn and seeding maize in bare soil in late spring, is known to favour nitrate losses. A green soil cover - either a catch crop seeded in autumn or an existing meadow - is cut for cattle fodder or flail chopped as green manure in spring before maize seeding. Maize is seeded with a specially constructed rotary band seeder into the living plant sward. The combination includes four band rotovators, working 15 cm deep and 30 cm wide, a winged tine 30 cm wide working at approximately 20 cm depth preceding the rotovator, a band fertiliser operator and a band sprayer to apply herbicides into the rotovated band (for details see Ammon and Bohren, 1996). Instead of herbicides, a band flamer can be used.

Regulation of catch crops and weeds between the maize rows

A tractor-mounted, four-row (flailer-type) mulcher was used between the maize rows to cut resprouting catch crop plants and weeds 1-3 cm above the soil surface. Normally two mulching procedures at the two- to three- and four- to six-leaf stages of maize are necessary. Such cutting is possible in regions with sufficient precipitation or in living mulch of low competitive ability (Ammon and Bohren, 1996). The regulation with herbicides is dealt with by Ammon *et al.* (1995).

Four year trial to compare living mulch with conventional tillage and to control the weed flora.

In a 4 year trial started in 1990, four maize cropping systems repeated on the same plots were tested (the details of method are in Bigler *et al.*, 1995a). The influence on important annual weeds in maize, e.g. *Echinochloa crus-galli* (L.) P. B. and late-emerging annual dicotyledoneous species known to be hard to control because of their ability to form atrazine-resistant biotypes, was evaluated from 1990 through 1993. The influence on the most import perennial weed, *C. sepium*, was evaluated from 1991 to 1993. The four cropping systems were as follows.

(1) Conventional tillage (ploughing in autumn) combined with pre-emergent herbicides according to the weed flora to achieve full weed control ("conventional").
(2) Conventional tillage with mechanical weed control and an underseeded grass-*Trifolium* mixture at the three- to four-leaf stage of maize ("underseeded").
(3) Rotary band seeding in rye (*Secale cereale* L.) catch crop, seeded in autumn, flail chopped as green manure in spring before maize seeding and interrow mulching at the three- to four-leaf stage of maize. Each year, after the maize harvest, the field was ploughed and the rye catch crop reseeded ("rye").
(4) Rotary band seeding in a grass-*Trifolium* meadow. Grass was harvested in autumn and in spring, resprouting grass-*Trifolium* being controlled by interrow mulching at the two- to three- and four- to six-leaf stages of maize. The grass sward was allowed to regrow after the maize harvest, no reseeding being done in the 3 years ("meadow").

One year trial

Conventional tillage was compared to maize underseeded with *Trifolium* (according to Ammon and Scherrer, 1995) and the influence on *C. sepium* was determined by counting the number and length of climbing and creeping shoots and the number of new stolons developed.

Results

The influence of the four cropping systems on the density of the annual weeds are presented in Figure 2, on the perennial weed *C. sepium* in Figure 3.

In the underseeded treatment the weed density reduction was variable, depending on the development of the grass-*Trifolium* sward (results not shown). In the fourth year with good, early development of the sward, the reduction was higher, but not sufficient, in particular concerning the dicotyledoneous species (Figure 2B). In the rye treatment, in the first years panicoid grasses and *Chenopodium polyspermum* L. were dominant. In subsequent years, these two species were reduced (Figure 2A). The control of the dicotyledoneous weeds was not sufficient particularly in the first 3 years (Figure 2B). Similarly, in the meadow treatment, but only in the first year, panicoid grasses, mainly

E. crus-galli (L.) P. B., dominated. In subsequent years, these species disappeared. In contrast to the rye treatment, in the meadow treatment the hard to control dicotyledoneous species were fully controlled from the first year on. Therefore this treatment is considered as a strategy to prevent or even control herbicide-resistant biotypes. In this meadow treatment the season-long, living mulch was obviously more competitive to annual weeds than rye, which dies out after the interrow mulching at the three- to four-leaf stage of maize.

In the conventional treatment, the herbicide combination of atrazine and alachlor was not effective against panicoid grasses and was replaced in the second year by atrazine and metolachlor. In the third year pendimethaline was added to control the atrazine-resistant species and, thus, to achieve a fully weed free soil, in contrast to the other systems.

One main perennial weed, *C. sepium*, was not effectively controlled in any of the four systems (Figure 3). Each year, in the conventional and rye treatments, a full dose of dicamba was applied as a spot treatment. Despite this, *C. sepium* remained an important weed, except in 1993 in rye. In underseeded and meadow systems, dicamba was also used, but at lower concentrations and as very restricted spot treatments in order not to harm the *Trifolium* living mulch. This gave some reduction of *C. sepium*, the greatest mean reduction over the 3 years occurring in the meadow system.

(1) Conventional tillage (ploughing in autumn) combined with pre-emergent herbicides according to the weed flora to achieve full weed control ("conventional").
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In the underseeded treatment the weed density reduction was variable, depending on the development of the grass-*Trifolium* sward (results not shown). In the fourth year with good, early development of the sward, the reduction was higher, but not sufficient, in particular concerning the dicotyledoneous species (Figure 2B). In the rye treatment, in the first years panicoid grasses and *Chenopodium polyspermum* L. were dominant. In subsequent years, these two species were reduced (Figure 2A). The control of the dicotyledoneous weeds was not sufficient particularly in the first 3 years (Figure 2B). Similarly, in the meadow treatment, but only in the first year, panicoid grasses, mainly

E. crus-galli (L.) P. B., dominated. In subsequent years, these species disappeared. In contrast to the rye treatment, in the meadow treatment the hard to control dicotyledoneous species were fully controlled from the first year on. Therefore this treatment is considered as a strategy to prevent or even control herbicide-resistant biotypes. In this meadow treatment the season-long, living mulch was obviously more competitive to annual weeds than rye, which dies out after the interrow mulching at the three- to four-leaf stage of maize.

In the conventional treatment, the herbicide combination of atrazine and alachlor was not effective against panicoid grasses and was replaced in the second year by atrazine and metolachlor. In the third year pendimethaline was added to control the atrazine-resistant species and, thus, to achieve a fully weed free soil, in contrast to the other systems.

One main perennial weed, *C. sepium*, was not effectively controlled in any of the four systems (Figure 3). Each year, in the conventional and rye treatments, a full dose of dicamba was applied as a spot treatment. Despite this, *C. sepium* remained an important weed, except in 1993 in rye. In underseeded and meadow systems, dicamba was also used, but at lower concentrations and as very restricted spot treatments in order not to harm the *Trifolium* living mulch. This gave some reduction of *C. sepium*, the greatest mean reduction over the 3 years occurring in the meadow system.

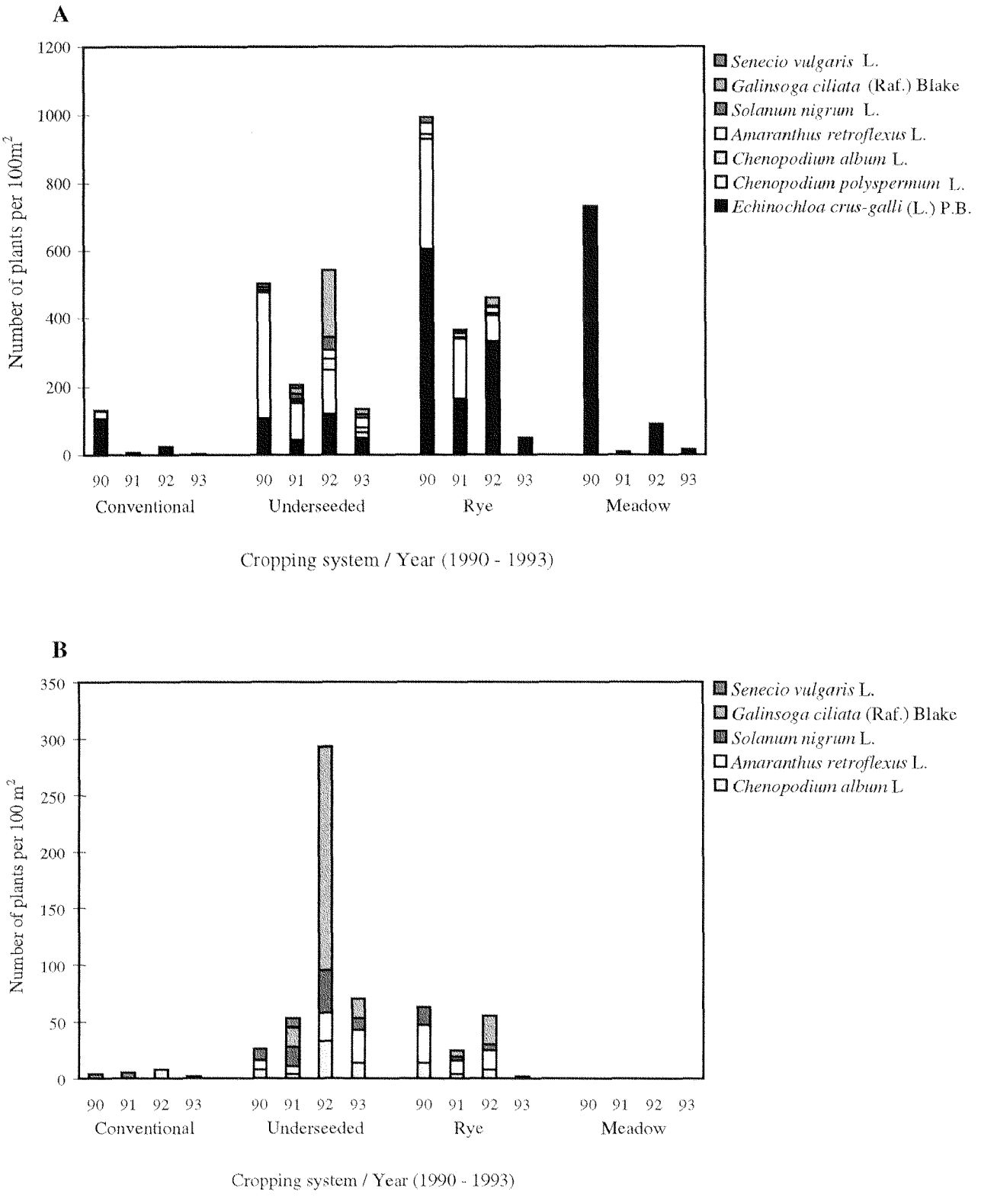

Figure 2: Densities of selected weeds at harvest in four cropping systems of maize in a 4 year trial (1990-1993). (A) All weeds. (B) Hard to control annual dicotyledoneous weeds.

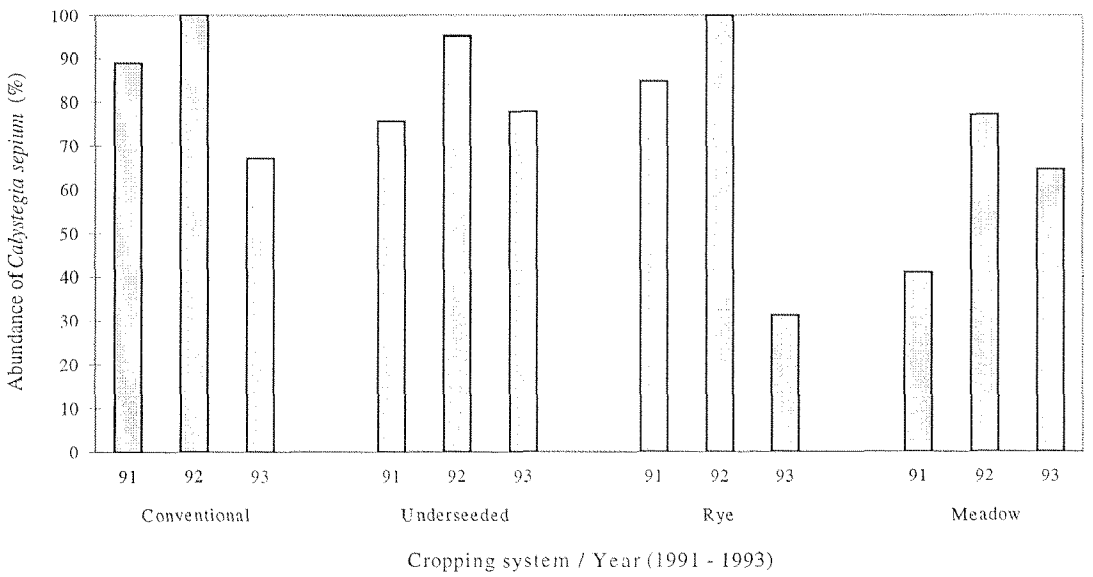

Figure 3: Abundance (%) of *C. sepium* in four cropping systems of maize rows (32 rows each 26m long with three repetitions; 100% represents *C. sepium* present in all the interrows).

The influence on the abundance in the 4 year trial agrees with the findings of the 1 year trial comparing the growth parameters of *C. sepium* in underseeded and conventional maize (Table 3). With a well-developed *Trifolium* cover, the development in the length and number of horizontally spreading shoots decreased and no new stolons were formed. The shoots climbing up the maize stems were not influenced in number, but their development was reduced.

Table 3: Development of *C. sepium* in maize with and without underseeded *Trifolium* (mean of 10 plants)

	"Climbing" shoots		**"Creeping" shoots**				**New stolons**
	Number	Ø height [m]	Number	Length [m]			
				Min.	Max.	Mean	
without *Trifolium*	1.7	1.2	6.7	0.3	5.6	2.5	25
with *Trifolium*	2.0	0.6	0.9	0.3	2.2	0.7	0

Discussion

Rotary band seeding into living mulch allows an ecologically sound maize cropping system. This includes a green ground cover before maize seeding to lower the nitrate leaching and a living or dead mulch during the maize cropping period to reduce soil erosion and water run-off. The ploughless seeding technique with living mulch reduces new weed emergence. The change of the weed flora prevents the build up of resistant weeds. A ground cover at harvest reduces soil compaction and, after maize harvest, the green cover re-establishes fully in the meadow or underseeded treatments and is therefore well suited for the following crop seeding in spring.

In the rye treatment regrowth is low and so suitable for autumn-sown rotational crops. Experience shows that grasses are good nitrate catch crops, but the release of the nitrate to maize is often too late. If abundant farm manure is not available, legumes should be chosen as catch crops to obtain normal maize yields (Ammon and Bohren, 1996).

According to Jäggi *et al.* (1995), a larger number of earthworms and collembola were found particularly in the meadow treatment. Bigler *et al.* (1995b) found a smaller number of corn borer, aphids and *Ustilago maydis* Corda in the rye and meadow treatments and more predators, in particular spiders and ants (Bigler *et al.*, 1995c).

The living mulch system described here can be used in regions with high precipitation. In dryer climates or with competitive living mulches, e.g. many grasses, such as *Lolium* species, mulch regulation with herbicides is necessary to avoid maize yield losses. Non-residual herbicides such as glyphosate or those used at pre-emergence of maize are preferable (Ammon *et al.*, 1995). The choice of catch crop, skilled seeding and the timely regulation of the living mulch are of key importance in the success of these systems.

One main maize weed, *C. sepium*, is suppressed, but not sufficiently controlled either by mulching or by herbicides applicable selectively in a living mulch system with maize. In these circumstances a specific control agent is necessary. A biological control agent would be an ideal solution. Often, biological methods are not suited because of their restricted weed control spectrum. However, this restriction is less problematic in ecological farming or in IPM, where conventional herbicides are proscribed or should be avoided as far as possible. The suppressing effect of the mulch may permit the use of

relatively low virulence pathogens, such as *Stagonospora* sp. as a parasite of *C. sepium* and *C. arvensis*. Current work suggests this approach is promising.

The example shows that the overall environmental and agronomic problems cannot be solved simply by changing conventional pest control to integrated control (IPM). New tillage techniques and sound crop rotations are also necessary. Weed control must be changed from weed eradication to a vegetation management system within integrated crop production (ICP).

Conclusions and outlook

Concern regarding the use of chemical herbicides has resulted in a demand for alternatives in weed control. New cultivation practices and crop management strategies may reduce the input to and impact of chemicals on agricultural crops. Underseeded green cover can reduce the need for chemical weed control. In maize underseeded with a living green cover, good control is achieved for a large spectrum of the typical weed flora known to develop with conventional tillage systems. Nevertheless *C. sepium* and *C. arvensis* remain as weed problems. The experiments presented show that *C. sepium* is only partly suppressed by the green cover, being able to escape control by climbing up the stems of the maize plants. Therefore, an additional control is necessary.

The control of a weed species with a fungal pathogen (mycoherbicide) has been successful in several cases (Greaves, 1996). Our research has shown that several isolates of *Stagonospora* sp. attack *C. sepium* and *C. arvensis* and field experiments have shown that they can reduce or stabilise ground cover by the weed. Further steps in the development of a possible mycoherbicide will be field trials under different environmental conditions. For this, studies of the efficacy of *Stagonospora* sp. to control bindweed will have to be performed in various European countries.

The effectiveness of a biocontrol agent can be increased by formulation, which should be designed to increase both the efficiency of application and efficacy of the control agent. One of the main limitations to mycoherbicides is the requirement of a lengthy dew period. We are now testing different formulations to reduce the dew period needed for a good infection process. Another possibility of increasing the effectiveness may be the combination of a control agent with low dosages of herbicides. Sharon *et al.* (1992) increased the susceptibility of the weed *Cassia obtusifolia* L. to the

mycoherbicide CASST® (*Alternaria cassiae* Jurair and Khan) by adding a sublethal dose of glyphosate, resulting in a specific suppression of the weed's elicited defence response.

The combination of a maize field underseeded with a living green cover with the application of spores of *Stagonospora* sp. may be a way of achieving an ecologically healthier production of maize. Future field experiments with this cropping system will show the effectiveness of this combination.

The underseeding of maize with a living green cover can be limited by environmental conditions. The main restriction appears to be water availability for the maize and the green cover together. Therefore the production system outlined will be practical only in areas with sufficient moisture during the vegetation period and will be used in the near future only in a small part of the maize production. It will remain a niche market. However, niche markets can be good areas for biological control agents. Other possible niche markets for the biological control of bindweed include forest nurseries, horticulture and non-crop situations such as gardens and parks.

After application in the field, the epidemiology and dispersal of the control agent must be studied. The use of DNA technology appears to be the most promising way. We are presently developing genetic markers, which will allow us to track the biocontrol agent released in the field.

Biological control of bindweed with *Stagonospora* sp. appears promising. Its integration in a weed management system may even improve the potential of the pathogen. In maize, the combination of the pathogen with cover crops may allow the control of typical weed flora.

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Chapter 2:

The potential of *Stagonospora* sp. as a mycoherbicide for field bindweed

Abstract

Field bindweed (*Convolvulus arvensis* L.) is one of the 12 most important weeds worldwide. *Stagonospora* sp. isolate LA39 was isolated from diseased field bindweed plants collected in Europe. No crop tested was susceptible to the fungus, but disease symptoms were observed on other *Convolvulaceae* species. On field bindweed, the fungus induces disease symptoms (i.e. lesions) mainly on leaves and less severely on stems. The application of spores in an oil emulsion (10% oil in water) enhanced the disease on field bindweed plants compared to spores suspended in a 0.1% aqueous solution of the surfactant agent, Tween 80. The necrotic leaf area of inoculated plants increased as the length of exposure to 100% relative humidity (RH) and spore density applied increased. Severe disease developed on plants inoculated with 1 x 10^7 spores/ml in oil emulsion, even in the absence of exposure to 100% RH. A delay of exposure to 100% RH (up to 8 h) did not have a significant effect on disease severity. Field bindweed was susceptible to the fungus at all growth stages tested, but older plants were more susceptible than younger ones. It was concluded that isolate LA39 has potential as a biocontrol agent of field bindweed, especially when applied in an oil emulsion. The oil emulsion maintains the aggressiveness of the pathogen during a dew-free period and provides a favourable micro-environment during the infection process.

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Introduction

Field bindweed (*Convolvulus arvensis* L.) is a perennial, noxious weed throughout the world. It is most troublesome in grapes, raspberries, beans and maize (Holm *et al.*, 1977; Maillet, 1988; Weaver and Riley, 1982), reducing crop value through competition and interference with harvest. Chemical and mechanical control is effective for seedlings and first-year plants, but destruction of the shoots of older plants leads to vigorous regrowth from root buds and rhizomes (Weaver and Riley, 1982).

The biological control of field bindweed involving insects, mites, and fungal pathogens as control agents has been investigated since 1970. The gall mite, *Aceria malherbae* Nuzzaci, originating from Greece, is now released as a potential biological control agent in some states of the USA (Boldt and Sobhian, 1993). The fungi *Phomopsis convolvulus* Ormeno (Morin *et al.*, 1989, 1990a; Ormeno-Nuñez *et al.*, 1988; Tsantrizos *et al.*, 1992) and *Phoma proboscis* Heiny (Heiny, 1990, 1994; Heiny and Templeton, 1991), which infect field bindweed, have also been extensively studied. These pathogens caused severe disease on bindweed seedlings, but only partly suppressed the growth of older plants.

One limitation to the use of mycoherbicides is that a long dew period is needed by the pathogen in order to cause sufficient infection of the target weed. An appropriate formulation of infective propagules which reduces or eliminates the dew requirement would greatly improve the potential of a pathogen as a mycoherbicide. Inert solid carriers, alginate granules, invert emulsions, and oil-in-water emulsions have all been considered (Boyette *et al.*, 1991; Daigle and Connick, 1990; Greaves, 1996).

In 1994 and 1995 several fungal species were isolated from diseased field bindweed plants. In this study, the potential of the most pathogenic isolate, a *Stagonospora* sp., is reported as a control agent of field bindweed. Research was conducted on the effect of an emulsion of vegetable oil in water on infectivity under various environmental conditions, on the effect of growth media on pathogenicity of spores, on the effect of plant growth stage on disease development and on the host range of the pathogen.

Materials and methods

Isolation of pathogens

Diseased leaves and stems of field bindweed were collected from natural infestations in Europe (mainly England and Romania) during 1994 and 1995. Diseased plants were air dried immediately after collection. In the laboratory, fungi were isolated using three different techniques. In the first method, samples were surface-sterilized in 5% sodium hypochlorite (v/v) for 5 min, washed in sterile water and placed on a glass rod above a wet filter paper in a closed petri dish (moist chamber). Mycelium of growing fungi was removed aseptically and transferred on to malt agar plates (1.5% malt extract [w/v; Oxoid], 1.2% agar [w/v; Oxoid, Basingstoke, UK]) containing 25 µl/ml of terramycin (MAT). In the second method, samples were surface sterilized in 96% ethanol (v/v) for 30 s, then in 14% sodium hypochlorite (v/v) for 30 s followed by another 30 s in 96% ethanol before being placed directly on MAT. In the third method, leaf samples were placed in moist chambers under continuous white fluorescent light at 20°C. Spore cirrhi produced by pycnidia were removed aseptically and streaked on to the surface of MAT. Stock cultures of every isolate were stored at 3°C on V8-juice agar slopes (10% V8-juice [v/v; Campbell, King's Lynn, Norfolk, UK], 30 mM $CaCO_3$, 1.2% agar [w/v; Oxoid]).

Plant production

Field bindweed seeds were obtained from MT Valley Seed Service, Fresno, CA, USA and also collected in different European countries. They were surface-sterilized in 1% sodium hypochlorite (v/v), rinsed with distilled water, scratched with a scalpel to break the seed coat and germinated on 0.85% water agar (w/v; Oxoid) in the dark for 2 days at room temperature. Each germinated seed was sown in a plastic pot (diameter 9 cm) in a 3:1 mixture of damp sterilized soil (4C from M. de Baat B. V. Coevorden, the Netherlands) and quartz sand (1.5-2.2 mm size). The pots were placed in a greenhouse chamber with 70% relative humidity (RH) and a 16 h photoperiod. The temperature used were 22°C (light) and 17°C (dark) respectively.

Inoculum production of *Stagonospora* sp. and plant inoculation

Spores from stock cultures of *Stagonospora* sp. isolate LA39 were streaked on to the surface of V8-juice agar plates. The plates were incubated for 2 weeks at 20°C under continuous white fluorescent light. In the experiment looking at the pathogenicity of spores produced on different media, the spores were also produced on potato dextrose agar (PDA) (3.9% [w/v; Difco, Detroit, MI, USA]) and malt-agar (1.5% malt extract [w/v; Oxoid], 1.2% agar [w/v; Oxoid]). Spores were harvested by flooding the plates with distilled water and lightly scraping the surface. The resulting spore suspension was filtered through cheesecloth and adjusted to the appropriate density using a hemacytometer. The suspension of freshly harvested spores was added to the oil emulsion in a 1:9 ratio (spore suspension:prepared emulsion) or to 0.1% aqueous Tween 80 (Fluka, Switzerland; v/v).

In all experiments, plants were sprayed to run-off using an aerosol atomiser, placed in the greenhouse chamber and covered with a plastic bag to maintain 100% RH for a defined time, depending on the experiment, after which RH was kept at 70%.

Preparation of vegetable oil emulsion

The method of Lawrie *et al.* (1997) was slightly modified by using Tween 80 instead of Tween 40. An aliquot of 0.1 ml Tween 80 was added to 10 ml pure, lightly warmed rape oil. The oil-surfactant mixture was then stirred using a magnetic stirrer while 90 ml of distilled water were added. The solution was emulsified using a laboratory mixer for a total of 3 min at low speed. Mixing was achieved with 20 s treatments on the mixer separated by 10 s breaks.

Pathogenicity of spores produced on different media

Plants of the leaf stage 3-5 were sprayed with a suspension of 10^7 spores/ml in oil emulsion containing spores produced on V8-juice agar, PDA or malt-agar. The plants were exposed to 100% RH for 48 h. The experiment was repeated three times.

Effect of plant growth stage on disease development

Plants at leaf stages 3-5, 7-9, or 11-13 were sprayed with a suspension of 10^7 spores/ml in oil emulsion and exposed to 100% RH for 24 h.

Effect of spore density, relative humidity, and vegetable oil emulsion on disease development

A two-by-four factorial experimental design was used to determine the effect of the oil emulsion and of various spore densities on disease development. Plants at leaf stage 3-5 were sprayed with 10^5, 10^6, 10^7 or 10^8 spores/ml, applied in either oil emulsion or 0.1% aqueous Tween 80 (v/v) and exposed to 100% RH for 24 h.

A two-by-four factorial experimental design was used to determine the effect of the oil emulsion and of the duration of exposure to 100% RH following plant inoculation. Plants at leaf stage 3-5 were sprayed with 10^7 spores/ml in either oil emulsion or 0.1% Tween 80 (v/v) and exposed to 100% RH for 0, 6, 24 or 48 h.

A two-by-five factorial experimental design was used to determine the effect of the oil emulsion and of delayed exposure to 100% RH of inoculated plants on disease development. Plants at leaf stage 5-7 were sprayed with 10^7 spores/ml in either oil emulsion or 0.1% Tween 80 (v/v). After inoculation, the plants were kept at 70% RH for 0, 1, 2, 4 or 8 h before they were subjected to 100% RH for 24 h.

Host range

Species of *Convolvulaceae* inoculated with *Stagonospora* sp. isolate LA39 were a range of ecotypes of field bindweed from different European countries and the USA, scammony (*C. scammonia* L.), small blue bindweed (*C. siculus* subsp. *agrestis* [Hochst. ex Schweinf.] Verdcourt), dwarf bindweed (*C. tricolor* L.), *Convolvulus humilis* Jacq., hedge bindweed (*Calystegia sepium* [L.] R. Br.), sweet potato (*Ipomoea batatas* var. *batatas*), tall morningglory (*I. purpurea* (L.) Roth [= *Convolvulus purpureus* L.]), cypress vine (*I. quamoclit* L. [= *Quamoclit pinnata* (Desr.) Bojer], scarlet creeper (*I. hederifolia* L.), and *I. versicolor* Meissner (= *Q. lobata* House).

The crops tested were maize (*Zea mays* L. cvs. Alpin, LG33.43, LG2253 and Tasty Sweet), wheat (*Triticum aestivum* L. cv. Arina), Italian ray-grass (*Lolium multiflorum* Lam. cvs. Ellire and Fedo), white mustard (*Sinapis alba* L. cv. Maxi), lucerne (*Medicago sativa* L.), clover (*Trifolium pratense* L. var. Ruttinova and *T. suaveolens* Willd. cv. Archibald), and grape (*Vitis* L.). All plants were sprayed to run-off with 10^7 spores/ml in the oil emulsion and exposed to 100% RH for 24 h.

Disease assessment

At 2 weeks after inoculation, the leaves of each plant sprayed with the spore suspension were counted and visually rated individually for disease symptoms using a 0-6 scale (0 = no disease; 1 = 0-5%; 2 = 6-25%; 3 = 26-75%; 4 = 76-95%; 5 = >95% of leaf surface with necrosis; 6 = leaf dead). Total necrotic leaf area was calculated as a percentage using the formula $(2.5 \times n_1 + 15 \times n_2 + 50 \times n_3 + 85 \times n_4 + 97.5 \times n_5 + 100 \times n_6) / N$ where n_x is the number of leaves with rating x and where N is the total number of leaves treated.

Data analysis

Unless stated otherwise, all experiments consisted of four replicates (i.e. pots) per treatment and were repeated four times. The total percentage necrotic leaf area was transformed to the arcsine of its square root to ensure normality. The data from all repeated experiments were pooled based on homogenous variances and then subjected to analysis of variance (ANOVA). Where appropriate, treatments were compared using Fisher's least significance difference (LSD) at P=0.05 using the Windows version 5.05 of the Systat program (SPSS Inc., Evanston, IL, USA).

Results

Isolation of pathogens

Fungi isolated from diseased field bindweed belonged mainly to the genera *Phoma, Septoria, Stagonospora, Alternaria,* and *Fusarium*. Only isolates of *Stagonospora* sp. were sufficiently pathogenic to be considered as potential control agents. In preliminary experiments, *Stagonospora* sp. isolate LA39, from Long Ashton, near Bristol, UK, was selected for further experiments. Identification of the fungus was confirmed by Dr. G.-A. Hedjaroude (University of Teheran). The fungus produces brown necrotic lesions on the leaves of field bindweed which cause chlorosis of leaves and defoliation. On stems, lesions are formed far less often and do not cause damage to the plant.

Pathogenicity of spores produced on different media

Spores grown on V8-juice agar produced a significantly higher necrotic leaf area on field bindweed than spores grown on PDA and malt agar (Table 1). The necrotic leaf areas (all leaves) resulting from spores produced on the three media were 91.5% (V8-juice agar), 59.2% (malt agar) and 27.2% (PDA) respectively.

Table 1: Effect of growth medium on pathogenicity of spores of *Stagonospora* sp. on field bindweed.

Growth medium	Necrotic leaf area (%)[a]	
V8-juice agar	91.5	a
Malt agar	59.2	b
PDA	27.2	c

[a] Data, for 2 weeks after inoculation, are means of three experiments with four replications/treatment. Values followed by the same letter are not significantly different ($P<0.05$) using Fisher's LSD test.

Effect of plant growth stage on disease development

All growth stages of field bindweed tested were susceptible to *Stagonospora* sp., older plants being slightly more susceptible than younger ones (Table 2). The necrotic leaf area was greater than 80% at every growth stage. Plants at leaf stage 11-13 (99.3% necrotic leaf area) were significantly more susceptible than plants at leaf stage 3-5 and 7-9, which had 87.5% and 94.4% necrotic leaf areas respectively.

Table 2: Effect of field bindweed growth stage on the disease caused by *Stagonospora* sp. 2 weeks after inoculation.

Growth stage (no. of leaves)	Necrotic leaf area (%)[a]	
3-5	87.5	a
7-9	94.4	a
11-13	99.3	b

[a] Each value is a mean of four experiments with four replications/treatment. Values followed by the same letter are not significantly different ($P<0.05$) using Fisher's LSD test.

Effect of spore density, length and delay of exposure to 100% relative humidity and vegetable oil emulsion on disease development

The severity of disease was dependent on the density of spores applied (Figure 1). Little disease resulted from low inoculum densities (10^5 and 10^6 spores/ml), the necrotic leaf area being less than 30% whether the pathogen was formulated in oil emulsion or in aqueous Tween 80 solution. At these densities, no statistical difference was found between the effects of the two formulations. In contrast, the oil emulsion significantly improved the effectiveness of the pathogen applied in higher doses compared with the Tween surfactant. Spores applied at 10^7 spores/ml formulated in the oil emulsion produced more than 80% necrotic leaf area of field bindweed, while spores applied at the same density in Tween produced less than 50% necrotic leaf area. The disease severity on plants sprayed with spores at 10^8 spores/ml in aqueous Tween solution or 10^7 spores/ml in oil emulsion was not statistically different. The highest necrotic leaf area was obtained on plants sprayed with 10^8 spores/ml in the oil emulsion.

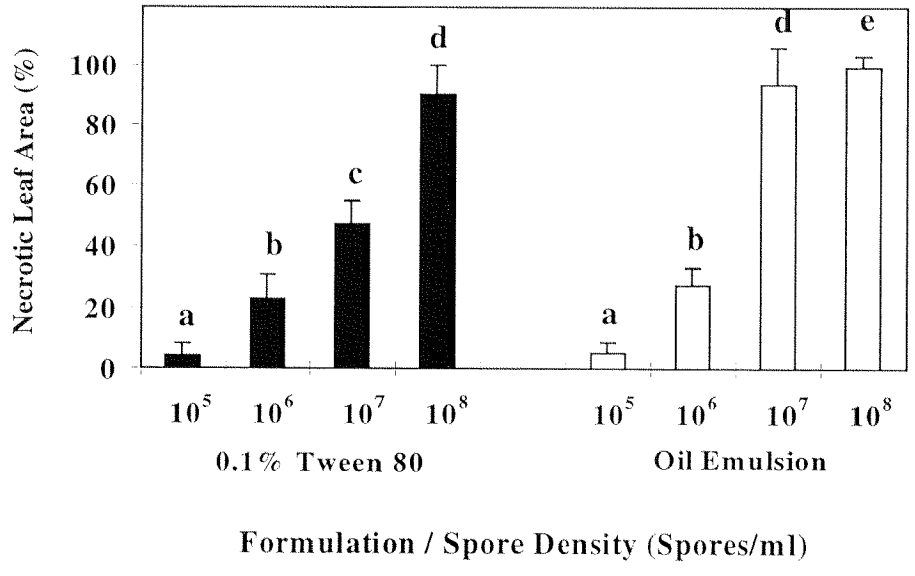

Figure 1: The effect of density of spores on infection of field bindweed by *Stagonospora* sp. 2 weeks after application. Means and standard deviations shown were calculated using data obtained in four independent experiments. Bars marked with the same letter are not significantly different. A similar relationship was found when statistical analyses were performed within each repeated experiment.

The disease severity in treatments with spores formulated in aqueous Tween 80 solution increased with increasing length of exposure to 100% RH. Disease severity ranged from 20% necrotic leaf area (without exposure to 100% RH) up to 80% necrotic leaf area (48 h exposure to 100% RH) (Figure 2). In contrast, the necrotic leaf area was always greater than 50% if the spores were applied in the oil emulsion. The elongation of the exposure to 100% RH up to 24 h did not affect disease severity induced by spores applied in the oil emulsion. The average necrotic leaf area over a dew period of 0, 6 and 24 h was 68.2%. Only an exposure of 48 h at 100% RH increased the necrotic leaf area significantly (94.5% necrotic leaf area) (Figure 2).

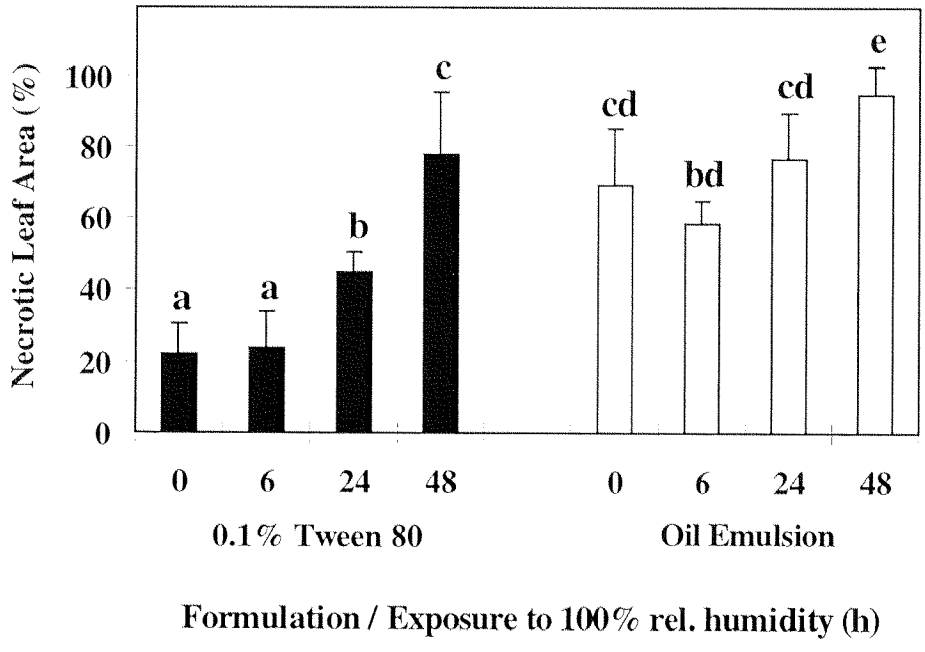

Figure 2: The effect of exposure to 100% RH on infection of field bindweed by *Stagonospora* sp. 2 weeks after application. Means and standard deviations shown were calculated using data obtained in four independent experiments. Bars marked with the same letter are not significantly different. A similar relationship was found when statistical analyses were performed within each repeated experiment.

In the experiment in which the exposure to 100% RH was delayed for up to 8 h, no differences were found between the treatments with spores applied in aqueous Tween 80 solution. The average necrotic leaf area of all the treatments with Tween was 37.7% (Figure 3). Necrotic leaf areas of plants sprayed with spores formulated in oil emulsion were significantly higher than those of plants sprayed with spores formulated in Tween 80, regardless of any delay in the exposure. In all the oil emulsion treatments the average necrotic leaf area was 77.1%. A delay in the exposure to 100% RH of 1 h led to the highest necrotic leaf area (86.0%). However, this was only statistically different to the treatment with a delay of 8 h (66.0% necrotic leaf area) (Figure 3).

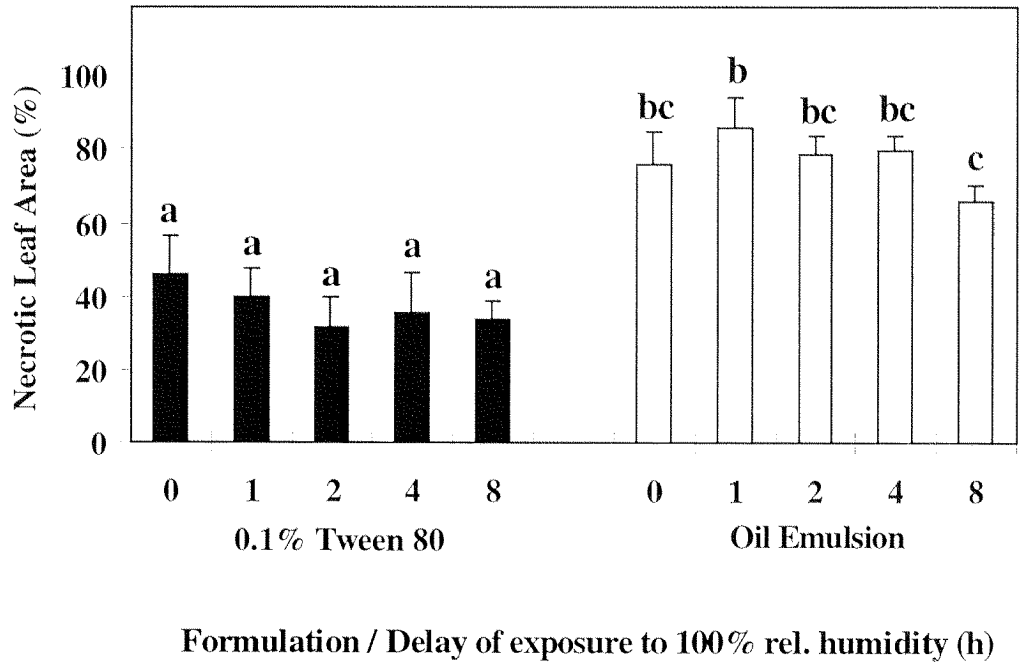

Figure 3: The effect of delay of exposure to 100% RH on infection of field bindweed by *Stagonospora* sp. 2 weeks after application. Means and standard deviations shown were calculated using data obtained in four independent experiments. Bars marked with the same letter are not significantly different. A similar relationship was found when statistical analyses were performed within each repeated experiment.

Host Range

Stagonospora sp. formed necrotic lesions on all *Convolvulaceae* species tested except sweet potato. Field bindweeds of different origins showed no difference in disease severity and symptoms. Necrosis observed on the other *Convolvulaceae* species, except *Calystegia sepium*, was far less severe than on field bindweed. The fungus had no effect on all the other plants tested.

Discussion

Stagonospora sp. isolate LA39 produces typical brown necrotic lesions on the leaves of field bindweed, causing extensive defoliation. Spores of the fungus produced on V8-juice agar were significantly more pathogenic than spores produced on PDA and malt agar, although they were not different morphologically. An opposite effect was found with *Phomopsis convolvulus*. No difference in pathogenicity on field bindweed was detected between conidia produced on half strength PDA, pot barley grains and in liquid culture (modified Richard's (V-8) medium) (Morin *et al.*, 1990b). The possible production (or absence) of certain metabolites on the different media may have caused the difference found in the present experiment. Such an influence of culture media may be a significant factor in the production of a highly effective inoculum needed for control of field bindweed. A similar effect was shown with the inoculum of *Septoria nodorum* Berk., a pathogen of wheat. Spores grown on barley kernels showed a higher deleterious effect on its host than inoculum produced on rye and wheat respectively (Tvaruzek, 1993).

Formulation is recognized as a way to increase both the efficiency of application and efficacy of the control agent. The formulation of spores of *Stagonospora* sp. in a vegetable oil emulsion significantly improved the effectiveness of the pathogen. The oil emulsion was easy to prepare, could be sprayed using standard equipment and was not toxic to *Stagonospora* spores. The oil emulsion may provide a favourable micro-environment around the spores during the infection process, either by retention of the water present in the emulsion or by inducing an exogenous supply of water, possibly from leaf tissue cells (Greaves *et al.*, 1998). The intensity of infection of spores of *Alternaria cassiae* Jurair & Khan on *Cassia obtusifolia* L. and *A. crassa* (Sacc.) Rands

on *Datura stramonium* L. was enhanced when applied in an invert emulsion (Amsellem *et al.*, 1990). The invert emulsion also abolished the selectivity of the two fungi (Amsellem *et al.*, 1991). Amsellem *et. al.* (1991) suggested that as well as retaining water for spore germination, the invert emulsion may cause cuticular damage and could also suppress the plant's elicited responses to infection. In addition, the oil may also attach the spores more strongly to the leaf surface, as was found with *A. cassiae* spores applied in emulsified oils (Bannon *et al.*, 1990).

The delay of exposure to 100% RH by up to 8 h did not reduce disease development, whether the spores were applied in the oil emulsion or in Tween 80. Thus, the potential of the pathogen to infect leaves is maintained without 100% RH for at least 8 h after inoculation. A similar effect, with a dew delay of 12 h, was observed with *P. convolvulus*, another possible candidate for biological control of field bindweed (Ormeno-Nuñez *et al.*, 1988). This resistance to short term desiccation is an important aspect of the suitability of an organism for field application, where dew may be an irregular feature. An oil emulsion formulation may also reduce the number of spores required, an important factor in reducing production costs (Amsellem *et al.*, 1990).

Host-range testing determined that most species of the *Convolvulaceae*, though susceptible to the fungus, were far less severely diseased than field bindweed. Most disease was observed on hedge bindweed, another weed of agricultural importance (Maillet, 1988). None of the crops tested, including sweet potato, were susceptible. The specificity of *Stagonospora* sp. isolate LA39 may be sufficient for its possible use as a mycoherbicide. However, the host-range test needs to be wider to ensure this.

Once applied as a mycoherbicide in the field, the effectiveness of *Stagonospora* sp. may be enhanced through weakening by crop shading or by adding sub-lethal dosages of herbicides. The possibility of enhancing the effectiveness of *Stagonospora* sp. by crop management (i.e. maize underseeded with a living green cover) is discussed elsewhere (Pfirter *et al.*, 1997).

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Chapter 3:

Mass production, storage and preservation of *Stagonospora convolvuli* strain LA39 pathogenic on field bindweed (*Convolvulus arvensis*)

Abstract

The mass production and storage is a crucial step in the development of a potential mycoherbicide. The mass production of *Stagonospora convolvuli* strain LA39, a promising biocontrol agent of field bindweed, on different solid substrates was investigated. The spore production was maximal on cous-cous (cracked hard wheat) yielding up to $4x10^8$ spores/g substrate. The spores were as pathogenic as those grown on agar media. Over 50% of spores air-dried on kaolin at room temperature and stored at 3°C remained viable for 180 days. The spore germination was higher than 70% for the first 140 days and then declined to 50% after 175 days. Less than 5% of spores were still viable after 17 months. No significant difference was found in pathogenicity between spores stored on kaolin for 168 days and freshly harvested spores. The preservation of the spores in 10% glycerine at –80°C and in liquid nitrogen did not affect viability and pathogenicity.

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Introduction

Field bindweed (*Convolvulus arvensis* L.), a climbing perennial, is one of the twelve most troublesome weeds world-wide (Holm *et al.*, 1977). A European weed survey in 10 major crop systems classified field bindweed seventh most important (Schroeder *et al.*, 1993). It is ranked first in grapes and wine-grapes and in orchards. The chemical and mechanical control is difficult as its extensive root system has a vigorous regeneration capacity (Swan and Chancellor, 1976) and there is increasing public pressure to reduce pesticide use. Thus, there is a growing interest in the biological control of weeds. The biological control of field bindweed has been reported previously for the gall mite *Aceria malherbae* Nuzzaci (Boldt and Sobhian, 1993) and the fungi *Phomopsis convolvulus* Ormeno (Morin *et al.*, 1989, 1990a; Ormeno-Nuñez *et al.*, 1988) and *Phoma proboscis* Heiny (Heiny, 1990, 1994; Heiny and Templeton, 1991).

Stagonospora convolvuli Dearness & House strain LA39 isolated from a diseased field bindweed plant collected in Long Ashton (UK) has been suggested recently as a potential control agent, specially when formulated in an oil emulsion (Pfirter and Défago, 1998). Its potential for field use was demonstrated by its control of field bindweed in a cemetery (Guntli *et al.*, 1998).

The formulation and inoculum production are often limiting factors in the development of a mycoherbicide. In small-scale experiments the method of spore production is often not important. However, large-scale application requires mass production of inoculum, which must be as inexpensive as possible, while product quality is maintained. Generally, spore production by liquid fermentation is the most economic option but, as *S. convolvuli* strain LA39 does not produce spores in liquid media, we have used solid agricultural products as substrates.

An adequate shelf-life is needed for mycoherbicides to be commercially viable. This will allow storage of the inoculum until environmental conditions are suitable for an application. Recently, the potential of a kaolin formulation to improve shelf life was reported by Norman and Trujillo (1995). Preservation should provide conditions in which the risk of phenotypic change is minimised. The methods of storage and preservation of fungi can be divided into three general groups: continuous growth, drying, and suspended metabolism (Smith and Onions, 1994). Whichever method used should maintain a fungus in a viable state with stable morphology, physiology and

genetics. Here we have examined the potential for prolonged storage on kaolin and preservation at –80°C or in liquid nitrogen.

Material and Methods

Plant production

Field bindweed was grown from seeds (MT Valley Seed Service, Fresno CA, USA). Seeds were scarified by exposure to concentrated sulphuric acid for 1 h, surface-sterilised in 5% sodium hypochlorite (v/v) and 10% hydrogen superoxide (v/v) for 10 min each and germinated on 0.85% water agar (w/v; Oxoid, Basingstoke, UK) in the dark for 2 days at room temperature. Each germinated seed was planted in a 9-cm-diameter plastic pot in a 3:1 mixture of soil (4C; de Baat BV, Coevorden, the Netherlands) and quartz sand (1.8-2.2 mm). Plants were grown in a greenhouse chamber with 70% relative humidity (RH) and a 16-h photoperiod at 22°C (light) respectively 17°C (dark).

Spore production on agar

Spores, from stock cultures of *Stagonospora convolvuli* strain LA39 stored at 3°C, were streaked on the surface of V8-juice agar plates (10% V8-juice [v/v; Campbell, King's Lynn, Norfolk, UK], 30mM $CaCO_3$, 1.2% agar [w/v; Oxoid, Basingstoke, UK]) and incubated for 2 weeks at 20°C under continuous white fluorescent light. Spores were harvested by flooding the plates with distilled water and lightly scraping the surface. The resulting spore suspension was filtered through cheesecloth, spore density determined using a haemacytometer and then adjusted to the desired value for inoculation.

Plant inoculation

Spores were formulated in a 10% oil-in-water emulsion (v/v; 0.1 ml Tween 80 [Fluka, Switzerland], 10 ml pure rape seed oil, 90 ml distilled water) as described by Potyka (1996). Spore suspension was added to the oil-in-water emulsion in a 1:9 ratio

(v/v) to give a final concentration of 10^7 spores/ml. In all experiments, plants were sprayed to run-off using an aerosol atomiser, placed in a greenhouse (16 h light at 22°C; 8 h dark at 17°C) and covered with a plastic bag for 24 h to maintain 100% RH. Subsequently, RH was kept at 70%.

Disease rating

The disease was evaluated two weeks after inoculation. All inoculated leaves of each plant were visually rated using a 0-6 scale (0 = no disease; 1 = 0-5%; 2 = 6-25%; 3 = 26-75%; 4 = 76-95%; 5 = >95% of leaf surface with necrosis; 6 = leaf dead). Total necrotic leaf area was calculated as a percentage using the formula $(2.5 \times n_1 + 15 \times n_2 + 50 \times n_3 + 85 \times n_4 + 97.5 \times n_5 + 100 \times n_6) / N$ where n_x is the number of leaves with rating x and where N is the total number of leaves treated.

Spore production on solid substrates

Mycelium growth, pycnidia formation and spore production were determined on17 different solid substrates (Table 1). Larger materials were cut to about 0.5-3 mm particle size. The substrates were put into 100 ml flask and autoclaved twice for 20 min at 121°C. A defined amount of sterile water (Table 1) and three 0.6-cm plugs from the fungal culture grown on V8-juice agar were then added to each flask. After incubation for 15 days at 20°C under continuous white fluorescent light the mycelium growth was visually assessed as percentage coverage of total surface. The pycnidia formation was determined using a microscope and classified (no pycnidia formation, pycnidia present on the surface of the substrate or pycnidia present on the surface of as well as in the substrate). The spore production was assessed using at least three pycnidia. The experiment was repeated four times and the five best substrates selected for further examination of mass production of *S. convolvuli* strain LA39.

Mass production on solid substrates

A spore suspension (20 ml) was added to 20 g of autoclaved cous-cous, hard wheat semolina, maize semolina, soya or 10 g wheat bran to give a final concentration of 10^5 spores/g substrate. After incubation for 15 days under continuous light, the substrate was suspended in approximately 200 ml water, mixed for 1 min using a laboratory mixer and the resulting spore suspension was filtered through cheesecloth. The spore concentration was determined using a haemacytometer. The experiment was repeated four times each with five replicates. Pathogenicity of the spores grown on cous-cous, hard wheat semolina and maize semolina was tested by inoculating bindweed plants at the 5-7-leaf stage as described before.

Storage on kaolin

The method of Norman and Trujillo (1995), described for *Septoria passiflorae*, a mycoherbicide effective against banana poka (*Passiflora tripartita* var. *tripartita*), was amended slightly. Kaolin was mixed into the spore suspension (1 g of kaolin per 10^9 spores), which was then centrifuged for 15 min at 3500 g. The pellet was air-dried in a sterile bench overnight, pulverised, placed in glass tubes and sealed. Samples were stored at 20°C or 3°C. The percentage of water content in the dried kaolin-spore mixture was 10%. To determine the spore viability a small sample of the kaolin-spore mixture was rehydrated in 30% sucrose (w/v; 1 ml per 0.1 g sample) by shaking for 1 h at 250 rpm (Mini-Shaker, Adolf Kühner AG, Switzerland) and inoculated onto 1.2% water agar (w/v; Oxoid). Plates were incubated in the dark at room temperature for 24 h and then stained with cotton-blue. Spore germination was determined microscopically for at least 100 randomly chosen spores for each replicate. The experiment was repeated twice with three (experiment 1) or four replicates (experiment 2). The spore germination in experiment 1 was measured at intervals for 113 days and, in experiment 2, for 175 days. In addition, the pathogenicity of spores stored for 168 days (experiment 2) was determined by inoculating 3-5-leaf bindweed plants by spraying to run-off with a suspension of rehydrated spores (10^7 spores/ml). One sample of the kaolin-spore mixture was stored at 3°C for 17 months before the viability and pathogenicity were determined on 4 replicates as described above.

Spore preservation

Spores of *S. convolvuli* strain LA39 were frozen at –80°C in 5, 10 or 20% glycerine (v/v) and in 10% glycerine (v/v) in liquid nitrogen. For storage at –80°C, the spore samples (2 ml at 10^6 spores/ml) were placed in sterile 4 ml sample vials (Wheaton, Milville NJ, USA) or, for storage in liquid nitrogen, in polypropylene cryotubes (Bibby Sterilin, Teddingten Middlesex, England). All samples were placed at room temperature for 1 h before transfer to the –80°C freezer (LCL Secfroid, Lausanne, Switzerland). The cryotubes were kept at –80°C for 24 h, cooled in the N_2-vapour phase for 1 min and then transferred into liquid nitrogen. The germination was determined after one week when samples were thawed in a water bath at 35°C and removed immediately all the ice has melted. The spore suspension was streaked on 1.2% water agar, incubated for 18 h, stained with cotton-blue and germination determined microscopically as described before. Thawed spores were also incubated on V8-juice agar for two weeks under continuos light, harvested and sprayed onto bindweed plants (5-7-leaf stage) to test pathogenicity. The experiment at –80°C was repeated four times with four replicates and that in liquid nitrogen twice with three replicates. The pathogenicity of spores preserved in liquid nitrogen was determined only once with four replicates each having two plants.

Data analysis

Data were subjected to analysis of variance (ANOVA) before treatments were compared using Fisher's least significance difference (LSD) at P=0.05 using the Windows version 5.05 of the Systat program (SPSS Inc., Evanston, IL, USA).

Each replicate in an assessment of spore pathogenicity consisted of four plants (i.e. pots) per treatment unless stated otherwise. The total percentage necrotic leaf area was transformed to the arcsine of its square root to ensure normality before data analysis.

Results

Mass production on solid substrates

Mycelium growth was observed on all solid substrates, except bean hulls and coffee, with coverage ranging from 35 to 100%. All mycelial growth was associated with pycnidia formation, but no spores were found in pycnidia on peanuts and popped corn (Table 1). On the basis of these results cous-cous, hard wheat semolina, maize semolina, soya, and wheat bran were selected for further experiments.

Table 1: Mycelium coverage, pycnidia formation and spore production by *Stagonospora convolvuli* strain LA39 on solid substrates 15 days after inoculation.

Substrate[a]	Quantity	Water added	Mycelium coverage[b]	Pycnidia formation[c]	Spore production[d]
Bean hulls	5 g	5 ml	0%	-	-
Bean seeds	5 g	5 ml	45 ± 5%	+	+
Carrots	10 g	-	97.5 ± 2.5%	+	+
Coffee	5 g	10 ml	0%	-	-
Cous-cous	5 g	5 ml	97.5 ± 2.5%	++	+
Crispbread	5 g	5 ml	70%	+	+
Hard wheat grits	10 g	10 ml	87.5 ± 2.5%	+	+
Hard wheat semolina	10 g	10 ml	97.5 ± 2.5%	++	+
Lentils	5 g	5 ml	35 ± 5%	+	+
Linseeds	10 g	10 ml	80%	+	+
Maize semolina	5 g	5 ml	95%	++	+
Peanuts	5 g	4 ml	60%	+	-
Popped corn	2 g	4 ml	92.5 ± 2.5%	+	-
Rice	5 g	5 ml	92.5 ± 2.5%	+	+
Soya	5 g	5 ml	65 ± 5%	++	+
Spaghetti	5 g	5 ml	92.5 ± 2.5%	+	+
Wheat bran	5 g	10 ml	65 ± 5%	++	+

[a] Substrate was filled into 100 ml flask and autoclaved twice before the sterile water was added. Particle sizes varied between 0.5 and 3 mm for the different substrates.

[b] Visual ratings of mycelium growth as percent cover of total surface.

[c] Formation of pycnidia determined microscopically (-: no pycnidia present, +: pycnidia present on surface of the substrate, ++: pycnidia present on the surface of as well as in the substrate).

[d] Spore production (-: no spores present, +: spores present) assess on at least three pycnidia.

A significantly higher spore production was found on cous-cous (4.8 x 10^8 spores/g substrate) than on the other four substrates and that on maize semolina (2.8 x 10^8 spores/g) was significantly higher than on the remaining three substrates (hard wheat semolina, 1.2 x 10^8 spores/g; soya, 0.76 x 10^8 spores/g; wheat bran, 0.35 x 10^8 spores/g) on which production was not significantly different (Figure 1). Spores from the different substrates were not different morphologically or in pathogenicity, which was similar to that of spores grown on V8-juice agar.

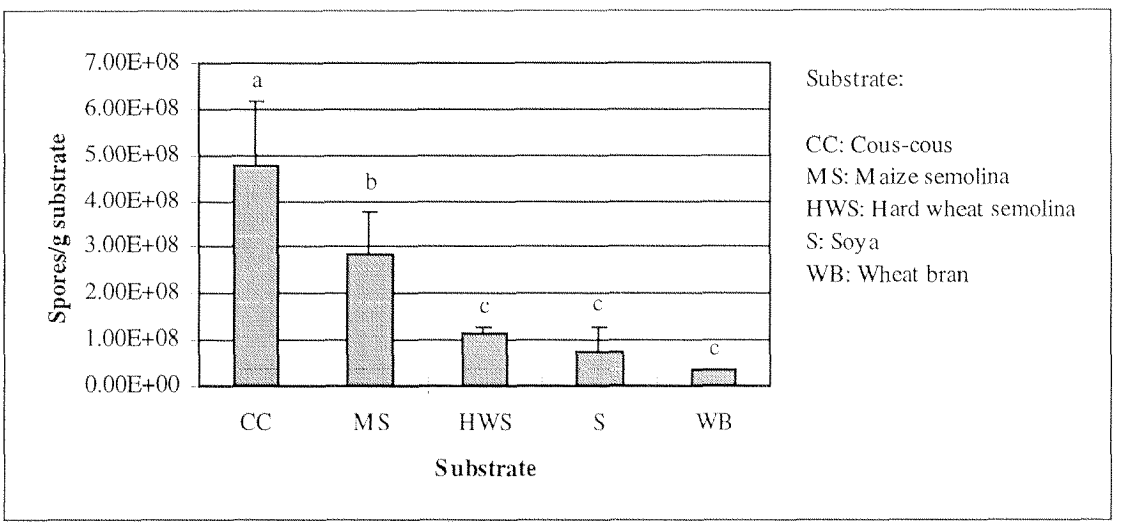

Figure 1: The spore production of *Stagonospora convolvuli* strain LA39 on 5 different solid substrates 15 days after inoculation. Means and standard deviations shown were calculated using data obtained in four independent experiments. Bars marked with the same letter are not significantly different ($P<0.05$) using Fisher's LSD test.

Storage on kaolin

The germination of spores dried on kaolin and stored at 20°C decreased below 20% within one week (data not shown). In contrast, the germination of spores stored at 3°C ranged between 60 and 80% for 160 days before decreasing to 50% by the end of the experiment (175 days) (Figure 2). Spores stored for 168 days at 3°C were still as pathogenic as freshly harvested spores from cous-cous, causing a mean necrotic leaf area of bindweed plants of 76.4% compared to 80.7% caused by freshly harvested spores. Prolonged storage (17 months) of dried kaolin-spore mixture reduced spore germination to 5.2%. The inoculation of bindweed plants with 10^7 spores/ml (i.e. total germinated and non-germinated spores) produced 50.6% mean necrotic leaf area, an effect achieved with 10^6 spores/ml when using freshly harvested spores.

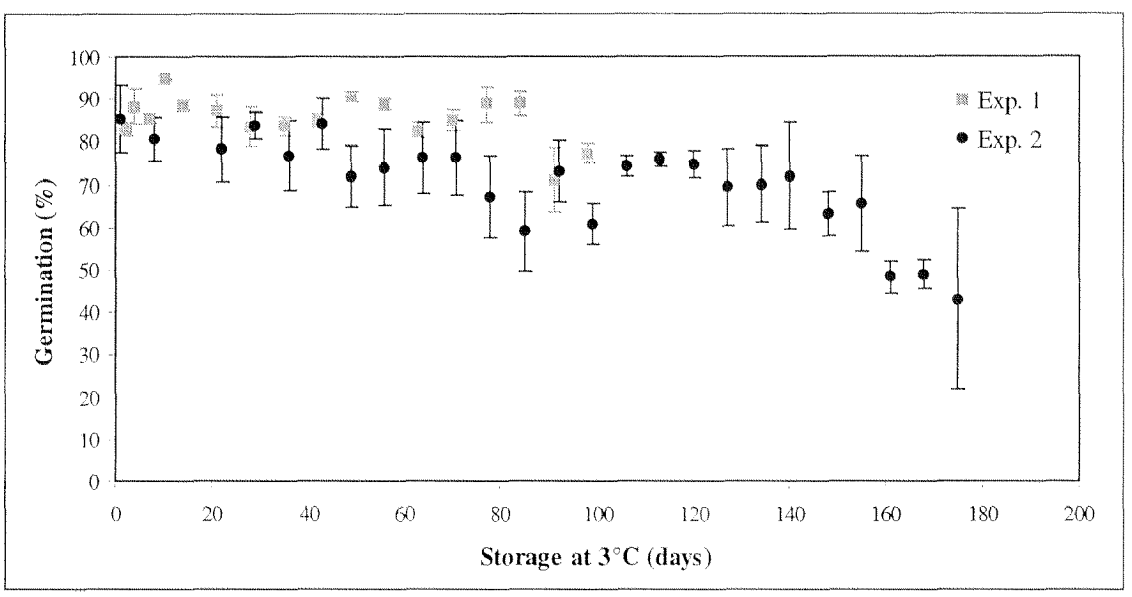

Figure 2: The germination of spores of *Stagonospora convolvuli* strain LA39 dried on kaolin and stored at 3°C for 113 days (experiment 1) or 175 days (experiment 2). Means and standard deviations shown were calculated using data obtained in two experiments with three (experiment 1) or four (experiment 2) replicates.

Preservation at low temperatures

While more than 80% of the spores frozen for one week at -80°C were viable, germination of those stored in 5 and 20% glycerine was statistically significantly less than for those stored in 10% glycerine (Table 2). The thawed spores were as pathogenic as spores from stock cultures stored at 3°C, always producing more than 80% necrotic leaf area (Table 2). Germination of spores stored in 10% glycerine at –80°C and in liquid nitrogen was not significantly different.

Table 2: The effect of preservation at low temperatures on germination of and disease induction by spores of *Stagonospora convolvuli* strain LA39.

Glycerine	Storage	Germination (%)[a]	Necrotic leaf area (%)[b]
5%	-80°C	81.70 ± 3.52 a[c]	87.8 ± 8.1 a
10%	-80°C	87.31 ± 4.07 b	86.0 ± 2.7 a
20%	-80°C	80.64 ± 5.14 a	81.1 ± 6.3 a
10%	liquid nitrogen	90.36 ± 7.23 b	75.8 ± 2.3 a
-	3°C (on agar)	-	80.7 ± 10.9 a

[a] Data from four experiments each with four replicates (-80°C) or from two experiments with three replicates (liquid nitrogen).

[b] Data from four experiments with four replicates/treatment and four plants/replicate (-80°C and 3°C) or one experiment with four replicates and two plants/replicate (liquid nitrogen).

[c] Values in one column followed by the same letter are not significantly different ($P<0.05$) using Fisher's LSD test.

Discussion

The large-scale production of spores should be efficient and give high yield of infective spores. *Stagonospora convolvuli* strain LA39 spores produced on V8-juice agar are highly aggressive (Pfirter and Défago, 1998), but this form of production is time-consuming, expensive and poor in yield. Therefore, it is not suitable for producing spores for large-scale application. In our study, the growth of *S. convolvuli* strain LA39 on solid substrates, especially on cous-cous, resulted in a high yield of aggressive spores. The yield from 1 g of cous-cous was equal to that from of 4 9-cm culture plates each containing 20 ml of media. The high yields of spores obtained with cous-cous may be due to its large surface area, good aeration and non-clumping, stable structure. As most mycoherbicide candidates do not produce spores in liquid media research is needed to establish appropriate solid substrate fermentation methods. Solid substrate processes are mainly used in the Orient to produce traditional foods and enzymes (Mudgett, 1986). For example, *Aspergillus oryzae* (Ahlburg) Cohn is cultivated on bran to produce amylolytic and proteolytic enzymes used in miso and sake fermentation (Hesseltine, 1977). Various solid substrates have been evaluated for spore production by *Phomopsis convolvulus*, a potential mycoherbicide for field bindweed (Morin *et al.*, 1990b). Pot barley grains produced a similar yield of virulent conidia ($5x10^8$ conidia/g substrate) as we found with *S. convolvuli* strain LA39 on cous-cous.

S. convolvuli strain LA39 spores can keep their viability and pathogenicity for over 6 months when stored on kaolin at 3°C and 10% RH. The loss of viability caused by storage at room temperature suggest that these conditions should be avoided for long term storage. Spores of *Colletotrichum gloeosporioides* f. sp. *clidemiae* and *Septoria passiflorae* stored on kaolin at –18°C and 1°C maintained their viability over 4 and 6 months respectively. Spores of both fungi stored at 22°C quickly lost their viability (Norman and Trujillo, 1995). Alves *et. al.* (1996) found no loss of viability of formulated *Beauveria bassiana* conidia, an entomopathogenic fungus isolated from the red fire ant (*Solenopsis invicta*), stored under refrigerator and freezer conditions for 7 years. In contrast, conidia stored at ambient temperatures (15-38°C) were totally unviable after 2 months.

Preservation should provide conditions in which micro-organism will maintain viability, morphology and physiology stable over a long period. No morphological or physiological change has been observed in over 7000 isolates stored in liquid nitrogen

at the International Mycological Institute (IMI) (Smith and Onions, 1994). The storage in liquid nitrogen often requires a special costly equipment, whereas generally storage at –80°C is less costly. In our experiments, no difference in viability and pathogenicity was found between spores stored at –80°C or in liquid nitrogen. Nevertheless, the recrystallization of ice, which may damage the spores, can occur during prolonged freezing at temperatures above –139°C (Smith and Onions, 1994).

This paper shows that there is clear potential for inoculum of *S. convolvuli* strain LA39 to be mass produced on cheap agricultural solid substrates. Also long term storage on kaolin kept refrigerated at low RH provides adequate shelf-life. These two factors greatly enhance the potential for large-scale (field) application of the fungus and, so, its successful development as a microbial herbicide.

Acknowledgement

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Chapter 4:

Genetic and pathogenic characterisation of different *Stagonospora* sp. isolated from bindweed

Abstract

The genetic variation among 38 isolates of *Stagonospora* sp. and 10 isolates of *Septoria* sp. from bindweed was studied using (a) the restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer (ITS) region and (b) the random amplified polymorphic DNA (RAPD) PCR. RFLP analysis revealed three types of fragment patterns among the isolates. A total of 26 groups of common patterns were distinguished based on cluster analysis of the RAPD-PCR data. Comparing the two methods, fragment pattern types and clusters were generally in agreement.

The degree of pathogenicity of six genetically characterised isolates of *Stagonospora* sp. was assessed on three ecotypes of field bindweed (*Convolvulus arvensis*). Disease was observed with all isolates on all ecotypes, but only *Stagonospora convolvuli* strain LA39, a potential biocontrol agent, showed a high degree of pathogenicity on all ecotypes.

A mixture of two *Stagonospora* sp. enhanced the mean necrotic leaf area on bindweed from 33.9% and 39.0% (when applied alone) to 64.9% applied together at the same final concentration of $5x10^6$ spores/ml. Molecular methods were used to identify the two pathogens. Both were present on the same plant when applied together, but never found in the same lesion.

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Introduction

Field bindweed (*Convolvulus arvensis* L.) is a hard to control, economically important weed in many agricultural areas (Holm *et al.*, 1977; Maillet, 1988; Weaver and Riley, 1982). Due to the poor control by herbicides and cultural methods (Derscheid *et. al.*, 1970; Westra *et al.*, 1992), natural enemies, such as fungal pathogens and arthropods, have been proposed as new control agents (Boldt and Sobhian, 1993; Heiny, 1990; Ormeno-Nuñez *et. al.*, 1988). Recently, the fungal pathogen *Stagonospora convolvuli* Dearness & House strain LA39 has been suggested as a mycoherbicide for field bindweed (Pfirter and Défago, 1998). During the development process towards this mycoherbicide, more than 600 fungi have been isolated and tested. The most promising candidates all belong to the genus *Stagonospora* (Pfirter *et al.*, 1997).

The taxa of the genus *Stagonospora* is only poorly described. The best known species is *S. nodorum* Berk. (telemorph *Leptosphaeria nodorum* Müller) the causal agent of wheat glume blotch. A few other *Stagonospora* species with an importance as crop disease have been reported: *S. tainanensis* Hsieh (telemorph *Leptosphaeria taiwanensis* Yen and Chi) causing sugarcane blight (Hsieh, 1979) and *S. meliloti* (Lasch) Petrak, causing root rot, crown rot, stem blight and leaf spot on diverse legumes species (Sivanesan, 1990). *Stagonospora* species form a septate, light-brown mycelium; pycnidia are brown to black. Conidia are typically 3- or more celled, cylindrical to elliptical (19 x 3.6 µm) (Smith *et al.*, 1988).

Taxonomic determination of the two species *Stagonospora* and *Septoria* is difficult. *Stagonospora nodorum*, the causal agent of wheat glume blotch, has only recently been classified as *Stagonospora*, before it was known as *Septoria nodorum*.

Identification and classification of organisms with DNA-based methods has become more and more common. Genes coding for RNA are found in multiple copies per genome and they are highly conserved. The slowly evolving nuclear rDNA sequences may be used for studying distantly related organisms, whereas the internal transcribed spacer region (ITS) of the nuclear rRNA repeat units can vary among individuals of the same species or genus (White *et al.*, 1990). One objective of this study was to determine the degree of detectable genetic variation among *Stagonospora* sp. and *Septoria* sp. isolated from bindweeds from different geographical locations. We present data on

variation among these assessed by DNA analyses (RFLP of amplified fragment of the ITS-region and RAPD-PCR).

So far, the effect of weed ecotypes on disease was rarely studied. Turner *et al.* (1981) compared 10 ecotypes of Canada thistle (*Cirsium arvense*) in their reaction to *Puccinia obtegens*. Susceptibility varied among and within ecotypes ranging from resistant to moderately susceptible. In this work we studied the interaction between three ecotypes of field bindweed and six strains of *Stagonospora* sp.. In addition, a mixture of two strains was tested for enhanced disease.

Materials and methods

Fungal isolates

Thirty-eight isolates of *Stagonospora* sp. from both hedge and field bindweed, 6 isolates of *Septoria septulata* (from field bindweed) and 4 isolates of *Septoria* sp. (from field bindweed) were studied genetically (Table 1). Fungi were isolated and maintained as described before (Pfirter and Défago, 1998). Fungal isolates were taxonomically determined by L. Sedlar and L. Petrini (*personal communication*).

DNA isolation

Fungal isolates were grown in Potato Dextrose Broth (2.4%, Difco, Detroit, MI, USA) for two weeks under continuos white fluorescent light at 20°C. The mycelium was harvested by centrifugation of the liquid medium, washed twice with sterile water, frozen at –80°C, lyophilised for 72 hrs and grounded. The DNA from ground samples of about 0.1 g was extracted using the QIAGEN Dneasy Plant Mini kit (Qiagen AG, Basel, Switzerland).

PCR-RFLP of the internal transcribed spacer (ITS) region of the nuclear rDNA

The ITS-region was amplified by the polymerase chain reaction (PCR). The reaction mixture (50 µl) contained 5 ng of genomic DNA, 100 µM dNTP's (Boehringer Mannheim, Germany), 0.2 µM each of primers ITS4 and ITS5 (MWG-Biotech, Münchenstein, Switzerland), 3.5 U *Taq* polymerase (Pharmacia Biotech, Uppsala, Sweden) and 1x reaction buffer (Pharmacia Biotech; 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl; pH 9.0 at room temperature). The conditions for ITS-amplification were the same as described by Tenzer and Gessler (1997). The sequence of the primers was 5'-TCCTCC-GCTTATTGATATGC-3' (ITS4) respectively 5'-GGAAGTAAAAGTCGTAACA-AGG-3' (ITS5) (White *et al.*, 1990). The PCR products were digested with the restriction enzyme *Hind*III (Boehringer Mannheim) in a final volume of 10 µl containing 1 U restriction enzyme, 1x reaction buffer (Boehringer Mannheim) and 4 µl amplification product. After incubation for at least 4 hours at 37°C the digested products were electrophoresed on a 2% agarose gel containing ethidium bromide.

Table 1: Isolates of *Stagonospora* and *Septoria* species analysed.

Isolate No.		Geographic origin	Year of isolation	Host plant
	Stagonospora convolvuli			
1	LA39	Long Ashton, UK	1994	*Convolvulus arvensis*
	Stagonospora sp.			
2	LA24	Long Ashton, UK	1994	*Convolvulus arvensis*
3	LA25	Long Ashton, UK	1994	*Convolvulus arvensis*
4	LA26	Long Ashton, UK	1994	*Convolvulus arvensis*
5	LA29	Long Ashton, UK	1994	*Convolvulus arvensis*
6	LA31	Long Ashton, UK	1994	*Convolvulus arvensis*
7	LA34	Long Ashton, UK	1994	*Convolvulus arvensis*
8	LA35	Long Ashton, UK	1994	*Convolvulus arvensis*
9	LA51	Long Ashton, UK	1994	*Convolvulus arvensis*
10	LA52	Long Ashton, UK	1994	*Convolvulus arvensis*
11	LA53	Long Ashton, UK	1994	*Convolvulus arvensis*
12	LA55	Long Ashton, UK	1994	*Convolvulus arvensis*
13	LA57	Long Ashton, UK	1994	*Convolvulus arvensis*
14	LA10A	Long Ashton, UK	1995	*Convolvulus arvensis*
15	LA10B	Long Ashton, UK	1995	*Convolvulus arvensis*
16	LA30B	Long Ashton, UK	1995	*Convolvulus arvensis*
17	A1	Yeorile, UK	1994	*Calystegia sepium*
18	A5	Yeorile, UK	1994	*Calystegia sepium*
19	A7	Yeorile, UK	1994	*Calystegia sepium*
20	B4	Yeorile, UK	1994	*Calystegia sepium*
21	B8	Yeorile, UK	1994	*Calystegia sepium*
22	C5	Yeorile, UK	1994	*Calystegia sepium*
23	C6	Yeorile, UK	1994	*Calystegia sepium*
24	D6	Yeorile, UK	1994	*Calystegia sepium*
25	D7	Yeorile, UK	1994	*Calystegia sepium*
26	92 Co a	Zürich, CH	1986	*Convolvulus arvensis*
27	174 Co b	Guebwiller, F	1988	*Convolvulus arvensis*
28	213 Co a	Brno, CZ	1988	*Convolvulus arvensis*
29	131 Ca f	Stein-Säckingen, CH	1988	*Calystegia sepium*
30	158 Ca a	Stein-Säckingen, CH	1987	*Calystegia sepium*
31	170 Ca a	Heuilley, F	1988	*Calystegia sepium*
32	172 Ca a	Staffelfelden, F	1988	*Calystegia sepium*
33	175 Ca a	Nambsheim, F	1988	*Calystegia sepium*
34	177 Ca b	Säckingen, D	1988	*Calystegia sepium*
35	178 Ca a	Säckingen, D	1988	*Calystegia sepium*
36	180 Ca a	Zurzach, CH	1988	*Calystegia sepium*
37	182 Ca a	Eschikon, CH	1988	*Calystegia sepium*
38	214 Ca a	Brno, CZ	1988	*Calystegia sepium*
	Septoria septulata			
39	71 Co	Wien, A	1986	*Convolvulus arvensis*
40	93 Co	Zürich, CH	1986	*Convolvulus arvensis*
41	120 Co a	Trajanova Tabla, YU	1987	*Convolvulus arvensis*
42	139 Co a	Brno, CZ	1987	*Convolvulus arvensis*
43	140 Co a	Zürich, CH	1987	*Convolvulus arvensis*
44	169 Co a	Bonboillon, F	1988	*Convolvulus arvensis*
	Septoria sp.			
45	131 Co a	Stein-Säckingen, CH	1987	*Convolvulus arvensis*
46	156 Co a	Calquier, F	1987	*Convolvulus arvensis*
47	166 Co a	Zurzach, CH	1988	*Convolvulus arvensis*
48	184 Co a	Stupice, CZ	1988	*Convolvulus arvensis*

Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

Amplification reaction volumes were 15 µl containing 5 ng of genomic DNA, 100 µM dNTP's, 0.3 µM primer, 1 U *Taq* polymerase and 1x reaction buffer. Amplification was performed as described by Koller *et al.* (1993). After screening of about 100 decamer primers the following four were selected based on the reproducibility of distinct, strongly stained bands: A04 (5'-AATCGGGCTG-3'), A13 (5'-CAGCACCCAC-3'), U10 (5'-ACCTCGGCAC-3') and U19 (5'-GTCAGTGCGG-3') (all from Operon Technologies Inc., Alameda, CA, USA). The amplification products were separated on 1% agarose gels containing ethidium bromide.

Data analysis

The WinDist program written by I.V. Yap (*personal communication*) was used for the distance and similarity matrix computation (Dice similarity). The distance matrix data were analysed by the unweighted-pair-group method with arithmetic average (UPGMA) of the neighbor program of the Phylogeny Inference Package (PHYLIP; developed by J. Felsenstein, version 3.5c).

Characterisation based on degree of pathogenicity

Six isolates of *Stagonospora* sp. were tested for different degree of pathogenicity on three ecotypes of field bindweed. The isolates used were *Stagonospora convolvuli* strain LA39 (Pfirter and Défago, 1998), *Stagonospora* sp. LA24, LA31 (both collected in Long Ashton, UK, 1994), LA10, LA30B (both collected in Long Ashton, UK, 1995) and 92 Co a (collected in Zürich, CH, 1986). The field bindweed ecotypes originated from the USA (seeds obtained from MT Valley Seed Service, Fresno, CA, USA), England (seeds from Herbiseed, Wokingham, England) and Switzerland (own seed collection).

Plant production

Seeds were scarified in concentrated sulphuric acid for 1 h, surface-sterilised in 5% sodium hypochlorite (v/v) and 10% hydrogen superoxide (v/v) for 10 min each and germinated on 0.85% water agar (w/v; Oxoid, Basingstoke, UK) in the dark for 2 days at room temperature. Each germinated seed was planted in a 9-cm-diameter plastic pot containing a 3:1 mixture of soil (4C; de Baat BV, Coevorden, the Netherlands) and quartz sand (1.8-2.2 mm). Plants were grown in a greenhouse chamber with 70% relative humidity (RH) and a 16-h photoperiod at 22°C (light) respectively 17°C (dark).

Effect of three bindweed ecotypes on disease caused by six different *Stagonospora* isolates

In a preliminary experiment, each of the first six leaves (without cotyledons) was inoculated separately with either strain LA39 or LA30B to test the influence of the leaf position on disease. The experiment was repeated once with the USA ecotype and with 8 plants per leaf position and isolate.

In the main experiment each of the first six leaves (without the cotyledons) of each plant was inoculated with another one of the six isolates (randomised). The experiment was performed with 60 plants of each ecotype.

Fungal inoculum was produced on V8-juice agar plates (10% V8-juice [v/v; Campbell, King's Lynn, Norfolk, UK], 30mM $CaCO_3$, 1.2% agar [w/v; Oxoid, Basingstoke, UK]) as described elsewhere (Pfirter and Défago, 1998) and formulated in a 10% oil-in-water emulsion (v/v; 0.1ml Tween 80 [Fluka, Switzerland], 10ml pure rape seed oil, 90ml distilled water) (Potyka, 1996). The spore suspension was added to the oil-in-water emulsion in a 1:9 ratio (v/v) to give a final concentration of 10^7 spores/ml. The leaves were sprayed until run-off using an aerosol atomiser, placed in a greenhouse (16h light at 22°C, 8h dark at 17°C) and covered with a plastic bag for 48h to maintain 100% RH. For the rest of the experiment, RH was kept at 70%.

Effect of a mixture of *Stagonospora convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B on disease

Field bindweed plants (USA-ecotype) at the leaf stage 5-7 were sprayed with a suspension of $5x10^6$ spores/ml of *S. convolvuli* LA39 and *Stagonospora* sp. LA30B alone and a 1:1 ratio of both pathogens ($2.5x10^6$ spores/ml each) in the oil emulsion as described before. Plants were exposed to 100% RH for 24h. The experiment was repeated four times with six replicates (i.e. plants) per treatment. The data from the four repetitions were pooled based on homogeneous variances and then subjected to analysis of variance as described below.

Disease assessment and data analysis

At 2 weeks after inoculation, the treated leaves of each plant were counted and visually rated individually for disease symptoms using a 0-6 scale (0 = no disease; 1 = 0-5%; 2 = 6-25%; 3 = 26-75%; 4 = 76-95%; 5 >95% of leaf surface with necrosis; 6 = leaf dead). Total necrotic leaf area was calculated as a percentage using the formula $(2.5 \times n_1 + 15 \times n_2 + 50 \times n_3 + 85 \times n_4 + 97.5 \times n_5 + 100 \times n_6) / N$ where n_x is the number of leaves with rating x and where N is the total number of leaves treated. The total percentage necrotic leaf area was transformed to the arcsine of its square root to ensure normality. Data were subjected to analysis of variance (ANOVA) and treatments were compared using Fisher's least significance difference (LSD) at P=0.05 using the Windows version 5.05 of the Systat program (SPSS Inc., Evanston, IL, USA).

Identification of *Stagonospora convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B in lesions derived from plants sprayed with a mixture of the two fungi

In a preliminary experiment, the purified DNA of the two strains was mixed *in vitro* at different ratio (1:1, 5:1, 10:1 and *vice versa*), amplified with the primers ITS4 and ITS5 and digested with *Alu*I and *Dra*I (Boehringer Mannheim) as described before. In the experiment *in vivo*, a total of 50 lesions cut off from bindweed-leaves sprayed with the mixture were frozen at –80°C, lyophilised for 72 hrs and grounded. The DNA from the grounded samples (i.e. leaf and pathogens) was extracted using the QIAGEN Dneasy Plant Mini Kit, amplified with ITS4 and ITS5 and digested with *Hind*III, as well as *Alu*I and *Dra*I. The digested products were separated on 1% agarose gels and classified as *S. convolvuli* strain LA39 or *Stagonospora* sp. isolate LA30B based on their known banding pattern.

Results

PCR-RFLP of the internal transcribed spacer (ITS) region of the nuclear rDNA

Three types of fragment patterns were found among the isolates (Figure 1). The apparent PCR product length was either 610 bp (base pairs) (Fragment pattern type A and B) or 590 bp (type C). Type A and B were identified after digestion with the enzyme *Hind*III (Figure 1). The amplification product of type B was cut into two pieces (430 and 180 bp), whereas there was no restriction site in type A. No further differentiation among the isolates was also found by digestion with other enzymes. Twenty-one of the 22 *Stagonospora* sp. collected in England in 1994 from both field and hedge bindweed, and one *Stagonospora* sp. isolate from Germany belonged to fragment pattern type A. Type B included the 3 *Stagonospora* sp. isolated in England in 1995, the three Central-European *Stagonospora* sp. isolated from field bindweed and three *Stagonospora* sp. isolated from hedge bindweed. All *Septoria* as well as 7 of the 10 *Stagonospora* sp. isolated in Central-Europe from hedge bindweed belonged to type C (Figure 1).

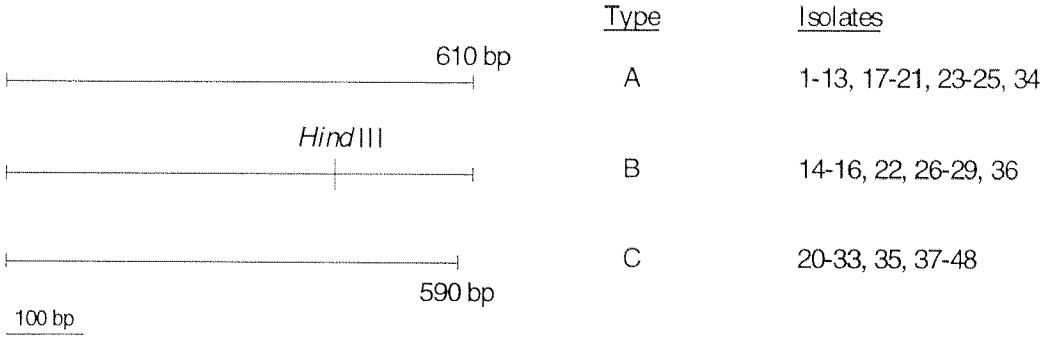

Figure 1. The fragment patterns found within 38 *Stagonospora* sp. and 10 *Septoria* sp. isolates. Amplified products of the ITS-region were cut with the restriction enzyme *Hind*III. The same grouping based on fragment patterns was also found with other restriction enzymes. Refer to Table 1 for isolate numbers.

Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

A total of 15 RAPD bands were selected that were reproducible in three replicates and were polymorphic among the isolates tested. Twenty-six groups were identified among the 48 isolates based on cluster analysis. One major group consisted of 10 of the 13 *Stagonospora* sp. from Long Ashton, UK (1994). The 3 *Stagonospora* from Long Ashton, UK (1995) belonged also to one separate group. In contrast, the 9 *Stagonospora* sp. from Yeorile, UK (1994) were less similar and formed 6 different groups. A high similarity was found among the *Septoria* isolates as well as among the *Stagonospora* sp. isolated from hedge bindweed collected in Central-Europe, forming two major subgroups.

The two main clusters found in the cluster analysis of the RAPD-PCR were in agreement with the PCR-RFLP assay (Figure 2). One cluster consisted of all the isolates with the ITS4/5 fragment of 590 bp whereas all the isolates with the ITS4/5 fragment of 610 bp belonged to the second cluster. The two subgroups within the second cluster were the same as in the PCR-RFLP assay. The *Septoria* sp. and *Stagonospora* sp. isolates belonging to the PCR-RFLP fragment pattern type C were separated with RAPD-PCR into two subgroups according to the genus, with the exception of isolate 71 Co, which was highly distinct from the others (Figure 2).

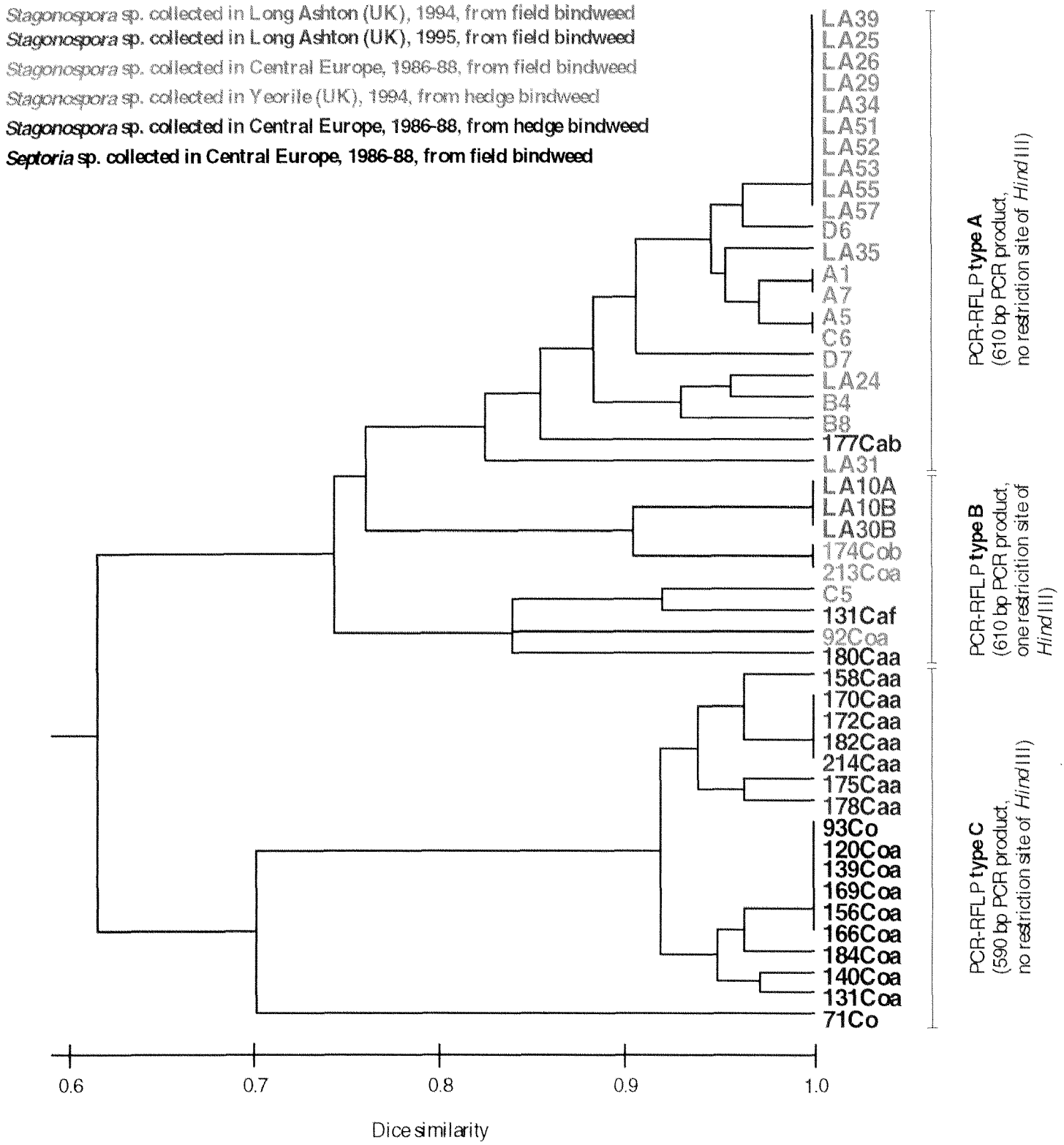

Figure 2: Dendogram of 38 *Stagonospora* sp. and 10 *Septoria* sp. isolates based on 15 polymorphic RAPD-PCR bands. The dendogram was constructed using the UPGMA method of the PHYLIP program. Fragment pattern type presented are based on PCR-RFLP of the ITS region. Description of isolates is presented in Table 1.

Characterisation based on degree of pathogenicity

No statistical difference was found regarding the influence of the leaf-position on disease severity. The necrotic leaf area caused by *Stagonospora* sp. LA30B was always higher than 97% whereas it ranged between 77.0% (leaf-position 1) and 99.8% (leaf-position 3) when *Stagonospora convolvuli* strain LA39 was applied (Table 2).

Table 2: The effect of the leaf position on the disease caused by *Stagonospora convolvuli* strain LA39 and *Stagonospora* sp. isolate 30B two weeks after inoculation.

	Leaf position / Necrotic leaf area (%)[a]					
	1	2	3	4	5	6
S. convolvuli LA39	99.8 a	86.7 a	77.0 a	96.5 a	96.0 a	99.2 a
Stagonospora sp. LA30B	99.6 a	99.8 a	99.8 a	99.5 a	100.0 a	97.8 a

[a] Data of one experiment with 8 plants/leaf position and isolate (USA ecotype). Values within one row followed by the same letters are not significantly different at $P > 0.05$ (Fisher's LSD test).

The degree of pathogenicity of six different isolates of *Stagonospora* sp. was tested on three ecotypes of field bindweed. In general, an effect of the bindweed ecotype as well as of the fungal isolate on disease was found. The CH–ecotype was most susceptible, the UK-ecotype the least. *S. convolvuli* strain LA39 showed a high degree of pathogenicity on all ecotypes, whereas it varied between the three bindweed ecotypes for the other five isolates (Table 3). The mean necrotic leaf area caused by LA39 was always higher than 94%, however the disease on the UK-ecotype was significantly higher than on the USA-ecotype (99.3% respectively 94.1% necrotic leaf area). The degree of pathogenicity of the isolates LA24, LA31 and LA30B was significantly reduced on the UK-ecotype compared to the CH- and USA-ecotype. The one of isolate LA10A differed significantly between all three ecotypes. The highest necrotic leaf area was found on the CH-ecotype, followed by the UK- and the USA-ecotype. The lowest necrotic leaf area on all bindweed-ecotypes was observed with isolate 92 Co a (Table 3).

Table 3: The effect of field bindweed ecotypes on the mean necrotic leaf area (%) caused by six isolates of *Stagonospora* sp. two weeks after inoculation.

	Bindweed ecotype / Necrotic leaf area (%)[a]			
	CH	UK	USA	Average
LA24	90.1 a A	32.6 b A	86.1 a A	69.6 A
LA31	99.0 a B	36.3 b AB	98.0 a B	77.8 B
LA39	97.1 ab AB	99.3 a C	94.1 b AB	96.8 C
LA10A	89.1 a A	53.6 b B	30.2 c C	57.6 D
LA30B	96.6 a AB	52.6 b B	95.3 a B	81.5 B
92 Co a	50.1 a C	25.3 b A	19.0 b C	31.5 E
Average	87.0 a	49.9 b	70.4 c	

[a] Data of one experiment with 60 plants/ecotype. Values within one column (capitalised letters) and within one row (small letters) followed by the same letters are not significantly different at $P > 0.05$ (Fisher's LSD test).

The field bindweed-ecotype from Switzerland was most susceptible with an average of 87.0% mean necrotic leaf area. The disease caused by the Long Ashton-isolates ranged between 89.1% (LA10A) and 99.0% (LA31) whereas it was 50.1% caused by the Swiss isolate (92 Co a). On the UK-ecotype, the disease of LA39 was significantly stronger (99.3% mean necrotic leaf area) compared with the other five isolates. No difference was found between LA10A and LA30B (53.6% and 52.6%), both isolated in Long Ashton in 1995. The disease of the other three isolates was less than 40%. On the USA-ecotype, the mean necrotic leaf area of the Long Ashton-isolates LA24, LA31, LA39 and LA30B was higher than 86%, while it was significantly less for LA10A (30.2%). The lowest disease on this ecotype was found with the isolate 92 Co a (19.0%).

Effect of a mixture of *Stagonospora convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B on disease

A mixture of *S. convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B produced a significantly higher disease on field bindweed than the two *Stagonospora* applied alone. The necrotic leaf area increased from 33.9% (LA39) and 39.0% (LA30B) to 64.9% when the two were applied together (Table 4).

Table 4: The effect of a mixture of *Stagonospora convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B on the disease of field bindweed two weeks after application.

	Necrotic leaf area (%)[a]	
Stagonospora convolvuli strain LA39	33.9 ± 9.8	a
Stagonospora sp. isolate LA30B	39.0 ± 8.3	a
Mixture	64.9 ± 17.5	b

[a] Each value is a means of four experiments with six replications/treatment. Values followed by the same letters are not significantly different at $P > 0.05$ (Fisher's LSD test).

Identification of *Stagonospora convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B in lesions derived from plants sprayed with a mixture of the two fungi

In a preliminary experiment, purified DNA from both strains was mixed at different ratio *in vitro*. After amplification of the DNA-mixture with the primers ITS4 and 5 and digestion of the amplification products, the banding pattern of both strains was always identified on agarose gels. The banding patterns of both strains were identified regardless of mixture ratio indicating an equivalent amplification of the DNA of both strains.

The same method was used to analyse 50 lesions of field bindweed-leaves sprayed with a mixture of both strains. In 35 lesions, the causal agent was identified as *S. convolvuli* strain LA39, in 15 lesions as *S. convolvuli* isolate LA30B. The two pathogens were never found in the same lesion.

Discussion

Three different fragment pattern types were found within 38 *Stagonospora* sp. and 10 *Septoria* isolates characterised with PCR-RFLP indicating little genetic variation. Type A consisted of one isolate from Germany (isolate 177 Ca b) and all but one *Stagonospora* sp.-isolates collected in Long Ashton and Yeorile, UK, during 1994. Only one isolate from Yeorile (isolate C5) belonged to type B formed by the isolates collected in Long Ashton in 1995 and all the isolates from field bindweed collected in Central Europe. No variation was found among the genus *Septoria*. These and all *Stagonospora* sp. isolated from hedge bindweed collected in Central Europe belonged to type C. Ueng *et al.* (1998) studied the intraspecific genetic variation of *Stagonospora avenae* and its differentiation from *S. nodorum, S. arenaria* and *Septoria tritici* with PCR-RFLP of the ITS-locus. In accordance with our results, a limited genetic variation was found within the genus, but the ITS sequence of *S. avenae* differed considerably from the other *Stagonospora* species. They concluded that the identification of *Stagonospora* on cereals could be facilitated with enzyme restriction (RFLP) of ITS PCR-amplified products.

More genetic variation was found within the RAPD-PCR assay. The polymorphism of 15 selected bands allowed the identification of 26 groups among the 48 isolates in the cluster analysis. No difference was found within 10 of the 13 *Stagonospora* from Long Ashton (1994). The *Stagonospora* from Long Ashton (1995) also formed a separate group. The cluster analysis was generally in agreement with the three fragment pattern types found in the PCR-RFLP assay. Furthermore, the *Septoria* isolates belonged to one major subgroup as well as the *Stagonospora* isolates from hedge bindweed collected in Central-Europe. Czembor and Arseniuk (1996) proposed in a study on genetic similarity among the three species *Septoria tritici*, *Stagonospora nodorum* and *Stagonospora avenae* f. sp. *triticea* that the RAPD-PCR assay may be of potential use in taxonomy of these species. The genetic characterisation of fungal strains with RAPD-PCR has been reported among others by Ouellet and Seifert (1993) for *Fusarium graminearum* and by Raina *et al.* (1997) for *Sclerotinia homoeocarpa*.

The isolates collected in Long Ashton in the same year (1994 or 1995) were homogenous among them, but different between the two years. This may suggest that there is a sexual reproduction within *Stagonospora* sp. pathogenic on bindweed. However, the sample size is very small to ensure this observation. So far, we did not

found the sexual stage of our isolates. In the case of *Stagonospora nodorum* and *S. avenae* the telemorph stage is known as *Phaeosphaeria nodorum* respectively *P. avenaria*.

Once applied in the field the epidemiology and dispersal of a control agent should be studied. With the findings presented in this paper we conclude that the molecular methods used will allow tracking of our *Stagonospora* sp..

We selected five *Stagonospora* sp. isolates from Long Ashton, UK (three found in 1994, two in 1995), and one isolate from Zürich, Switzerland, to study their degree of pathogenicity on three different bindweed ecotypes. The six fungal isolates belonged, with the exception of the isolates LA10A and LA30B, to different clusters found in the RAPD-PCR assay. Only *Stagonospora convolvuli* strain LA39, recently suggested as a potential control agent (Pfirter and Défago, 1998), led to high necrotic leaf surface regardless of the ecotype, whereas the other isolates reacted differently on the different ecotypes. The relatively wide host-range of *S. convolvuli* strain LA39 on most species of the *Convolvulaceae* (Pfirter and Défago, 1998) may be a reason for this observation. The other isolates collected in Long Ashton, except LA10A, showed a high degree of pathogenicity on the CH- as well as on the USA-ecotype, but not on the UK-ecotype.

The molecular markers we used are supposed to be neutral. Therefore, no correlation between the degree of pathogenicity and these genetic markers should be found. LA10A and LA30B, belonging to the same group found in the cluster analysis of the RAPD-PCR data, varied in the degree of pathogenicity, whereas LA31 and LA30B, different in the RAPD-PCR assay, did not. In addition, LA39 was more aggressive on bindweed than the other isolates belonging to the same group (data from previous screening experiments, not shown). However, a weak correlation between RAPD and virulence data was found in a study of wild populations of *Colletotrichum lindemuthianum* from common bean (Sicard *et al.*, 1997).

The isolates from Long Ashton, beside LA39, showed in general a lower degree of pathogenicity on the UK-bindweed ecotype compared with the two other ecotypes. Co-evolution with the host may have led to a certain resistance. Plants propagated vegetatively would be expected to have little genetic diversity and therefore host-resistance should not be a main problem. Nevertheless, a collection of different isolates should be maintained to overcome the uncertainty of distinct ecotype susceptibility. A mixture of genetically different spores may be applied against various ecotypes. In

1971, the release of the rust *Puccinia chondrillina* in Australia reduced the density of skeleton weed dramatically (Cullen *et al.*, 1973). However, the strain used was aggressive only on one form of skeleton weed ("narrow-leaved"). The two other forms ("intermediate-leaved" and "broad-leaved") present remained a problem. It was solved by the release of two other strains of the same fungi (Hasan, 1984).

Our study demonstrated that a mixture of two pathogens (*S. convolvuli* strain LA39 and *Stagonospora* sp. isolate LA30B) can enhance the disease on field bindweed. The PCR-RFLP method was used to identify the two pathogens when applied together. Mixed *in vitro*, the DNA of both strains was equivalent amplified even in a ratio of 10:1. The same method was used to determine the pathogen(s) in lesions derived from plants sprayed with the two pathogens. Thirty-five of the 50 lesions analysed were caused by LA39, 15 by LA30B. Both pathogens were found on the same plant but never in the same lesion. This result seems to exclude direct interaction between the two pathogens as an explanation for the synergism observed. However, production and segregation of different toxins into the weed may weak the plant and therefore facilitate the infection process of the two pathogens. *S. convolvuli* strain LA39 produces a range of toxins, one of them identified as leptosphaerodione (B. Nicolet, *personal communication*).

Synergism involving a combination of fungi has been reported among others for *Botrytis cinerea* and *Puccinia lagenophorae* on groundsel (Hallett *et al.*, 1990) by giving *B. cinerea* direct access to the interior of the leaf through rust infection. Not only pathogen-mixtures but also adding of sub-lethal dosages of herbicides may enhance the effectiveness of a mycoherbicide. Heiny (1994) improved the effect of *Phoma proboscis* Heiny in the field against field bindweed by adding a sublethal dose of 2,4-D and MCPP. The susceptibility of the weed *Cassia obtusifolia* L. to the mycoherbicide CASST® (*Alternaria cassiae* Jurair & Khan) was increased by a combination with a sub-lethal dose of glyphosate resulting in a specific suppression of the weed's elicited defence response (Sharon *et al.*, 1992). Another way may be the integration of biological weed control into a weed management system. The possibility of improving the potential of *S. convolvuli* strain LA39 by cover crops is discussed elsewhere (Pfirter *et al.*, 1997; our own unpublished results).

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Final discussion and outlook

As long as crops were planted, man fought weeds. Since some weeds have become difficult to control by traditional methods, there is need for new strategies. Field bindweed (*Convolvulus arvensis* L.), a perennial, is hard to eradicate mechanically and chemically. The aim of this study was to estimate the potential of controlling field bindweed biologically. The inundative, or bioherbicide, approach was chosen as the principal method. In this approach, the fungal pathogen is applied into a specific weed-infested field similarly to a chemical herbicide. Several crucial steps were taken in the development of a mycoherbicide against field bindweed. The starting point was the collection of diseased bindweed plants all around Europe to identify a suitable control agent; *Stagonospora convolvuli* Dearness & House strain LA39 was chosen. Next came preliminary field experiments, studies on dew requirement, spore concentration, formulation, mass production and preservation of the pathogen. Finally, the control agent and other *Stagonospora* sp. isolates were genetically and pathogenically characterised and the impact of a mixture of two isolates on disease was evaluated. The results suggest that *S. convolvuli* strain LA39 has the potential to become a bioherbicide for field bindweed.

The control agent

Stagonospora convolvuli strain LA39, found in Long Ashton (UK) in 1994, was selected out of about 600 isolates as a possible biological control agent of field bindweed (*Convolvulus arvensis*) (Chapter 1). On field bindweed, *S. convolvuli* strain LA39 causes typical brown lesions followed by defoliation resulting in the reduction of plant growth. Several phytotoxins, which might be used as herbicides, are produced in liquid media (i.e. Potato Dextrose Broth and V8-juice media); among others one was identified as leptosphaerodione (B. Nicolet, *personal communication*). Field bindweed was susceptible to the pathogen at all growth stages tested (Chapter 2). The genus *Stagonospora* belongs to the *Deuteromycota (Fungi imperfecti)* and is classified in the class *Coelomycetes*, order *Sphaeropsidales* and the family *Sphaeropsidicaceae*.

In 1991, Charudattan listed in general more than 100 agents possible for biological weed control. Most of them belonged to the genus *Colletotrichum* spp. (18), followed

by *Fusarium* spp. (13) and *Alternaria* spp. (12). In the literature, there are only two others *Stagonospora* sp. described as possible control agents: *Stagonospora* sp. against bracken (*Pteridium aquilinum*) (Petrini *et al.*, 1992) and *Stagonospora apocyni* against hemp dogbane (*Apocynum cannabinum* L.) (Venkatasubbaiah *et al.*, 1992).

Stagonospora convolvuli strain LA39 was not only pathogenic on field bindweed but also on several other species of the Convolvulaceae; however, only hedge bindweed, another important weed, was highly susceptible (Chapter 2). A lack of specificity to the target species is a known feature in the development of mycoherbicides. Under laboratory conditions, *Phytophthora palmivora*, the active ingredient in the commercial product DeVine© used for control of stranglervine, is pathogenic to several crops (onion, citrus, pea and others). Nevertheless, selective site-specific application should guarantee safety of the non-target plants (Ridings, 1986). In the case of *S. convolvuli* strain LA39, none of the crops tested, including sweet potato, is susceptible. The susceptible species of the Convolvulaceae are not very common, and host specificity of the control agent seems to be sufficient.

No difference in disease was found among field bindweed ecotypes from the UK, USA and Switzerland (Chapter 4). These facts may increase the commercial potential of *S. convolvuli* strain LA39, because it can be applied to areas infested either by both field and hedge bindweed or by different ecotypes of field bindweed. Besides *S. convolvuli* strain LA39, some other isolates were highly pathogenic on at least one of the field bindweed ecotypes tested. As an example, *Stagonospora* sp. isolate LA30B (found in Long Ashton, UK, in 1995) was highly pathogenic on the USA- and CH-ecotypes, but not on the UK-ecotype. The possible synergism of spore mixtures was studied. The two strains *S. convolvuli* LA39 and *Stagonospora* sp. LA30B applied together increased control of the weed (Chapter 4).

Formulation, mass production and storage of *Stagonospora convolvuli* strain LA39

A formulation should make the active ingredient convenient to handle and facilitate its application. In the case of a mycoherbicide, formulation may be used additionally to enhance the efficacy of the control agent. As an example, the reduction of the dew requirement during infection would improve the potential of a mycoherbicide. The formulation of spores of *S. convolvuli* strain LA39 in a 10% vegetable oil emulsion significantly increased their effectiveness. The amount of spores needed to reach 80% necrotic leaf area was reduced by a factor of 10. Delay of exposure to 100% relative humidity by up to 8 h did not affect disease development; furthermore, severe disease was found even in absence of exposure to 100% relative humidity (Chapter 2). As dew is an irregular feature in the field, resistance to short-term desiccation greatly increases the suitability of a pathogen as a control agent. The favourable effect of the oil emulsion can be explained either by retention of the water present in the formulation or by the induction of water from leaf tissue cells. Oil-in-water formulations are cheap, easy to prepare, and can be applied with standard equipment. The same formulation was used for *Mycocentrospora acerina* against field pansy (*Viola arvensis*). The dew period needed to infect and kill field pansy was reduced from >36 h to 12 h (Potyka, 1996). Electron microscope examination of transverse sections of field pansy leaves showed that the oil phase had penetrated into the leaf tissue and contained many small droplets of water. This fact was observed in the deposits on the leaf as well as in the intercellular oil (Greaves, *personal communication*). Klein *et al.* (1995) reported that canola and soybean oil (at rates of 0.5, 1, 5, and 10% in the formulation) improved the control of bathurst burr (*Xanthium spinosum*) compared to water with *Colletotrichum orbiculare* in the field in 1991-1992, but not in 1992-1993.

Solid state fermentation with *S. convolvuli* strain LA39 on cous-cous (cracked hard wheat) highly improved its potential to become a bioherbicide. Cous-cous was selected out of 17 different solid substrates, because it is rather cheap, easy to get and easy to handle. Up to $4x10^8$ spores/g substrate was produced, 80 times more than on V8-juice agar, a standard medium for the cultivation of *Stagonospora* spp. (Chapter 3). Spores dried on kaolin and stored at 3°C kept their viability for 180 days; however, spore germination declined to 70% after 140 days and 50% after 175 days. No loss in pathogenicity was observed (Chapter 3). A potential bioherbicide should have an

appropriate shelf life in order to pass through the market network and to allow a period of storage before application. Spores of *S. convolvuli* strain LA39 dried on kaolin keep their pathogenicity over a sufficient time and can be easily rehydrated and directly formulated in the oil-in-water emulsion. This strategy seems therefore suitable for commercial use. Nevertheless, further studies may be focused on production of granules, which can be directly applied on the soil. Solid granules protect and provide nutrients for the incorporated fungal spores. An interesting approach is the so-called "Pesta" (Connick *et al.*, 1991), containing organic and inorganic fillers, i.e. wheat flour and kaolin. It may be possible to mix dried spores of *S. convolvuli* strain LA39 (on kaolin) directly with wheat flour and thus produce infective granules. So far, solid carriers have mostly been used with soil-borne pathogens. The pathogen may either sporulate on the substrate or directly infect the host by mycelial growth, as described for *Sclerotinia sclerotiorum* against Canada thistle (*Cirsium arvense*) (Brosten and Sands, 1986). The application of *S. convolvuli* strain LA39 on solid carriers is possible. However, rain splash would be needed to distribute the spores grown on the granules.

Field release and tracking

The most important step in the development of a bioherbicide is its application in the field under the same conditions as it would be used commercially. In 1995, *S. convolvuli* strain LA39 was studied in a field trial in maize. Ground cover with bindweed in the *Stagonospora*-treated plots did not increase. Necrotic leaf area reached 78% (45.4% of leaves dead). In the control plots (treated with benomyl), ground cover increased by 115%, and necrotic leaf area was 13.8% (6% of leaves dead) (Chapter 1). During the summers of 1996 and 1997, field trials with *S. convolvuli* strain LA39 in maize were continued, with red clover (*Trifolium pratense*) as an additional competition factor for bindweed (Guntli *et al.*, submitted). In both years, a high disease level was observed and led to defoliation of the weed. Ground cover of bindweed was significantly reduced. The undersowing with red clover had no positive effect on the weed control. Living green cover used in maize fields (called "maize meadow") can suppress many weeds but does not control bindweed (Garibay *et al.*, 1997; Hall and Hartwig, 1990; Chapter 1). A green cover controlling most weeds with application of *S. convolvuli* strain LA39 on the escaping bindweed would be perfectly suited for an

integrated pest management system. Application of the mycoherbicide in a place with a broad weed spectrum, where bindweed would be replaced instantly by another weed, seems unwise. A mixture of different mycoherbicides could be taken into account. However, these should be formulated and applied in the same way to become a real alternative to chemical herbicides with a broad range. Therefore, the integration of bioherbicides into a pest management system, as in the afore-mentioned maize meadow, is absolutely necessary for a successful product promotion.

The potential of *S. convolvuli* strain LA39 as a bioherbicide was also demonstrated in a non-crop situation when applied in a cemetery, where cotoneaster (*Cotoneaster dammeri*) was heavily infested by field bindweed. Within 20 days after application, 60% of the bindweed leaf surface was necroted, increasing to over 80% after 40 days. Groundcover density of bindweed decreased from 40 to 17% in the plots infested with *S. convolvuli* strain LA39 (Guntli *et al.,* 1998).

Bindweeds are difficult to control chemically. The deep root system allows regrowth when aerial parts are killed by herbicides. Bindweeds emerge in many cases with or even after the crop. Therefore the use of a non-selective herbicide is problematic. Furthermore bindweeds can even be favoured by certain herbicides. Triazine and urea herbicides have a broad action against many weeds but do not control bindweed and thus providing a selective advantage by reducing competition from other weeds (Stalder *et al.*, 1974). In Switzerland, only a limited number of herbicides are in practical use for bindweed control, among others 2,4-D, Dicamba and Glyphosate (Guntli, 1998).

Public concern about environmental problems produced demands for lower input of pesticides in agriculture and, in particular, of non-cropped areas. Forest nurseries, gardens, and parks call for alternative strategies. Control of bindweed with chemicals may affect many other plants. Hand-weeding is expensive and time-consuming and therefore not very suitable. Selective application of an environmental-safe bioherbicide is one solution. In the beginning, such markets are probably the best places to establish a commercial product based on our mycoherbicide.

Once a control agent is released in the environment, its dispersal and epidemiology can be studied with molecular methods. *S. convolvuli* strain LA39 was characterised using PCR-RFLP and RAPD-PCR (Chapter 4). The identification and tracking of the strain applied as a mycoherbicide now seems possible. In addition, the data obtained plus further research on disease development under different environmental conditions

would allow building a computer model of the epidemic of *S. convolvuli* strain LA39. This model may help to optimise time of the application and development of the disease resulting in better control of field bindweed.

With the development of DNA markers of the host plants analogous to the ones described for the pathogens, studies on the impact of the pathogens on bindweed population structure will become feasible. Such information (clarification of pathogen taxonomy, tracking of released bioherbicide, impact on host population structure), achieved with molecular methods, might facilitate standardisation of protocols for the development and the release of biological control agents.

Genetic and pathogenic differences among different *Stagonospora* isolates

The genetic relationship among 38 *Stagonospora* sp. from field and hedge bindweed, and 10 *Septoria* sp. (from field bindweed) were studied using PCR-RFLP of the internal transcribed spacer (ITS)-region and RAPD-PCR. Little variation was found in the PCR-RFLP assay where only three types of fragment pattern were identified. In contrast, a total of 26 groups were distinguished based on cluster analysis of the RAPD-PCR data (Chapter 4). Comparing the two methods, fragment pattern types and clusters were generally in agreement. Clusters found can be explained by genus, collection site and year, and host plant. The variation between isolates from the same place collected in different years suggests that there is sexual reproduction within *Stagonospora* sp. pathogens on bindweed. However, the sample size was rather small, and a sexual stage of our strains was not observed yet. In the case of *Stagonospora nodorum* and *S. avenae*, the two best known species in the genus, the sexual stages are known as *Phaeosphaeria nodorum* and *P. avenaria*, respectively.

Six *Stagonospora* isolates were tested for pathogenicity on three field bindweed ecotypes. Differences were found between the pathogens as well as in the susceptibility of the hosts. Only *S. convolvuli* strain LA39 was highly pathogenic on all ecotypes (Chapter 4). These results and the lack of unique specificity for field bindweed may implicate that a development of a resistance against this pathogen will be unlikely. Nevertheless a collection of several pathogenic isolates of *Stagonospora* sp. should be maintained to overcome the uncertainty of distinct field bindweed susceptibility or of resistance development.

There was only a weak correlation, if any, between pathogenicity and genetic markers. It must be mentioned that the RAPD loci and the ITS-region of the rDNA are supposed to be selectively neutral and therefore might not tell anything about pathogenicity. However, DNA markers associated with the pathogenicity to bindweed may be used to clarify the taxonomy of the species of *Stagonospora* (and other genera) used to control bindweed. Czembor and Arseniuk (1996) concluded in their study that PCR-RAPD is of potential use in taxonomy of *Stagonospora* spp. and of *Septoria tritici*, as well as in molecular identification of causal disease agents.

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Curriculum vitae

Hanspeter Andreas Pfirter

30.06.68	Born in Zürich, Switzerland
1974-80	Primary school in Seegräben, ZH
1980-87	Gymnasium in Wetzikon, ZH
1987	Federal Matura type B
1988-93	Studies of Natural Science at the Swiss Federal Institute of Technology (ETHZ), Zürich
1992-93	Diploma thesis at the Institute of Plant Science/Phytopathology group (ETHZ), Zürich
1993	Diploma in Natural Sciences, section biology
1993	Scientific work at the Swiss Federal Institute for Environmental Science and Technology (EAWAG) in Dübendorf, ZH (preparation of a handbook for practical studies in limnology; in co-operation with the University of Munich, Germany)
1995	Scientific studies at the Institute of Arable Crops Research (Department of Agricultural Sciences, University of Bristol) in Long Ashton, England (formulation of mycoherbicides)
1997	Scientific studies at the Weizmann Institute of Science in Rehovot, Israel (weed-pathogen interactions, i.e. *Fusarium* spp. on *Orobanche* spp.)
1994-98	Dissertation at the Institute of Plant Science/Phytopathology group, at the Swiss Federal Institute of Technology (ETHZ), Zürich, supervised by Prof. Dr. G. Défago.

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