Doctoral Thesis

Biological control of bindweeds using a mycoherbicide, plant competitors and a rhizobacterium degrading alkaloids

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Biological control of bindweeds using a mycoherbicide, plant competitors and a rhizobacterium degrading alkaloids.

A dissertation submitted to
Swiss Federal Institute of Technology
for the degree of
Doctor of Natural Sciences

presented by
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Dipl. sci. nat. ETH
born 9. December 1969
citizen of Vilters SG

accepted on the recommendation of
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Prof. Dr. Yvan Moënne-Loccoz, co-examiner
Dr. Hans Ulrich Ammon, co-examiner
Prof. Dr. Peter Stamp, co-examiner

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Summary

Hedge bindweed (*Calystegia sepium*) and field bindweed (*Convolvulus arvensis*) are among the most important weeds in temperate and subtropical areas world-wide. Chemical control is difficult, mainly because the weeds emerge in phase with the crop and while the crop is sensitive to many herbicides active for bindweed control. The objective of this thesis was to study the interactions between bindweeds and several plants and microorganisms to determine their potential for the biological control of bindweeds. These organisms were the mycoherbicide *Stagonospora convolvuli* strain LA39, the plant competitors *Lolium multiflorum* or *Trifolium pratense* and the rhizobacterium *Sinorhizobium meliloti* strain Rm41, which can degrade calystegines (bindweed alkaloids).

At first, the impact of the mycoherbicide LA39 on *C. sepium* was studied in an outside pot experiment. Treatment of *C. sepium* with LA39 resulted in increased biomass allocation to stems and leaves but it reduced root biomass and carbohydrate reserves of the rhizomes. Rhizome carbohydrate reserves determine the extent of vegetative propagation and of emergence of the weed. Therefore, LA39 could be useful to reduce spring emergence of *C. sepium* under these conditions.

Possibly, the use of a mycoherbicide alone may not be sufficient for bindweed control and therefore plant competitors were tested as well, with the objective of combining them with *S. convolvuli* LA39. In greenhouse experiments, *L. multiflorum* and *T. pratense* reduced the growth of *C. sepium* and *C. arvensis*, respectively, when they were cultivated in competition. The negative effect of *T. pratense* on *C. sepium* resulted mainly from competition for light (shading). In experiments carried out in a maize field, underseeded *T. pratense* failed to control bindweed, probably because the Legume was not well developed. Fully-developed plant competitors were found in an amenity area, where a green cover of the perennial *Cotoneaster dammeri* was established. However, the site was severely infested by bindweeds, indicating that established plant competitors may be insufficient to control them. The usefulness of combining plant competitors and a mycoherbicide was assessed in the greenhouse and the field. In the greenhouse, the combination of plant competitors (*L. multiflorum* or *T. pratense*) and mycoherbicide (LA39) killed most bindweed plants. In a maize field underseeded with *T. pratense* and treated with LA39, bindweed displayed severe leaf necrosis and ground coverage by the weed was reduced. This effect was observed as soon as two weeks after application of LA39 and throughout the ten weeks of the
Zusammenfassung


Vermutlich genügt aber für die Windenkontrolle ein Mykoherbizid alleine nicht. Deshalb wurden in einer nächsten Phase dieser Arbeit Konkurrenzpflanzen getestet, mit dem Ziel sie mit *S. convolvuli* LA39 zu kombinieren. Im Gewächshaus reduzierten konkurrierende *L. multiflorum* oder *T. pratense* das Wachstum beider Winden. Die Wirkung von *T. pratense* war vor allem auf eine verringerte die Menge an Licht das für die Winden verfügbar war zurückzuführen. Im Feldversuch hatte *T. pratense* als Untersaat im Mais keine Wirkung auf die Winden, was vermutlich auf sein geringes Wachstum zurückzuführen war. Eine Situation mit ausgewachsenen Konkurrenzpflanzen fand sich in einer Parkanlage mit einer Grünbedeckung von *Cotoneaster dammeri*. Allerdings war diese stark mit Winden verseucht, was auf eine ungenügende Konkurrenzkraft dieser Grünbedeckung hindeutet. Die Möglichkeit, Winden mit einer Kombination von Konkurrenzpflanzen und Mykoherbizid zu bekämpfen, wurde im Gewächshaus und im Feld getestet. Im Gewächshaus tötete die Kombination von Mykoherbizid (LA39) und Konkurrenzpflanzen (*L. multiflorum* oder *T. pratense*) die meisten Winden. In Versuchen in einem Maisfeld mit einer Untersaat von *T. pratense*, verursachte *S. convolvuli* eine schwere Nekrose der Windenblattfläche und reduzierte den Windenbedeckungsgrad. Dieser Effekt wurde bereits ab zwei
experiments. Bindweed control was better in 1997 compared to 1996, probably because of more humid and cool conditions in 1997. In the amenity area with an established C. dammeri ground cover, treatment with LA39 resulted in death of most of the aboveground parts of the large population of C. arvensis and in a decrease of ground coverage within 40 days. However, the emergence of the bindweeds in the next growing season was not affected by the LA39 treatment.

The ecological success of the bindweeds may be due in part to the presence of calystegines which are produced in large amounts by the plants. Biological functions of these alkaloids include toxicity to mammals, insect deterrence, antivirosis and allelopathy. In the last stage of this work, the microbial degradation of the calystegines was studied. Investigations at the level of rhizosphere microbial communities indicated that, in natural fields, microorganisms capable of calystegine degradation were present not only in the rhizosphere of C. sepium but also in that of maize which is not producing calystegines. However, the proportion of maize rhizospheres containing calystegine-degrading microorganisms was higher in plots where C. sepium was present than in bindweed-free plots. The plasmid pRme41a of S. meliloti strain Rm41 contains genes for calystegine degradation (cac). In experiments in controlled environment a spontaneous rifampicine-resistant mutant of Rm41 containing the cac plasmid reached population levels in the rhizosphere of calystegine-positive plants that were several orders of magnitude higher than those of the same strain without the plasmid, when each was coinoculated with a derivative of Rm41 cured of pRme41a. Inoculation of the calystegine degrader Rm41 had no effect on the efficacy of the leaf pathogen LA39 in the greenhouse. However, the specific advantage for rhizosphere colonisation conferred by pRme41a and presumably due to genes cac could be used for the improvement of microbial biocontrol agents of bindweeds.

On the way towards biological control of the bindweeds, the different strategies investigated in this thesis should be optimised, further developed and implemented into integrated pest management (IPM) systems.


Im Hinblick auf die biologische Windenkontrolle sollten nun die verschiedenen Strategien, die in der vorliegenden Arbeit untersucht wurden, optimiert und weiterentwickelt werden, um sie schliesslich im Integrierten Pflanzenschutz (IPM) einsetzen zu können.
Leer - Vide - Empty
Chapter I.

Introduction

Weeds are among the plant species living in quite adverse conditions, because man is efficient in controlling plants he does not want. Still weeds were present throughout the history of agriculture. With the development of modern selective chemical herbicides in the middle of this century, conditions became particularly unfavourable for weeds – resulting in lower weed competition in crops and an increase in crop yields. Nevertheless, some weed species are equipped to cope with the conditions encountered today in crops. One example are the bindweeds, *Convolvulus arvensis* L. (wild morning glory or field bindweed) and *Calystegia sepium* (L.) R. Br. (hedge bindweed), perennial weeds with a great plasticity, which are poorly controlled by many selective herbicides, and they often fill the niche left by chemically controlled species.

Bindweed biology

*C. arvensis* and *C. sepium* belong to the large *Convolvulaceae* family which consists of about 900 species. In this family, *Ipomea batatas* (L.) Lam. is the most important crop, which is cultivated in many tropical areas of the world and 129.1×10^7 t are produced on an area of 9.1×10^7 ha (annual average of 1987-1998; FAO statistical database). *Ipomea tricolor* Cav. and *Pharbitis nil* are well known ornamental plants. Because of their similarity, *C. sepium* and *C. arvensis* are often confounded, but their separation into two genera has been confirmed (Lewis and Oliver, 1965; Wang and Kok, 1985). The genus *Calystegia* R. Br. is separated from the genus *Convolvulus* L. by its unilocular ovary, blunt stigmas, distinct pollen morphology, and, usually, by having large involucral bracteoles (Ogden, 1978). Furthermore, the anatomy of underground storage organs of *C. sepium* corresponds to rhizomes (stem structures) and that of *C. arvensis* to roots (Klimeš and Klímešová, personal communication).

*C. arvensis* and *C. sepium* are anatomically very plastic and can adapt to many different environments (Kennedy and Crafts, 1931). They occur in many temperate,
Mediterranean and tropic areas of the world (Weaver and Riley, 1982; Holm et al., 1977; Kogan, 1986). Both plants are perennial and propagate vegetatively by roots (C. arvensis) or by rhizomes (C. sepium) (Kennedy and Crafts, 1931; Torrey, 1958; Klimeš and Klimešová, 1994). Seed production of the self-incompatible C. arvensis can reach up to 300 seeds per plant, some of which can remain viable in soil for more than 30 years (Timmons, 1949). Both plants produce stolons and climbing stems which do not survive winter. A new field bindweed transplant may develop shoots as far as 2.89 m away from the parent plant within 15 months of introduction and form up to 45 new shoots within 4 months of introduction (Best, 1963). Towards the end of the growing season, the stolons of C. sepium penetrate into the soil and transform into rhizomes (Klimeš and Klimešová, 1994). The root system of C. arvensis may occupy an area of 6 m in diameter and 3 m in depth (Holm et al., 1977). Stolons of C. sepium can reach up to 6 m in length in a year leading to an important vegetative dispersal of the plants (van Ast and van Groenendael, 1993; Neubauer, 1988). Stolons and rhizomes of C. sepium have many properties in common. Stolons may develop from rhizomes, seeds or flowering branches and rhizomes may develop from stolons or from cotyledonary buds (Vakili-Nejad, 1976).

C. arvensis does not grow very well in areas with fewer than 3000 degree-days above 5°C (Rousseau, 1968). Seed dormancy of C. sepium and C. arvensis can be broken by injury to the hard seed coat, caused by chemical or mechanical effects (Hunyadi, 1992; Jordan and Jordan, 1982).

The roots and rhizomes of the bindweeds contain large amounts of reserves, which are used for plant regrowth in the next spring or after mechanical destruction of the plants. C. arvensis can contain 58% readily available carbohydrates at the end of a growing season for undisturbed plants (Barr, 1940). Frazier (1943) reported a much lower content of 28% at the end of the growing season.

Calystegines, important secondary metabolites of bindweeds

The glycoretines contained in the Convolvulaceae have been used as purgatives by various peoples. Furthermore, alkaloids, polyphenols and reserve carbohydrates are often found (Hegnauer, 1964). More recently, water soluble tropane alkaloids, calystegines, have been extracted from C. sepium and C. arvensis (Tepfer et al., 1988). This alkaloid is abundantly produced in rhizomes, roots and aerial parts of the plants (Tepfer et al., 1988; Dräger, 1995; Dräger, personal communication). Calystegine
production has also been detected in *Atropa belladonna* L. (Tepfer et al., 1988), *Hyoscyamus niger* L. (Asano et al., 1996), *Morus alba* L. (Asano et al., 1994), *Physalis alkekengi* var. francheti L. (Asano et al., 1995), *Solanum tuberosum* L., *Solanum dulcamara*, *Solanum dimidiatum*, *Solanum kwebense*, *Solanum melongena* L. and *Datura wrightii* (Nash et al., 1993). The fact that so far members of three families Convolvulaceae, Solanaceae and Moraceae have been shown to produce these alkaloids, suggests, that there are more taxa with plants containing calystegines (Goldmann et al., 1996). Chemical synthesis of some calystegines has been achieved by different groups (Johnson and Bis, 1995; Boyer and Lallemand, 1994). Two tropinone reductases in the biosynthesis pathway of tropane alkaloids have been cloned from *D. stramonium*. They reduce tropinone to tropine and then to pseudo-tropine (ψ-tropine) (Nakajima, 1993; Portsteffen, 1992). It is assumed that calystegines are formed from ψ-tropine (Nakajima, 1993). Some properties of a bacterial tropinone dehydrogenase are described by Bartholomew et al. (1995). Rabot et al. (1995) recently purified tigloyl-CoA:pseudotropine acyl transferase from *D. stramonium*. This enzyme is further downstream in the tropane alkaloid biosynthetic pathway. So far, no enzymes of the tropinone alkaloid biosynthesis have been purified from *C. sepium* or *C. arvensis*. Calystegines are glycosidase inhibitors (Molyneux et al., 1993; Asano et al., 1994a). Their properties include inhibition of glycosidases, insect feeding deterrence, toxicity to cattle, anti-viral effects, reduction of metastatic potential of cancers, stimulation of the immune system, inhibition of other types of hydrolases (Fuhrmann et al., 1985; Fellows et al., 1992; Nash et al., 1993; Todd et al., 1995; Bridges et al., 1995; Asano et al., 1995a; Goldmann et al., 1996). Calystegines may be responsible for specific nutritional interactions between calystegine producing plants and rhizosphere microorganisms capable of calystegine degradation (Tepfer et al., 1988; Tepfer et al., 1988a; Boivin et al., 1990; Molyneux et al., 1993; Goldmann et al., 1996).

**The bindweeds as weeds and their economic impact**

The morning glories are very well adapted to the conditions in agricultural fields. They start to grow in phase with or later than many crops and weeds. Therefore, they are difficult to distinguish within the crops, precluding hand weeding, and are difficult to control because of selectivity, shading by other weeds as well as their regrowth potential after treatment with non-translocated, leaf applied herbicides. Vegetative propagation is guaranteed for small fragments of roots or rhizomes. Morning glories are difficult to target with herbicides, because of their deep roots. The plants grow very
quickly, and can strangle crops. Bindweeds are favoured when the herbicides eliminate mainly annual weeds as well as by certain tillage practices (Maillet, 1988; Ammon, 1986; van Ast and van Groenendael 1993; Ammon, personal communication). Triazine and urea herbicides, which have a broad action against many other weeds, did not control morning glories providing a selective advantage because of reduced competition from the other weeds (Stalder et al., 1974). A large part of the research conducted on C. sepium and C. arvensis is dedicated to the control of these plants as weeds and much less to their biology.

Table 1. Selected studies on chemical herbicides for the control of bindweeds.

<table>
<thead>
<tr>
<th>Herbicide / Herbicide combination</th>
<th>Crop / Crop rotation</th>
<th>Application method and/or additional weed control methods</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulphate</td>
<td>-</td>
<td>-</td>
<td>Crafts, 1960</td>
</tr>
<tr>
<td>2,4-D</td>
<td>-</td>
<td>-</td>
<td>Hammer and Tukey, 1951</td>
</tr>
<tr>
<td>2,4-D</td>
<td>continuos wheat, wheat-fallow, wheat-sorghum, wheat-sorghum-fallow</td>
<td>tillage</td>
<td>Derscheid et al., 1970</td>
</tr>
<tr>
<td>Fluroxypyr</td>
<td>corn</td>
<td>preemergence, at different growth stages of corn</td>
<td>MacDonald et al., 1993</td>
</tr>
<tr>
<td>Chlorothiamid; Dichlobenil; MCPB</td>
<td>gooseberries</td>
<td>between crop rows</td>
<td>Davison, 1970</td>
</tr>
<tr>
<td>Glyphosate; MCPB; Paraquat; Atrazin</td>
<td>vineyards (Vitis vinifera)</td>
<td>between crop rows</td>
<td>Stalder et al., 1974, 1977</td>
</tr>
<tr>
<td>2,4-D</td>
<td>fallow; winter wheat</td>
<td>tillage, competitive crops</td>
<td>Wiese and Rea, 1959</td>
</tr>
<tr>
<td>Imazazylone, metsulfuron</td>
<td>fallow</td>
<td>-</td>
<td>Heering and Peeper, 1991</td>
</tr>
<tr>
<td>2,4-D; MCPA; Glyphosate; Imazapyr (and many more)</td>
<td>fallow</td>
<td>-</td>
<td>Matic and Black, 1994</td>
</tr>
<tr>
<td>Imazapyr; Dicamba; 2,4-D; Picloram; combinations</td>
<td>fallow</td>
<td>-</td>
<td>Schoenhals et al., 1990</td>
</tr>
<tr>
<td>Dicamba; 2,4-D</td>
<td>fallow</td>
<td>combination with Phoma proboscis (bindweed pathogen)</td>
<td>Schweizer et al., 1978</td>
</tr>
<tr>
<td>2,4-D and MCPB combination</td>
<td>fallow</td>
<td>-</td>
<td>Heiny, 1994</td>
</tr>
<tr>
<td>2,4-D, Glyphosate</td>
<td>fallow</td>
<td>-</td>
<td>Gigax and Messersmith, 1978</td>
</tr>
<tr>
<td>Trifluralin</td>
<td>-</td>
<td>- (greenhouse studies)</td>
<td>Siczek et al., 1995</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>-</td>
<td>- (greenhouse studies)</td>
<td>Dall'Armellina and Zimdahl, 1989</td>
</tr>
<tr>
<td>Glyphosate; 2,4-D; Dicamba; combinations</td>
<td>-</td>
<td>- (greenhouse studies)</td>
<td>Flint and Barret, 1989</td>
</tr>
</tbody>
</table>

C. sepium and C. arvensis are important weeds world-wide (Holm et al., 1977). According to Oerke et al. (1994) bindweeds are major weeds in wheat, potatoes, soybean and cotton all over the world. C. arvensis is found in vegetable and arable crop fields, cereals, vineyards, citrus, potatoes and olive groves and can infest practically any crop (Americanos, 1994). In the Near East, C. arvensis is causing problems in many crops, including vegetables, orchards, cereals, fruit, citrus, cotton, sugar cane, lentils, legumes (FAO, 1987). In Spain, C. arvensis was present in 84% of 591 citrus orchards.
surveyed in 1977 (Costa, 1997). Field bindweed is also widely distributed in South America, Australia and the Indian subcontinent (Kogan, 1986).

Bindweeds have an important economic impact (Weaver and Riley, 1982). In vineyards, yields can be reduced by half (Juliard, 1971). According to Malicki and Berbeciowa (1986) field bindweed is among the most dangerous competitors to winter wheat, spring barley, sugar beet and rape. *C. arvensis* halved the yields of processing tomatoes (Lanini and Miyao, 1987, 1989). In addition to interference with harvesting procedures, Maw (1981) observed yield reductions of 20% for rye and of 78% for grain sorghum caused by field bindweed. Bindweeds can disturb apple development by shading the fruit and hamper harvesting by interference with picking (Davison, 1976). Saghir (1987) reported that *C. arvensis* may cause lodging of cereals and that there is interference with harvesting procedures. Matic (1994) observed a 180% yield increase of barley and 264% for field pea, if the bindweeds were removed chemically. Maillet (1988) reported yield reductions caused by bindweeds of 30% in barley and in rye, 40% in wheat, 70% in maize and more than 80% in sorghum.

Schweizer et al. (1978) reported that an annual investment of $ 54 ha$^{-1}$ for chemical bindweed control raised the gross crop value from $ 2906 \text{ ha}^{-1}$ to $ 2970 \text{ ha}^{-1}$ in a sorghum/corn rotation. In that work, herbicide treatments increased yields of corn but not of sorghum. In wheat, Wiese et al. (1996) observed a $ 110 \text{ ha}^{-1}$ increase in income but invested $ 77 \text{ ha}^{-1}$ for chemical bindweed control. In a wheat sorghum rotation, Wiese et al. (1997) netted $ 226 \text{ ha}^{-1}$ more income with an additional investment of $ 132 \text{ ha}^{-1}$ for chemical bindweed control in their two years study. For California, the estimated annual total costs for bindweed control and yield loss were about $ 30$ million, corresponding to about $ 37 \text{ ha}^{-1}$ of infested land (Rosenthal, 1983).

**Chemical weed control**

Only few chemical herbicides were available until 1940. Important milestones in the discovery of modern selective herbicides were marked by work on bindweed control. Thus in the 1920s, a dilute solution of carbon arsenite was used for the control of field bindweed (Crafts, 1960). After the release of 2,4-D (Pokorny, 1941), the first agricultural field tests were conducted for the control of bindweeds (Hammer and Tukey, 1944). A next important step is documented by the discovery of the phenylurea herbicides (Bucha and Todd, 1951). A large number of herbicide methods, partly in combination with other methods have been investigated for the control of bindweeds (Table 1). However, only a limited number of herbicides are in practical use in
Switzerland and these rely just on three different chemical modes of action (Table 2). The decision about which herbicide is used in a specific case depends on its activity, on economical constraints and on considerations of how long the compound will stay in the environment before its degradation.

Table 2. Herbicides in practical use for bindweed control in different cultures in Switzerland and their mode of action.1

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>HRAC Group</th>
<th>WSSA Group</th>
<th>Cereals</th>
<th>Maize</th>
<th>Soya</th>
<th>Peas</th>
<th>Potato</th>
<th>Sugar</th>
<th>Beet</th>
<th>Rape</th>
<th>Vine</th>
<th>Fruit</th>
<th>Fallow</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>O1</td>
<td>44</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Dicamba</td>
<td>O</td>
<td>4</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MCPA</td>
<td>O</td>
<td>4</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MCPB</td>
<td>O</td>
<td>4</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mecoprop</td>
<td>O</td>
<td>4</td>
<td>++</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Clopyralide</td>
<td>O</td>
<td>4</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fluroxypyr</td>
<td>O</td>
<td>4</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Metsulfuron</td>
<td>B</td>
<td>2</td>
<td>++</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Amidoclefone</td>
<td>B</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Triasulfuron</td>
<td>B</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>G</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

1Data from Brenner et al., 1998, Ammon, pers. com.
2Mode of action group according to the Herbicide Resistance Action Committee (HRAC, 1997).
3Mode of action group according to the Weed Science Society of America (HRAC, 1997).
5++ recommended use; +: restricted use; -: not registered in this crop

In 1995, 251’000 tons of active ingredient of herbicides were sold worldwide (Aspelin, 1997). In the US agricultural crop production, the herbicide atrazine was the most used conventional pesticide. Herbicides constituted 70 % of all pesticides used in the US and 45 % world-wide. These figure demonstrate the great importance of the herbicides for today’s crop production (Aspelin, 1997). One main threat to weed management with herbicides is the evolution of weed resistance to herbicides. Where chemical control is no longer effective, weeds compete with crop plants and reduce yields. The first documented resistance were triazine resistant Senecio vulgaris in the US and Amaranthus retroflexus in Austria in 1973 (De Prado et al., 1997), although there were earlier undocumented reports. Today many weed species are reported resistant to many different herbicides (Powles and Holtum, 1994; De Prado et al., 1997a). Substantial variation in response to 2,4-D has been found for different strains of C. arvensis (Whitworth, 1964). Two cases of resistance to 2,4-D have been reported (Mouch, 1987; Schroeder et al., 1993). Known variabilities in quantitative levels of resistance to glyphosate (Gressel, 1996) are a sign that resistance is more likely to develop than it was thought before (Dyer, 1994; Darmercy, 1994). Causes of variations in response to glyphosate of C. arvensis may include greater DAHPS and EPSPS activity and higher concentrations of phenolic compounds (Westwood and Weller,
1997), but probably are not caused by differences in leaf stomatal or epidermal cell numbers (DeGennaro and Weller, 1984) nor by differential absorption and translocation of the herbicide (Westwood et al., 1997). Comparison of imazapyr control of field bindweed populations differing in vigour showed, that better herbicide action was achieved for more vigorous populations (Schoenhals et al., 1990).

Chemical control of bindweeds is particularly difficult, because in many cases they emerge with or even more often after the crop itself. Chemical control therefore is often not possible without damage to the crop, except when the herbicides are very selective. Consequently, bindweed control often is a trade-off between crop damage and adequate weed control.

The development and the use of selective chemical herbicides did not solve the world-wide problems with weeds. The Weed Science Society of America lists 2076 plants as weeds and this list is growing (Composite List, 1989), yet only about 200 are of global importance (Holm et al., 1997). The development of herbicide resistant weed populations, public health concerns and soil degradation lead to the call for alternative methods.

In the last decades, the understanding of weed science as the science of weed control has evolved to the science of weed biology, ecology and management (Radosevich et al., 1997; Smith, 1995; Aldrich and Kremer, 1997). Gressel (1997) proposed that where crop rotation is not possible, chemical abstinence using other techniques of weed control as a long term strategy for adequate weed control is advisable. New tools are needed for weed management combining chemistry, mechanics, agronomy, biocontrol and regulation in integrative ways (Gressel 1997). There is a clear linkage between herbicide use and the number of reported cases of resistance which occurred most often in major crops, in monocultures and where the same herbicide was used repeatedly (Rubin, 1997). A risk assessment can be used to predict if resistance may become a problem in a specific situation (Moss, 1997).

**Weed control by cultural practices**

Because modern agriculture is facing an increasing number of problems with chemical herbicide weed control, weed science is investigating the large number of alternatives (Table 3). Hand weeding, the traditional physical practice to cope with weeds, is back breaking, very labour intensive, too expensive in industrialised agriculture. Hand weeding for bindweed control is practised mainly in home gardens or amenity areas in the first world (personal observation).
Table 3. Methods of weed management their use, advantages and disadvantages.

<table>
<thead>
<tr>
<th>Method</th>
<th>Use</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hand pulling, hoeing</td>
<td>traditional</td>
<td>very specific</td>
<td>tedious, back-breaking, expensive</td>
</tr>
<tr>
<td>Fire</td>
<td>traditional</td>
<td>cheap</td>
<td>unspecific</td>
</tr>
<tr>
<td>Flame</td>
<td>special applications for</td>
<td>specific</td>
<td>expensive</td>
</tr>
<tr>
<td></td>
<td>woody crops or protected crop meristems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tillage (mouldboard plough)</td>
<td>depletion of weed reserves,</td>
<td>flexible use, may be combined with</td>
<td>weed germination, weed dispersal, soil compaction,</td>
</tr>
<tr>
<td></td>
<td>weed burial, perennial weed control,</td>
<td>chemical control</td>
<td>erosion</td>
</tr>
<tr>
<td></td>
<td>seadbed preparation, burial of crop</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>residue, altering soil surface state,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>incorporation of fertilisers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tillage (minimum,</td>
<td>in dry climate, for soil</td>
<td>erosion control, cheap</td>
<td>perennial weeds not controlled, crop residues on</td>
</tr>
<tr>
<td>conservation reduced, ridge)</td>
<td>conservation</td>
<td></td>
<td>soil surface</td>
</tr>
<tr>
<td>Mowing, Shredding</td>
<td>roadsides, vacant lots,</td>
<td>reducing vegetation, without</td>
<td>increase of weed seed abundance</td>
</tr>
<tr>
<td></td>
<td>abandoned cropland</td>
<td>killing</td>
<td></td>
</tr>
<tr>
<td>Chaining, Dredging</td>
<td>rangeland, aquatic weed control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water management</td>
<td>flooding (terrestrial weeds) or draining</td>
<td>long time unproductive</td>
<td>1-2 months submergence</td>
</tr>
<tr>
<td></td>
<td>(aquatic weeds)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mulches, Solarisation</td>
<td>annual weeds in cash crops and</td>
<td>effective</td>
<td>expensive, needs sunshine</td>
</tr>
<tr>
<td></td>
<td>organic food growing</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cultural methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weed prevention</td>
<td>widely used</td>
<td>effective</td>
<td>economic trade-offs</td>
</tr>
<tr>
<td>Crop rotation</td>
<td>cereals, fodder</td>
<td>effective</td>
<td>yield trade-offs</td>
</tr>
<tr>
<td>Crop competition</td>
<td>widely used</td>
<td>effective</td>
<td>competition on crop</td>
</tr>
<tr>
<td>Living mulches, cover crop</td>
<td>integrated weed management</td>
<td>elevate biodiversity</td>
<td></td>
</tr>
<tr>
<td>Harvesting</td>
<td>fodder, frequent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed crops / Intercropping</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biological control</td>
<td>annual weeds</td>
<td>reduced erosion</td>
<td>sophisticated machinery</td>
</tr>
<tr>
<td>Grazing</td>
<td>traditional</td>
<td>natural, low impact, specificity</td>
<td></td>
</tr>
<tr>
<td>Inoculative biocontrol</td>
<td>imported weeds</td>
<td>specificity, low impact</td>
<td>possibility of host range changes</td>
</tr>
<tr>
<td>Insects</td>
<td>imported weeds, indigenous</td>
<td>specificity, low impact</td>
<td>possibility of host range changes</td>
</tr>
<tr>
<td></td>
<td>weeds</td>
<td></td>
<td>expensive, allergenicity, seldom competitive with</td>
</tr>
<tr>
<td>Mycoherbicides</td>
<td>in activity windows of</td>
<td>specificity, interest in niche</td>
<td>herbicides</td>
</tr>
<tr>
<td></td>
<td>herbicides, few products,</td>
<td>markets</td>
<td>effects on non-target spp.</td>
</tr>
<tr>
<td></td>
<td>niche markets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allelopathy</td>
<td>not widely used, potential</td>
<td>natural</td>
<td></td>
</tr>
<tr>
<td></td>
<td>for suppression of weed growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chemical Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herbicides</td>
<td>almost every culture, most</td>
<td>effective, cheap</td>
<td>resistances, can have adverse effects on</td>
</tr>
<tr>
<td></td>
<td>widely applied weed control method</td>
<td></td>
<td>environment, health</td>
</tr>
</tbody>
</table>

*Data from Smith, 1995; Aldrich and Kremer, 1997; Radosevich et al., 1997

Frequent cultivation of bindweed is known to reduce carbohydrate reserves (Barr, 1940). Mechanical methods are labour saving and effective, sometimes specificity may be insufficient and frequent treatments may disturb the soil too much, and promote compaction and erosion (Radosevich et al., 1997; Wicks et al., 1995).
Buhler et al. (1994) observed in a 14-year field experiment, that in crop rotations of maize and soybeans, field bindweed increased in various tillage systems. However, it is known, that extensive tillage may reduce field bindweed infestation (Buhler et al., 1994; Timmons and Bruns, 1951; Timmons 1949). Usually tillage every 12 days after weed emergence about 10 cm deep is needed for up to two years until near eradication of a bindweed infestation is achieved (Timmons and Burns, 1951). A large part of the weed management traditionally relies on cultural practices (Radosevich et al., 1997; Aldrich and Kremer, 1997).

**Biological weed control**

In biological weed control, the interaction between the weed and another organism results in a reduction of weed development. Geese, sheep, goats and cattle are widely used to graze a broad range of weed species (Radosevich et al., 1997; Gillen and Scifres, 1991).

Allelopathy for weed control receives increasing attention and is not yet widely in use (Smith, 1995; Lovett 1991; Radosevich et al., 1997; Aldrich and Kremer, 1997). Allelopathy has been proposed as a method of control for *Ipomea hederacea* and *Ipomea lacunosa* (Pue et al., 1995; White et al., 1989; Blum et al., 1993; Blum et al., 1992). However, *Convolvulaceae* do seem to have allelopathic properties themselves. Several studies have been conducted on allelopathic properties of the *Ipomea batatas* (Reinhardt et al., 1993; Peterson and Harrison, 1991; Harrison and Peterson, 1991) and of *Ipomea tricolor* (Anaya et al., 1995; Pereda-Miranda et al., 1993; Anaya et al., 1990). In Mexico, *I. tricolor* is used in traditional weed control in sugar cane fields (Anaya et al., 1990). Allelopathic activity of *C. sepium* was proposed by Quinn (1974) and by Maillet (1988). Possibly, calystegines are allelopathic substances because they hinder germination of seeds of *Medicago sativa* (Goldmann et al., 1996).

Intercropping and living mulches for weed biocontrol are increasingly getting attention for integrated weed management (Radosevich et al., 1997). In vineyards, Stalder et al. (1974) observed excellent control of bindweed if *Stellaria media* was used as a living green mulch below vines. Likewise, living mulches are being introduced into maize cropping (Koskinen and McWorther, 1986; Garibay et al., 1997; Burgos and Talbert 1996; Hall and Hartwig, 1990). These authors observed reduced erosion, improved accessibility, reduced need for herbicide input and a reduction of most annual and many perennial weeds normally accompanying maize. Unfortunately, the green covers do not affect the density of *C. sepium* (Pfirter et al., 1997).
Generally two approaches are discerned for biological weed control using insects or fungi: the classical or inoculative strategy and the inundative strategy (Boyetchko, 1997). The inoculative approach is applicable where an introduced, foreign ('alien') weed species lacks its natural enemy, which then is inoculated at the place of introduction. This approach proved particularly successful with *Phragmidium violaceum* for the control of blackberry infesting large areas in Chile (Oehrens, 1977), but there are a number of other examples of successes of the inoculative approach (Smith, 1995). In cases, where the weed is global or endemic and/or possesses a wide genetic variability, the inundative approach to biological weed control is often used. It involves the repeated application of massive inoculum of the control agent.

In the case of fungal pathogens, the inundative approach became known as 'mycoherbicide approach' (Charudattan and Walker, 1982). In the U.S., 21 patents on bioherbicides or mycoherbicides are held (Table 4). Currently, one classical (DeVine, Abbott Laboratories, IL) and one inundative product is sold, even though four products are registered and active in the United States (Table 5). In the rest of the world two more products are registered, and one of them is sold (Table 5). This seems little success compared to the promises and hopes raised in the scientific literature (Charudattan and Walker, 1982; TeBeest et al., 1992; Wapshere et al., 1989). However, the biological control of weeds has still a long way to go if one considers that ca. 100000 chemicals need to be tested to find one commercial product in the herbicide industry. Of the 20 most noxious weeds in agriculture in Europe (Schroeder et al., 1993), only *Cirsium arvensis* (Wan et al., 1996) and *Convolvulus arvensis* (Pfirter and Défago, 1998) received major attention in biological weed control research. However, this is not very surprising as biological weed control will probably always remain a niche market for very specific conditions, e.g. where chemical herbicides are not available (Auld and Morin, 1995), are too expensive (e.g. on rangelands or forests) or in urban situations where there is a social demand to avoid their use.

**Choice of the target weed and selection of the agent**

Biological, environmental, technological and commercial aspects must be considered in the development of an agent for the biological control of a weed species (Auld and Morin, 1995). Weed targets should be selected in function of need, i.e. their abundance or the availability of effective control methods (Schroeder et al., 1993). The strategy chosen is mainly dependent on the width of the genetic basis of the target, i.e. whether it is an endemic weed or an alien species (Charudattan and Walker, 1982).
<table>
<thead>
<tr>
<th>Date</th>
<th>Number</th>
<th>Organism</th>
<th>Adjuvant</th>
<th>Target</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sept. 1996</td>
<td>5,559,079</td>
<td><em>Xanthomonas campestris</em></td>
<td>sulfonylurea herbicides</td>
<td>annual bluegrass (<em>Poa annua</em>)</td>
<td>Imazumi, S., Yamada, M. and Nishino, T.</td>
</tr>
<tr>
<td>July. 1996</td>
<td>5,538,890</td>
<td><em>Sclerotinia sclerotium</em></td>
<td>-</td>
<td>broad spectrum</td>
<td>Sande, D.C., Miller, R.V., Ford, E. andKennet, G.</td>
</tr>
<tr>
<td>Dec. 1995</td>
<td>5,472,690</td>
<td><em>Fusarium nivalis, Colletotrichum calagrostis</em></td>
<td>allelopathic agent (e.g. straw, extracts)</td>
<td><em>Clamagrostis canadensis</em></td>
<td>Winder, R.</td>
</tr>
<tr>
<td>Mar. 1994</td>
<td>5,296,369</td>
<td><em>Colletotrichum gloeosporioides f. sp. malvae</em></td>
<td>-</td>
<td><em>Malva pusilla</em> Sm.</td>
<td>Mortensen, K., Makowski, R.M.D., Cunningham, J.E. and Carmichael, R.E.</td>
</tr>
<tr>
<td>Sept. 1993</td>
<td>5,244,659</td>
<td><em>Colletotrichum dematiu</em></td>
<td>-</td>
<td>fireweed</td>
<td>Watson, A.K. and Winder, R.S.</td>
</tr>
<tr>
<td>Jun. 1993</td>
<td>5,221,314</td>
<td><em>Colletotrichum cocodes</em></td>
<td>chemical herbicides</td>
<td>velvetleaf</td>
<td>Watson, A.K. and Gotlieb, A.R.</td>
</tr>
<tr>
<td>May 1993</td>
<td>5,200,026</td>
<td><em>Colletotrichum sp. G1</em></td>
<td>-</td>
<td>Plantago major</td>
<td>Watson, A.K., Tourgny, G. andWinder, R.S.</td>
</tr>
<tr>
<td>Mar. 1990</td>
<td>4,909,626</td>
<td><em>Amphobotrys ricini</em></td>
<td>-</td>
<td><em>Caperonia palustris</em></td>
<td>Whitney, N.G.</td>
</tr>
<tr>
<td>Oct. 1989</td>
<td>4,871,386</td>
<td><em>Alternaria euphorbiocola Simmons and Engelhard</em></td>
<td>-</td>
<td>wild poinsettia, weedy spurges</td>
<td>Riley, J.A.</td>
</tr>
<tr>
<td>Aug. 1989</td>
<td>4,767,441</td>
<td><em>living fungus</em></td>
<td>alginate gel pellets</td>
<td>corresponding weed</td>
<td>Walker, H.L., Connick Jr., W.J. andQuimby Jr., P.C.</td>
</tr>
<tr>
<td>Jul. 1988</td>
<td>4,755,207</td>
<td><em>Alternaria cassiae</em></td>
<td>crop oil, surfactants</td>
<td>sicklepod</td>
<td>Bannon, J.S.</td>
</tr>
<tr>
<td>Dec. 1987</td>
<td>4,715,881</td>
<td><em>Colletotrichum cocodes</em></td>
<td>-</td>
<td><em>Solanum pycanthum</em></td>
<td>Andersen, R.N. and Walker, H.L.</td>
</tr>
</tbody>
</table>
In the development of the biological control of the bindweeds, extensive surveys of organisms, insects and pathogens, associated with the bindweeds have been conducted (Rosenthal 1982; Rosenthal 1985; Mohyuddin, 1969; Pfirter et al., 1997). Among the insects tested for biocontrol of bindweeds were Megacerus discoidus (Say) (Wang and Kok, 1986) and Aceria malherbae Nuzzaci (Boldt and Sobhian, 1993). Pathogens proposed as mycoherbicides include Phomopsis convolvulus Ormeno (Ormeno-Nuñez et al., 1988; Vogelsang et al., 1998 and 1998a), Phoma proboscis Heiny (Heiny, 1990) and Stagonospora convolvuli Dearness and House (Pfirter and Défago, 1998).

Table 5. Registered bioherbicides.

<table>
<thead>
<tr>
<th>Bioherbicide</th>
<th>Organism</th>
<th>Target weed</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collego¹</td>
<td>Colletotrichum gloeosporioides f. spp. aescynomeone (# ATCC 20358)</td>
<td>northern jointvetch; Aescynomeone virginica (L.) B.S.P. (AESVI) in rice and soybean</td>
<td>USA, Canada</td>
</tr>
<tr>
<td>DeVine²</td>
<td>Phytophthora palmivora</td>
<td>strangler or milkweed vine; Morenia odorata (H. &amp; A.) Lindl. (MONOD)</td>
<td>USA, Canada</td>
</tr>
<tr>
<td>BioMal³</td>
<td>Colletotrichum gloeosporioides f. sp. mala (ATCC 20767)</td>
<td>round leaved mallow, Malva pusilla Sm.</td>
<td>USA, Canada</td>
</tr>
<tr>
<td>Dr. Biosedge⁴</td>
<td>Puccinia canaliculata (Schweinitz) Lagerheim (# ATCC 40199)</td>
<td>Yellow nutsedge, Cyperus esculentus L. (CYPES)</td>
<td>USA, Canada</td>
</tr>
<tr>
<td>BioChon⁵</td>
<td>Chondrostereum purpureum (Pers: Fr.) Pouzar</td>
<td>hardwood weeds e.g. quaking aspen, alders, birch, pincherry and maples</td>
<td>Netherlands</td>
</tr>
<tr>
<td>Camperico⁶</td>
<td>Xanthomonas campestris pv. poae (JT-P482)</td>
<td>annual bluegrass, Poa annua L., (POAAN)</td>
<td>Japan</td>
</tr>
<tr>
<td>Stump Out</td>
<td>Cylindrobasidium laeve</td>
<td>Prevents regrowth of tree stumps</td>
<td>South Africa</td>
</tr>
</tbody>
</table>

¹ Ecogen Inc., 2005 Cabot Blvd., West Langhorne, PA 19047-1810, Tel ++ 215 757 15 90
² Abbot Laboratories, 14 Street & Sheridan Road, North Chicago, IL 60064, USA
³ Philom Bios Inc., 15 Innovation Blvd., Saskatoon, Saskatchewan, S7N 2X8, Canada
⁴ Tifton Innovation Corp., 1753 Tifton, GA 31793/753, USA
⁵ Koppert Biological Systems; Postbus 155; NL-2650 AD Berkel en Rodenrijs
⁶ Japan Tobacco Inc, 6-2 Umagaoka, Aoba-ku, Yokohama, Kanagawa 227, Japan

If a mycoherbicide is developed, several technical aspects must be considered. Often the feasibility of mass production of the biocontrol agent is a crucial point (Auld and Morin, 1995). Liquid fermentation proved successful for mass production of the mycoherbicide Collego from Colletotrichum gloeosporioides f. spp. aescynomeone (TeBeest and Templeton, 1985). However, fungal spore production often requires solid substrates. Western science has little experience with solid state fermentation, but in eastern societies this is a commonly used technique (Kennedy and Couch, 1981; Hesseltine, 1977; Aidoo et al., 1982). Thus, S. convolvuli does not produce spores in liquid culture but if cultivated on cracked wheat, ample spore production is observed (unpublished data).

Another, some say the most important, technical hurdle to surpass in the development of a mycoherbicide is its formulation for application (Fravel et al., 1985;
Connick, 1991; Egley and Boyette, 1995; Auld and Morin, 1995; Boyetchko, 1997). Once the fungal spores are produced, they are transported to the place of use, applied to the target, they have to outdo the defence mechanisms of the target weed and infect it. Formulations proposed include invert emulsions, oil emulsions, dry granules (Pesta) and spreading agents (Auld and Morin, 1995; Lawrie et al., 1997; Pfirter and Défago, 1998).

In many cases there are commercial limitations to the development of a biological control agent, like limited market size, patent protection, high production costs and extensive regulations. Some of these latter constraints could be overcome by using a strategy of mixing public and private investment for the development of bioherbicides (Auld and Morin, 1995).

**Microbial degradation of bindweed root alkaloids, the calystegines**

As calystegines deter insects and have antiviral properties (Fellows et al., 1992), their production may be a defence mechanism of the bindweeds. This might be the reason why rhizomes and roots do not seem to be attacked by pathogens or insects (personal observation). In biological bindweed control, such a defence might be overcome be the use of organisms tolerating the calystegines or even catabolising them. Furthermore, if the calystegines are allelopathic substances (as proposed by Goldmann et al., 1996), their degradation by microbes may weaken the competitiveness of the bindweeds towards crops or other weeds.

One example of a calystegine-catabolising organism is the *Sinorhizobium meliloti* strain Rm41(abc) (Tepfer et al., 1988). Rm41(abc) is not pathogenic towards bindweeds, but the efficacy of a biocontrol agent may be improved if applied in combination with Rm41(abc). The 225 kb, non-symbiotic plasmid pRme41a of Rm41(abc) contains genes cac for calystegine degradation (Tepfer et al., 1988; Tepfer et al., 1988a; Boivin et al., 1990). Those genes could be used to establish a metabolic link between the calystegine producing plant and a biocontrol agent of bindweeds, with the objective of improving the ability of the biocontrol agent to colonise bindweeds or its pathogenicity towards bindweeds. Several studies have demonstrated the possibility of constructing such specific trophic interactions between rhizosphere microorganisms and plants (Savka and Farrand, 1997; Guyon et al., 1993; Oger et al., 1997; Murphy et al., 1995).

These hypotheses indicate, that studies on the biological properties of the agents and the targets would provide information on the Achilles heel of the target weed, and
provide spearheads for the biocontrol agents. The potential of this approach is examplified by the breakdown of resistance of *Cassia obtussifolia* to *Alternaria cassiae* due to the application of reduced rates of glyphosate which inhibits the production of specific phytoalexins (Gressel *et al.*, 1996a).

**Literature cited**


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Chapter II.

Scope of the thesis

This thesis examines the interactions between the bindweeds *Calystegia sepium* and *Convolvulus arvensis* and several types of microorganisms and plants. The objective was to assess the potential of these interactions for the biological control of the bindweeds.

The first objective was to study the effects of the mycoherbicide *Stagonospora convolvuli* strain LA39 on its bindweed hosts. This fungus was described by Dearness and House (1916). Specificity of strain LA39, its cultivation and formulation for application have been investigated by Pfirter and Défago (1998). In this thesis, the impact of LA39 on growth of the bindweed *C. sepium* was assessed in an outdoor pot experiment.

The second objective was to study the effect of plant competitors on the biocontrol of bindweeds in the greenhouse and the field. As one biocontrol agent may provide insufficient control when used alone, the benefit of combining the mycoherbicide with plant competitors was also investigated. The plants *Lolium multiflorum* and *Trifolium pratense* were chosen as competitors because they are often used as ground cover plants. Field trials included *Zea mays* with underseeded *T. pratense* as competitor plants. In an amenity area, the effect of the mycoherbicide was tested in combination with the perennial green ground cover plant *Cotoneaster dammeri*.

The third objective was to study the interaction of the bindweeds with rhizosphere microbes capable of degrading calystegines, a type of bindweed alkaloids. In this thesis, plants producing calystegines or unable to do so were used to investigate the occurrence of calystegine-degraders in the rhizosphere and to compare bacteria differing in calystegine degradation capacity for rhizosphere colonisation.


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Chapter III.

Effect of mycoherbicide

*Stagonospora convolvuli* strain LA39
on rhizome- and seed-propagated
*Calystegia sepium*, grown in soils of contrasting fertility status in a non-crop situation.
Summary

The effect of the mycoherbicide *Stagonospora convolvuli* on growth and development of the perennial weed *Calystegia sepium* was studied. The plants, either propagated as seeds or from rhizomes, were cultivated at two nutrient levels in an outside, one-season pot experiment. Increased nutrients resulted in bigger plants. Plants grown from rhizomes invested more biomass in structures of sexual reproduction compared to seed derived plants. Mycoherbicide application resulted in leaf necrosis and loss of leaves. However, twining and stolon axes were elongated due to the mycoherbicide application and biomass allocation to these structures was increased. Even though above-ground biomass was not affected, the biomass of the below-ground structures and the content of carbohydrate reserves of the rhizomes were reduced as an effect of the mycoherbicide. In general, the activity of this foliar pathogen was independent of the propagation method and of the nutrition of the plant. Therefore, in different environmental conditions, repeated application of this mycoherbicide may continuously reduce the biomass of the structures of vegetative reproduction and eventually lead to the biological control of *C. sepium*. 
Introduction

*Calystegia sepium* (L.) R. Br. (hedge bindweed), an important weed in temperate climates all around the world (Holm *et al*., 1977), is a problem in maize, grapes and cereals. It can cause yield reductions of up to three quarters (Maillet, 1988; Oerke *et al*., 1994). *C. sepium* emerges after these crops and therefore only herbicides selective to the crops may be used for bindweed control (Weaver and Riley, 1982; Westra *et al*., 1992). Furthermore, the large amount of reserves stored in the rhizomes of *C. sepium* make it difficult to kill more than the aerial parts of the plants (Timmons, 1970).

One goal of weed control consists in hindering propagation and thereby reducing the density of the weed. Vegetative propagation by rhizomes is more important to *C. sepium* than sexual propagation by seeds (Neubauer, 1988; van Ast and van Groenendael, 1993). Allocation of biomass to stolons promotes vegetative propagation because stolons penetrate into the soil and form rhizomes towards the end of the growing season (Klimeš and Klimešová, 1994). In contrast, sexual reproduction is favoured by biomass allocation to climbing stems (Klimeš and Klimešová, 1994).

Seed derived plants invest more biomass into stolons (vegetative propagation), while plants grown from rhizomes allocate more to climbing stems (sexual propagation) (Klimeš and Klimešová, 1994). Vegetative propagation is considered responsible for the multiplication of *C. sepium* in an bindweed-infested field whereas bindweed-free fields are colonised by seeds which are more easily transmitted and remain viable for up to 30 years (Barr, 1945; Timmons, 1949).

The difficulties with chemical control led to research on biological control which concentrated on isolation and identification of promising pests and pathogens, and on the formulation of these agents (Rosenthal, 1985; Wang and Kok, 1986; Ormeno-Núñez *et al*., 1988; Heiny, 1990; Boldt and Sobhian, 1993). Among the *Stagonospora convolvuli* Dearness & House tested in our laboratories, strain LA39 was the most promising candidate as a mycoherbicide for the control of *C. sepium* (Pfirter and Défago, 1998). *S. convolvuli* strain LA39 is specific to *Convolvulaceae* and it attacks both bindweeds, *C. sepium* and *Convolvulus arvensis* L.

Environmental conditions may also be influential on the development of *C. sepium*. Thus, bindweeds produce more biomass if they grow in intraspecific shoot competition (Klimeš and Klimešová, 1994). Likewise, a larger proportion of biomass is allocated to stolons under nutrient poor conditions than under nutrient rich conditions, a
behaviour possibly elevating the chances of finding an environment rich in nutrients, as suggested by Klimeš and Klimešová (1994).

The goal of this study was to evaluate the effect of the mycoherbicide S. convolvuli strain LA39 on development, biomass allocation and propagation of C. sepium in outdoor climatic conditions with plants propagated from seed or from rhizomes and grown in soils of different nutrient status.

Material and Methods

The effects of the mycoherbicide S. convolvuli strain LA39, plant nutrition and propagation method on C. sepium plants were studied in a factorial experiment. Two levels of nutrients, two propagation methods and two levels of mycoherbicide were employed and each treatment was studied in 12 pots (i.e. 12 replications). The statistical design was completely randomised.

C. sepium was propagated as seeds or rhizomes which originated from many plants in Třeboň (Czech Republic). Seeds were collected at the beginning of April 1996 and rhizomes about 12 cm long with a single growing tip were procured at the start of the experiment. On May 9, seeds were scarified and germinated on Petri dishes. On May 20, a seedling or a rhizome was planted per pot. The plants were on terrain outside of the Institute of Botany of the Academy of Sciences of the Czech Republic in Třeboň. Throughout the experiment, the plants were watered daily with tap water and excess water flowed out through the bottom of the pots. Pots (15×15×17 cm³; 3.3 l volume) were filled with standard garden soil (350 mg/l N, 300 mg/l P₂O₅, 400 mg/l K₂O, pH 6.0) and pure washed quartz sand, mixed at a ratio of three to one for the high nutrient availability treatments, and one to three for the low nutrient availability treatments. No fertilisation was applied.

For inoculations, the fungus S. convolvuli strain LA39 was grown on V8 agar plates (10% v/v Campbell V8 juice, 30 mM CaCO₃, 1.2% w/v Oxoid agar No. 1). They were incubated either at room temperature in the daylight in Třeboň or at 20°C with continuous artificial light in Zürich. At 19 days, the spores were dislodged from the medium using sterile distilled water and the suspension was filtered through cheesecloth. In Zürich, the suspension was adjusted to 10⁹ spores/ml using a haemacytometer, filtered onto finely ground caolin clay mineral at a rate of 1 ml/g and subsequently dried overnight under the sterile hood to give a dry powder. At use, the powder was rehydrated with 10 ml/g sterile distilled water yielding a suspension of 10⁸
spores/ml. Spores produced in Třeboň were not formulated as a powder and directly adjusted to the density of $10^8$ spores/ml. The spore suspension was incorporated into an oil emulsion (0.1 ml of Tween-80, 10 ml pure rapeseed oil, 90 ml sterile distilled water) in a 1:9 (v/v) ratio, as described by Lawrie et al. (1997). On June 6, the spores produced at Třeboň were added to half the pots by spraying the spores onto the leaves until run-off (i.e. about 8 ml per pot) with a handsprayer. For the second inoculation, the spores were produced at Zürich and added to the same pots on July 23.

On August 26 and 27, from each treatment half of the plants were randomly chosen and harvested. Remaining plants were harvested on November 15 and 16. Leaves, stolons and flowers and fruits were counted for each plant. In the August harvest, the nodes of main stem and the nodes of stolons were also counted. The root system was extracted from the pot and washed thoroughly. Next, the length of the main stem, the length of all stolons combined and the length of the rhizome was measured. Disease severity was estimated from the August harvest - each leaf was visually assessed for the necrotic leaf surface (nls) according to the following scale: 0: 0% nls; 2.5: (0<nls≤ 5%); 15: (5<nls≤ 25%); 50: (25<nls≤ 75%); 85: (75<nls≤ 95%); 97.5: (95<nls≤ 100%); 100: 100% nls (leaf dead). *S. convolvuli* as well as leaf senescence contributed to nls. The main stem, the stolons, the leaves from the main stem and from the stolons, the flowers and fruits, the roots and the rhizomes were separately dried at 80°C and weighed. Rhizomes were extracted twice with 80% (v/v) aqueous ethanol for 30 min for the analysis of the water soluble carbohydrate content (WSC) and digested with H$_2$SO$_4$ for analysis of the starch content, according to the method of Fischer et al. (1997).

For statistical analysis, proportional data were arcsine transformed. The data from each harvest were subjected to ANOVA with the treatments as factors. Biomass allocation to stolon axes, climbing axes, rhizomes and reproductive structures was studied using ANCOVA with plant size as a covariable and a size-dependent allocation approach based on regression analysis, as suggested in several works (Samson and Werk, 1986; Dong and de Kroon, 1994). Plant size was expressed as the dry mass of structures responsible for acquisition of resources, i.e. roots and leaves. Data were analysed using Systat 5.05 (SPSS Inc., Evanston, IL).

**Results**

In this experiment, plants were harvested in August, when the plants had attained the maximum biomass, and in November, when the plants were overwintering. The
disease severity of plants treated with *S. convolvuli* was three times as high as for untreated plants (Figure 1). Between 18 and 32 % of the leaf surface of treated plants was necrotic depending on plant nutrition and propagation. Furthermore, the mycoherbicide defoliated *C. sepium* and reduced the number of leaves by 25 to 30% in August (Figure 2; Table 1).

![Figure 1](image_url)  
**Figure 1.** Disease severity on *C. sepium* leaves estimated in August as mean percentage of necrotic leaf surface. Treatments were nutrition (L: low; H: high); propagation (S: from seeds; R: from rhizomes) and mycoherbicide (0: not applied; 1: applied). Error bars indicate standard error.

Effects of the mycoherbicide on the development of the above-ground structures of *C. sepium* included the increase in the length of climbing stems as well as the number of nodes but only in August (Figure 2; Table 1). The mycoherbicide increased biomass allocation to stolons in August and in November and to climbing stems only in August (Figure 3; Table 2). Even though the mycoherbicide increased biomass allocation to the flowers and fruit in August (Table 2), it had no effect on the absolute biomass of these sexual reproduction structures (Figure 4; Table 3). However, the mycoherbicide reduced the biomass of the below-ground structures by 5 to 50 % (Figure 4; Table 3). The mycoherbicide increased the content of water soluble carbohydrates of the rhizomes in August, whereas in November, the mycoherbicide reduced these as well as the total content of carbohydrate reserves (Figure 5; Table 4).

The disease severity of mycoherbicide treated plants was the highest for vegetatively propagated plants grown in high nutrients (Figure 1; Table 1). Surprisingly, in low nutrients the number of leaves was independent of the extent of disease severity, while in high nutrients the number of leaves decreased with increasing disease severity (Figure 6). The mycoherbicide reduced the biomass of below-ground structures of plants propagated vegetatively more than that of seed grown plants (Figure 4; Table 3).
Overall, the effects of plant nutrition or propagation method rarely interacted significantly with those of mycoherbicide application (Tables 1-4).

Several characteristics of the vegetatively propagated plants distinguished them from seed grown plants: (i) They had longer climbing stems, reduced stolon lengths and in August they had fewer leaves (Figure 2; Table 1). (ii) They allocated more biomass to rhizomes and to flowers and fruit (Table 2). (iii) They produced more biomass in climbing stems, in below-ground structures, in flowers and fruit and for the whole plant (Figure 4; Table 3). (iv) In their rhizomes, starch content was reduced in November (Figure 5; Table 4).

Overall, high nutrients increased the biomass of most plant parts compared to low nutrients with the exception of leaves on climbing stems and, in August, of rhizomes (Figure 4; Table 3). Higher nutrients resulted in longer climbing stems and...
stolons as well as an increased number of leaves (Figure 2; Table 1). In August, higher nutrients promoted biomass allocation to stolons while they reduced biomass allocation to rhizomes (Table 2). In November, lower nutrients reduced the content of starch in rhizomes, while higher nutrients reduced the content of water soluble carbohydrates (Figure 5).

**Figure 4.** Dry mass fractions of *C. sepium* at the harvests in August and November. The treatments were nutrients (L: low; H: high), propagation (S: from seeds; R: from rhizomes) and mycoherbicide (0: not applied; 1: applied). Error bars indicate standard error.

**Figure 5.** Percent content of starch and water soluble carbohydrates (WSC) in rhizomes of *C. sepium* in August and November. Treatments were nutrition (L: low; H: high), propagation (S: from seeds; R: from rhizomes) and mycoherbicide (0: not applied; 1: applied). Error bars indicate standard error.

**Discussion**

The goal of this work was to test the usability of *S. convolvuli* as a mycoherbicide for the control of *C. sepium*. The plants were grown outside to mimic field conditions. The mycoherbicide attacked young and old plant leaves causing foliar disease and defoliation (Figure 1), while without artificial inoculation *S. convolvuli* attacks *C. sepium* only towards the end of the growing season when leaves are senescing (personal observation). However, in this work the disease severity was lower compared to our experiments in maize fields (unpublished data). Possibly, in these single pots the microclimate was too dry for the mycoherbicide, whereas in the maize field humid conditions contributed to increased disease of *C. sepium*. 
Table 1. Statistical effects of treatments on *Calystegia sepium* in August and in November according to three-way ANOVA. Treatments were nutrient level (N: low or high), propagation method (P: seed [-] or rhizome [+]) and mycoherbicide (M: untreated or *S. convolvuli* applied). Disease severity was estimated on leaves of *C. sepium* in August and arcsine transformed before testing.

<table>
<thead>
<tr>
<th>Plant characteristic</th>
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<sup>a</sup>Significance levels are indicated by the number of signs (x): P<0.1, x: P<0.05, xx: P<0.01, xxx: P<0.001, where x is +, - or * and ns: P≥0.1.

<sup>b</sup>Disease severity was estimated as percentage necrotic surface on leaves of *C. sepium* and arcsine transformed before testing.

Table 2. Statistical effects of treatments on biomass allocation in *C. sepium* in August and in November according to three-way ANCOVA, where the dry mass of leaves + roots was used as covariate. Treatments were nutrient level (N: low or high), propagation method (P: seed [-] or rhizome [+]) and mycoherbicide (M: untreated or *S. convolvuli* applied).

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<sup>a</sup>Significance levels are indicated by the number of signs (x): P<0.1, x: P<0.05, xx: P<0.01, xxx: P<0.001, where x is +, - or * and ns: P≥0.1.
Table 3. Statistical effects of treatments on dry mass of *C. sepium* in August and in November according to three-way ANOVA. Treatments were nutrient level (N: low or high), propagation method (P: seed [-] or rhizome [+] ) and mycoherbicide (M: untreated or *S. convolvuli* applied).

<table>
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<td>Climbing stem: leaves</td>
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<td>Climbing stem: axes and leaves</td>
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<td>Axes of climbing stems and stolons</td>
<td>+++</td>
</tr>
<tr>
<td>Leaves of climbing stems and stolons</td>
<td>+++</td>
</tr>
<tr>
<td>Total of climbing stems and stolons</td>
<td>+++</td>
</tr>
<tr>
<td>Rhizomes</td>
<td>ns</td>
</tr>
<tr>
<td>Roots</td>
<td>+++</td>
</tr>
<tr>
<td>Total of rhizomes and roots</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Total biomass</strong></td>
<td>+++</td>
</tr>
<tr>
<td><strong>November</strong></td>
<td></td>
</tr>
<tr>
<td>Climbing stems: axes</td>
<td>+++</td>
</tr>
<tr>
<td>Climbing stem: leaves</td>
<td>ns</td>
</tr>
<tr>
<td>Climbing stem: axes and leaves</td>
<td>+++</td>
</tr>
<tr>
<td>Stolons: axes</td>
<td>+++</td>
</tr>
<tr>
<td>Stolons: leaves</td>
<td>+++</td>
</tr>
<tr>
<td>Stolons: axes and leaves</td>
<td>+++</td>
</tr>
<tr>
<td>Flowers and fruits</td>
<td>+++</td>
</tr>
<tr>
<td>Axes of climbing stems and stolons</td>
<td>+++</td>
</tr>
<tr>
<td>Leaves of climbing stems and stolons</td>
<td>+++</td>
</tr>
<tr>
<td>Total of climbing stems and stolons</td>
<td>+++</td>
</tr>
<tr>
<td>Rhizomes</td>
<td>+</td>
</tr>
<tr>
<td>Roots</td>
<td>+++</td>
</tr>
<tr>
<td>Total of rhizomes and roots</td>
<td>(+)</td>
</tr>
<tr>
<td><strong>Total biomass</strong></td>
<td>+++</td>
</tr>
</tbody>
</table>

<sup>a</sup> + denotes an increase of mean value with the level of a factor, − a decrease and * significant interactions.

<sup>b</sup> Significance levels are indicated by the number of signs, (x): ns: P>0.1, −: P<0.05, ++: P<0.01, +++: P<0.001, where x is +, − or * and ns: P≥0.1.

Table 4. Statistical effects of treatments on carbohydrate reserves in rhizomes of *C. sepium* in August and in November according to three-way ANOVA. Treatments were nutrient level (N: low or high), propagation method (P: seed [-] or rhizome [+] ) and mycoherbicide (M: untreated or *S. convolvuli* applied). *Calystegia sepium* in August and November. The data was arcsine transformed before testing by three-way ANOVA.

<table>
<thead>
<tr>
<th>Carbohydrate fraction</th>
<th>Significance of factors&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td><strong>August</strong></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>ns</td>
</tr>
<tr>
<td>Water soluble carbohydrates</td>
<td>(+)</td>
</tr>
<tr>
<td>Total non-structural carbohydrates</td>
<td>ns</td>
</tr>
<tr>
<td><strong>November</strong></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>++</td>
</tr>
<tr>
<td>Water soluble carbohydrates</td>
<td>−</td>
</tr>
<tr>
<td>Total non-structural carbohydrates</td>
<td>(+)</td>
</tr>
</tbody>
</table>

<sup>a</sup> + denotes an increase of mean value with the level of a factor, − a decrease and * significant interactions.

<sup>b</sup> Significance levels are indicated by the number of signs, (x): P<0.1, ±: P<0.05, ++: P<0.01, +++: P<0.001, where x is +, − or * and ns: P≥0.1.
The effects of the mycoherbicide on the above-ground structures of *C. sepium* included the reduction of the leaf number and the necrosis of the leaf surface. Nevertheless, *C. sepium* was able to keep at least 50 leaves (Figures 2), the mycoherbicide had no effect on the above-ground biomass (Table 3), and it caused increased biomass allocation to the above-ground structures (Table 2). The mycoherbicide reduced below-ground structures including the biomass of the rhizomes and their content of carbohydrate reserves (Tables 3 and 4). Apparently, *C. sepium* compensated the impact of the mycoherbicide on the above-ground structures with resources from the below-ground structures. Rhizomes are the only plant structures surviving winter and ensuring the vegetative propagation of *C. sepium*. Their reduction may delay spring growth of *C. sepium* and lower the number of shoots produced, as suggested by van Ast and van Groenendael (1993) and Buhler et al. (1994).

In the current work, interactions between mycoherbicide and propagation or nutrition were either non-significant or very weak for the parameters investigated, suggesting that the mycoherbicide may control *C. sepium* in a broad range of environmental conditions (Tables 1 and 3). However, plants grown with nutrient rich soil displayed a more severe disease development compared to those grown in nutrient poor soil (Table 1). This finding is encouraging because today nutrient concentration is usually high in agricultural fields where bindweed control is necessary. According to Huber and Watson (1974), nutrient concentration may increase or decrease disease development in different host-pathogen systems. However these authors observed, that disease severity increases with increasing amounts of nitrogen for many plant pathogen interactions. Similarly, Fagan (1985) showed that nitrogen fertiliser increased disease severity of *Drechslera* leaf spot on coconut seedlings. Yamazaki and Hoshina (1995) showed that increased calcium nutrition increased the resistance of tomato seedlings to bacterial wilt.

In this work, the method used for plant propagation influenced plant development. Thus, seed derived *C. sepium* developed fewer flowers and produced longer stolons compared to rhizome derived plants (Table 1). New rhizomes were grown by vegetatively and by sexually propagated plants, but only the former flowered (Table 1 and 3). This finding is consistent with the observation by Klimeš and Klimešová (1994), that seed derived plants invest more biomass in vegetative compared to sexual propagation structures. Furthermore, they confirm that vegetative propagation is more important to *C. sepium* than sexual reproduction as suggested by Neubauer (1988) and van Ast and van Groenendael (1993). In the current work, nutrient rich soil increased the dry mass of most parts of *C. sepium* compared to nutrient poor soil. This was in contrast to the study of Klimeš and Klimešová (1994), where nutrient poor soil did not increase biomass allocation to stolons. However, environmental conditions and
absolute nutrient content differed widely between the work of Klimeš and Klimešová (1994) and the current study.

Overall, the findings of this work confirm the usability of *Stagonospora* spp. for the biological control of *C. sepium*, as it has been suggested by Hasan et al., (1992) and by Pfirter and Défago (1998). In the environmental conditions applied here, the mycoherbicide reduced below-ground biomass and reserve carbohydrate storage in rhizomes of *C. sepium*. Continuous reduction of the biomass stored in the rhizomes may reduce vegetative propagation of the weed, and consequently repeated application of *S. convolvuli* might help to control *C. sepium* in the long term. Possibly, bindweed control could be achieved in shorter time, if other biological control methods were used simultaneously. Therefore, future work should investigate the effectiveness of combinations of *S. convolvuli* with other methods of biological weed control including living green cover plants, as suggested by Pfirter et al. (1997), or specific gall mites, as suggested by Rosenthal and Platts (1990).

**Acknowledgements**

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**Literature cited**


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Chapter IV.

Combination of mycoherbicide and plant competitors.
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Part 1 Combination of mycoherbicide *Stagonospora convolvuli* strain LA39 and the green cover plant *Lolium multiflorum* for control of bindweeds (*Calystegia sepium* and *Convolvulus arvensis*) in the greenhouse

**Summary**

The two bindweeds *Calystegia sepium* (L.) R. Br. and *Convolvulus arvensis* (L.) cause important yield losses in many crops world-wide. In this study, we investigated the combination of the green cover plant *Lolium multiflorum* Lam. and the bindweed fungal pathogen *Stagonospora convolvuli* Dearness & House strain LA39 for the biological control of bindweeds in the greenhouse. Very efficient control of *C. arvensis* was obtained with *S. convolvuli* strain LA39. It reduced biomass, stem length and number of leaves of *C. arvensis* regardless of whether *L. multiflorum* was present or not. On *C. sepium*, disease severity caused by *S. convolvuli* was lower than on *C. arvensis*. The combination of *L. multiflorum* and *S. convolvuli* reduced the biomass, stem length and number of leaves of *C. sepium* more than each factor alone. Combining the fungal bindweed pathogen *S. convolvuli* and the green cover plant *L. multiflorum* is promising for effective biological control of bindweeds in integrated weed management systems.
Introduction

Field and hedge bindweeds (Convolvulus arvensis L. and Calystegia sepium (L.) R. Br., respectively) are present in all parts of the world with a temperate climate. They are important weeds in Europe and the Middle East (Holm et al., 1977), and in many agricultural areas of the central and southern USA (Weaver and Riley, 1982; Rosenthal, 1985). Bindweeds compete with crops for water, nutrients, space and light (Frazier, 1943; Stahler, 1948). In addition, they may strangle crops, delay ripening and hinder harvesting procedures (Maw, 1984). Yield loss can be as high as 30% for barley, 40% for wheat, 70% for maize and 80% for sorghum (Maillet, 1988; Oerke et al., 1994). Adequate control of bindweeds is required to ensure economic yield.

Dissemination of bindweeds is facilitated by the fact that seeds can remain viable for up to 50 years (Timmons, 1949). Once a field is colonised, the population dynamics of bindweeds depends mainly on their ability to regrow from rhizomes. Optimum regrowth of C. sepium was observed from segments of rhizome as small as two nodes in length (van Ast and van Groenendael, 1993).

Colonisation of bindweed-free fields can be hindered by the use of composts and manure which are free of bindweed seed and rhizome (Koch and Hurle, 1978). In fields already colonised by bindweeds, minimising tillage limits cutting and redistribution of rhizome segments to other parts of the field. On the other hand, cutting the shoots of bindweeds repeatedly exhausts the plant in the long term (Timmons, 1970), although it fails to deplete the amounts of reserves in the rhizomes in the short term (Barr, 1940). The development of bindweeds starts in phase with that of many crop plants, which limits the range of chemical herbicides available for use, e.g. 2-4-D, Dicamba and glyphosate (Westra et al., 1992). Furthermore, the deep-rooted bindweeds can escape chemical control.

Difficulties encountered with these traditional methods of bindweed control have stimulated the search for alternative, effective strategies against these weeds. Biological control has been proposed using specific fungal pathogens such as Phomopsis convolvulus Ormeno (Ormeno-Nuñez et al., 1988) or Phoma proboscis Heiny (Heiny, 1990), or herbivorous insects like Megacerus discoidus (Say) (Wang and Kok, 1986) or Aceria malherbae Nuzzaci (Boldt and Sobhian, 1993). However, only partial control has been achieved using these agents.

Green covers help to suppress many weed species, thereby enabling a reduction in herbicide usage (Hartwig, 1977). Indeed, Pfrirter et al. (1997) observed that growth of
bindweeds in maize fields was reduced in presence of a green cover (grass-Trifolium mixture). However, bindweeds were not controlled as well as other weeds by green covers in these experiments.

Since neither a green cover nor the use of fungal pathogens alone are sufficient to achieve effective control of bindweeds, a combination of these two approaches was investigated in this study. Experiments made use of the fungal pathogen Stagonospora convolvuli Dearness & House strain LA39 which has demonstrated potential as mycoherbicide for biological control of bindweed (Pfirter and Défago, 1998). Unlike other fungal pathogens of bindweeds, LA39 is pathogenic both to C. arvensis and C. sepium and this wider range of target bindweeds may be useful where infestation involves more than one bindweed. Italian ryegrass (Lolium multiflorum Lam.), widely used in green covers for maize cultivation in Switzerland (Garibay et al., 1997), was selected as the green cover plant.

Materials and methods

Plants

Seeds of L. multiflorum Lam. cv. Ellire were obtained from the Swiss Federal Research Station for Agroecology and Agriculture (FAL, Reckenholz, Switzerland) and were not surface disinfected prior to sowing. Seeds of C. arvensis (MT Valley Seed Service, Fresno, CA) and those of C. sepium (collected in Zürich city) were scarified by stirring them in 95% sulphuric acid for one hour and surface-disinfected by stirring them successively in 5% sodium hypochlorite (15 min) and 10% hydrogen peroxide (15 min). Seeds were then germinated on 0.85% water agar in the dark for 2 days at 24°C before planting.

Inoculum preparation

Spores of the fungus S. convolvuli strain LA39 from stock cultures stored at 3°C on V8 agar slants (10% v/v Campbell V8 juice, 30 mM CaCO₃, 1.2% w/v Oxoid agar No. 1) were streaked onto V8 plates and incubated at 20°C under continuous white fluorescent light. After 2 weeks, the spores were collected using sterile distilled water, the suspension filtered through cheesecloth and adjusted to contain 10⁸ spores/ml using a haemacytometer. Spores were formulated in an oil emulsion (0.1 ml of Tween 80, 10 ml pure rapeseed oil, 90 ml sterile distilled water) as described by Lawrie et al. (1997).
The spore suspension was added to the oil emulsion in a 1:9 (v/v) ratio (final density $10^7$ spores/ml).

**Experimental design**

The effects of *S. convolvuli* strain LA39 and of competition with the green cover *L. multiflorum* on the bindweeds *C. arvensis* and *C. sepium* were studied in a factorial experiment. Plastic pots (20 cm Ø, 20 cm depth) were filled with 4.8 l sandy loam soil from Eschikon (2.9 % organic matter content; Wüthrich and Défago, 1991). The green cover plant *L. multiflorum* was sown at the rate of about 30 (*C. sepium*) or 20 (*C. arvensis*) seeds per pot. One week after sowing, *L. multiflorum* seedlings were thinned to 20 plants (*C. sepium*) or 10 plants (*C. arvensis*) in each pot. One week later, two seedlings of *C. sepium* or *C. arvensis* were planted per pot. Two weeks after planting the bindweeds, the mycoherbicide was applied to half the pots by spraying the spores, using an aerosol atomiser, until run-off from the leaves took place (i.e. about 7 ml per pot). Immediately after spraying, the plants were placed inside a wet plastic bag for 24 h to maintain a high relative humidity and ensure homogenous rates of fungal infection.

All pots were placed into a controlled greenhouse with a relative humidity of 70%, 16 h of light (22°C) and 8 h of dark (17°C). Artificial light of about 70 μmol/m²/s photosynthetic photon flux density (PPFD) was supplemented when daylight intensity fell below 195 μmol/m²/s PPFD. The soil water content was adjusted to 15% w/w (i.e. 30% saturation of soil porosity) with tap water three times per week.

**Data assessment**

The plants in each treatment were harvested two weeks after inoculation. Immediately before harvest, each pot was placed in turn at a same location in the greenhouse where light intensity was measured using a quantometer (Li-cor, Li-189; LI-COR Inc., Lincoln, USA) above the plant canopy and at soil surface level, in the centre of the pot.

At harvest, the shoots of *L. multiflorum* were cut and weighed. Leaves and stems of bindweeds were weighed separately. Carefully, bindweed roots were extracted from the soil, washed, blotted dry and weighed. To assess fungal disease severity, necrotic leaf surface (NLS) was rated visually for each leaf using the following scale: 0 = 0% NLS; 2.5 = (1% to 5%) NLS; 15 = (6% to 25%) NLS; 50 = (26% to 75%) NLS; 85 = (76% to 95%) NLS; 97.5 = (96% to 99%) NLS; 100 = 100% NLS (leaf dead).
Data analysis

Each combination of treatments (mycoherbicide × green cover) was replicated six times for each bindweed and the statistical design was completely randomised. The data of average NLS per plant were transformed to the arcsine of the square root prior to analysis. Data were subjected to analysis of variance and treatments were compared with Fisher’s LSD test, using Systat 5.05 (SPSS Inc., Evanston, USA). All analyses were performed at \( P=0.05 \) level. The experiments were repeated and statistical analyses performed within each repeated experiment. The data from each experiment alone, as well as after pooling all data from both repeated experiments, yielded the same results. Means and standard errors shown were obtained after pooling all data.

Results

Biomass and light intercepted by *L. multiflorum*

The average biomasses per pot of *L. multiflorum* in *C. arvensis* and in *C. sepium* pots were similar (Table 1). Light intensity at the soil surface was also similar in the *C. arvensis* and *C. sepium* pots (Table 1). In both experiments, the amount of light intercepted by the green cover plant was about 31%. No statistical differences in light intensity or *L. multiflorum* biomasses were observed between the treatments.

<table>
<thead>
<tr>
<th>Bindweed</th>
<th>Light intensity (( \mu \text{mol/m}^2/\text{s PPFD} \pm \text{SE} ))</th>
<th>Fresh weight of <em>L. multiflorum</em> (g/pot ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. arvensis</em></td>
<td>50.6 ± 4.4</td>
<td>20.5 ± 2.8</td>
</tr>
<tr>
<td><em>C. sepium</em></td>
<td>49.0 ± 3.5</td>
<td>23.2 ± 2.9</td>
</tr>
</tbody>
</table>

*C. arvensis* experiment

*S. convolvuli* strain LA39 caused severe necrosis of almost 100% of the leaf surface of *C. arvensis* whether *L. multiflorum* was present or not (Figure 1), whereas uninoculated plants displayed no disease symptom. The plants treated with *S. convolvuli* strain LA39 produced significantly fewer leaves than the control plants (Table 2). Total leaf, stem and root biomasses were significantly reduced in plants sprayed with the
fungus. The presence of *L. multiflorum* plants did not lead to a reduction in the number of leaves, length of stems and biomasses of leaves, stems and roots (Table 2).

![Figure 1](image.png)

**Figure 1.** Percentage necrotic leaf surface of *C. arvensis* and *C. sepium* caused by *S. convolvuli* strain LA39, in presence or absence of the green cover plant *L. multiflorum*. Means followed by the same letter were not significantly different. Error bars represent standard error.

### C. sepium experiment

No disease developed on *C. sepium* plants not sprayed with the fungus, as observed in the *C. arvensis* experiment. Plants not associated with *L. multiflorum* and sprayed with the fungus developed less disease than plants grown with the green cover plant (Figure 1). In absence of *L. multiflorum*, the fungal pathogen reduced significantly the number of leaves per plant, length of stems and total leaf, stem and root biomasses (Figure 2). Similar reductions were obtained when non-inoculated *C. sepium* plants were grown with *L. multiflorum*. The combination of green cover and fungal inoculation resulted in an even lower number of leaves per plant, length of stems and total leaf, stem and root biomasses.

**Table 2.** Effects of the green cover and of the mycoherbicide on biomass and length of stems, biomass and number of leaves and biomass of roots per plant of *C. arvensis*. Means are followed by the standard error.

<table>
<thead>
<tr>
<th>Green cover</th>
<th>Mycoherbicide</th>
<th>Stem Biomass (g)</th>
<th>Stem Length (cm)</th>
<th>Leaf Biomass (g)</th>
<th>Leaf Number</th>
<th>Root Biomass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>Not applied</td>
<td>0.166 ± 0.013</td>
<td>39.4 ± 2.0</td>
<td>0.311 ± 0.034</td>
<td>12.4 ± 0.5</td>
<td>0.214 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>Applied</td>
<td>0.037 ± 0.012</td>
<td>13.3 ± 1.9</td>
<td>0.017 ± 0.006</td>
<td>4.8 ± 0.7</td>
<td>0.019 ± 0.006</td>
</tr>
<tr>
<td>Present</td>
<td>Not applied</td>
<td>0.170 ± 0.013</td>
<td>42.0 ± 2.9</td>
<td>0.304 ± 0.031</td>
<td>11.5 ± 0.9</td>
<td>0.196 ± 0.024</td>
</tr>
<tr>
<td></td>
<td>Applied</td>
<td>0.021 ± 0.007</td>
<td>12.2 ± 1.2</td>
<td>0.017 ± 0.005</td>
<td>5.4 ± 0.5</td>
<td>0.016 ± 0.004</td>
</tr>
</tbody>
</table>
Figure 2. Effects of the fungal pathogen *S. convolvuli* strain LA39 and of competition with *L. multiflorum* on biomass and total number of leaves, biomass and length of stems and biomass of roots per plant of *C. sepium*. Means followed by the same letter are not significantly different. Error bars represent standard error.

### Discussion

In Switzerland, the two bindweeds *C. arvensis* and *C. sepium* can be found in the same field plot, although *C. arvensis* is often prevalent in drier, warmer areas (e.g. Valais county) and *C. sepium* may dominate in cooler parts of the country like the midlands. *C. arvensis* grows slower than *C. sepium*, as was confirmed in the current work (Table 2, Figure 2). Therefore, competition experiments between bindweeds and the green cover plant were performed at ratios of 2:10 (*C. arvensis*: *L. multiflorum*) or 2:20 (*C. sepium*: *L. multiflorum*).

Competition for light is often considered as the main factor in the interaction between bindweeds and other plants (Stahler, 1948; Dall’Armellina and Zimdahl, 1988). Indeed, light availability was more influential on growth of *C. sepium* than was competition with the roots of *Trifolium pratense* L. in greenhouse experiments (our unpublished data). In this study, the green cover *L. multiflorum* produced similar biomasses and caused a similar reduction in light availability, regardless of whether 10 or 20 plants were used per pot (Table 1). Similarly, Massion and Lindow (1986) observed that reductions of aboveground biomass and rhizome length of *Sorghum halepense* (L.) Pers. were dependent on the presence of *Medicago sativa* L. rather than on the density of *M. sativa*. However, in our study only *C. sepium* was affected by the presence of *L. multiflorum* regarding leaf and stem parameters (Table 2, Figure 2),
suggesting that *C. sepium* is more sensitive to a reduction in light availability than *C. arvensis*.

In the current work, *S. convolvuli* strain LA39 effectively infected both bindweeds, although disease severity was higher on *C. arvensis* than on *C. sepium* (Figure 1). Likewise, the percentage reduction in leaf number, stem length and biomasses of stems and leaves caused by the fungus were higher on *C. arvensis* than on *C. sepium* (Table 2, Figure 2). The effort to extract the roots of the bindweeds from the pots was hindered as the roots tended to break easily, even in the treatments without green cover. However, the root biomass data correlated well with stem or leaf biomass for *C. arvensis* (r>0.94*) and *C. sepium* (r>0.92*), suggesting that the fungal pathogen affected the development of the entire plant.

The extent of disease afforded by the inoculation with *S. convolvuli* strain LA39 alone was sufficient to control *C. arvensis* (Table 2, Figure 1) and addition of the green cover plant *L. multiflorum* did not hinder this under the experimental conditions tested. In contrast, this strain of *S. convolvuli*, which had been isolated from *C. arvensis*, was less effective on *C. sepium* (Figure 1). However, the latter bindweed was affected significantly by the presence of *L. multiflorum*, and combining fungus and green cover resulted in improved control of *C. sepium* compared with the treatments in which *S. convolvuli* or *L. multiflorum* were used alone (Figure 2). Interestingly, the severity of the fungal disease was enhanced when *C. sepium* was grown in competition with *L. multiflorum*. Pennypacker et al. (1994) reported that a reduction of photosynthetic photon flux density caused the loss of resistance of *M. sativa* against *Verticillium albo-atrum* Rke. et Berth. These authors suggested that a reduction in carbon assimilation caused by reduced light might cause a reduction in synthesis of defence compounds leading to a breakdown of resistance. In our study however, the quantitative effects on the leaf and stem parameters studied were only additive, perhaps because the full potential of these biocontrol measures was achieved in the conditions of the experiment. Similar results were described for skeleton weed grown in competition with subterranean clover and infected with *Puccinia chondrillina* Bubak & Syd. (Groves and Williams, 1975). Also *S. halepense* reacted similarly to stresses caused by plant competition and infection with *Sphacelotheca holci* Jack. (Massion and Lindow, 1986).

Overall, this study shows that the potential mycoherbicide *S. convolvuli* strain LA39 and the green cover plant *L. multiflorum* are compatible for biocontrol of bindweeds. For one of the two bindweeds (*C. sepium*), presence of *L. multiflorum* even promoted fungal infection. Combining the mycoherbicide and the green cover appears to be a promising strategy for effective control of bindweeds in the field, in line with current concerns regarding the development of integrated weed/pest management systems.
Acknowledgements

This work was supported in part by the COST Action 816. We are grateful to N. Boll for discussions and technical assistance, M. Hildmann for technical assistance, and Y. Moënne-Loccoz from our laboratory for discussions and help with writing the manuscript.

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Groves, R.H. and Williams, J.D. 1975. Growth of skeleton weed (Chondrilla juncea L.) as affected by growth of subterranean clover (Trifolium subterraneum L.) and infection by Puccinia chondrillina Bubak & Syd. Australian Journal of Agricultural Research 26, 975-983.


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Part 2 Biological control of hedge bindweed (*Calystegia sepium*) with mycoherbicide *Stagonospora convolvolii* strain LA39 in combination with competition from red clover (*Trifolium pratense*) in the greenhouse and the field.

**Summary**

The so called 'maize meadow' is a maize cropping system with a living green ground cover. This ground cover controls soil erosion and also suppresses many weeds. However, *Calystegia sepium* is able to escape control. In this work, the potential for biological control of *C. sepium* was assessed using the bindweed pathogen *Stagonospora convolvolii* strain LA39 as a mycoherbicide in combination with competition by the green cover plant *Trifolium pratense*. In a greenhouse experiment, the competition from shoots of *T. pratense* caused a strong reduction of the biomass of *C. sepium* and combined competition from shoots and roots had the same effect. In a second, factorial greenhouse experiment, competition by *T. pratense* again reduced *C. sepium* biomass. However, *S. convolvolii* did not influence the number of leaves or the biomass of *C. sepium* in the greenhouse even though severe necrosis was observed on inoculated bindweed leaves. In contrast, in a two year field study, *S. convolvolii* caused severe disease and a strong reduction of *C. sepium* ground coverage in maize. Underseeding with *T. pratense* had no effect on disease severity, but *T. pratense* reduced ground coverage at two of eight samplings in the first year. In conclusion, this mycoherbicide is useful in the field and a competitive green cover might help for the biological control of *C. sepium*.

This chapter is submitted for publication: D. Guntli, S. Burgos, I. Kump, M. Heeb, H.A. Pfrirter and G. Défago. Biological control of hedge bindweed (*Calystegia sepium*) with mycoherbicide *Stagonospora convolvolii* strain LA39 in combination with competition from red clover (*Trifolium pratense*).
Hedge bindweed (*Calystegia sepium* (L.) R. Br.) is a weed often associated with maize cultivation (Holm *et al.*, 1977; Oerke *et al.*, 1994). It is a perennial plant with climbing stems, fast growing stolons, a large rhizome system and a facility to produce abundant long living seeds (Timmons, 1949; Klimeš and Klimešová, 1994). In Switzerland, hedge bindweed is more prevalent in maize than the closely related field bindweed (*Convolvulus arvensis* L.). Both species compete for water, nutrients and light with the crops (Frazier, 1943; Stahler, 1948) and cause significant yield reductions (Maw, 1984; Maillet, 1988). The bindweed emergence occurs in phase with and more often after that of many crop plants, including maize. Since postemergence herbicides cause damage to the crops and preemergence herbicide treatments do not accomplish bindweed control, the chemical control of bindweeds is difficult. Difficulties also arise because of the large amounts of reserves stored in the rhizomes (Barr, 1940) which are often not affected by herbicides (Timmons, 1970; Weaver and Riley, 1982; Westra *et al.*, 1992). Furthermore, biotypes of bindweeds may be tolerant to various amounts of herbicides (DeGennaro and Weller, 1984; Whitworth and Muzik, 1967).

In the search for alternatives, a number of biological control agents have been investigated (Rosenthal, 1985; Wang and Kok, 1986; Ormeno-Nuñez *et al.*, 1988; Heiny, 1990; Boldt and Sohbian, 1993). Several *Stagonospora* spp. were isolated which proved promising as mycoherbicides for the biological control of bindweeds (Pfirter *et al.*, 1997; Pfirter and Défago, 1998). However, only partial control of the bindweeds was achieved with these biological control agents. This encouraged the search for additional strategies which could be combined with biocontrol agents to support or complement the control.

In Switzerland, in maize one such strategy for weed control is the use of living green covers (Pfirter *et al.*, 1997). Such maize production systems harbour several advantages compared to conventional fields, that are kept free of all vegetation except the crop with the help of herbicides (Garibay *et al.*, 1997; Burgos and Talbert, 1996; Hall and Hartwig, 1990). In comparison to conventional maize fields, these authors observed reduced erosion, improved accessibility, and a reduced need for herbicide input as well as a substantial reduction of the annual and perennial weeds which normally accompany maize. Unfortunately, *C. sepium* remains a dominant weed in maize cropped with living green covers (Pfirter *et al.*, 1997).
The selectivity of biological control agents make them unsuitable for normal, diverse weed communities as they occur in conventional maize production. However, they can be useful in integrated weed management systems, where one dominant weed species must be controlled (Müller-Schärer and Scheepens, 1997). The use of living green covers can create a situation where most weed species are suppressed and remaining weeds could be controlled using a biological control agent (Pfirter et al., 1997). One green cover plant, known to be useful, is the red clover, *Trifolium pratense* L. (Garibay et al., 1997). The objective of the current work was to study the effects of competition of a green cover plant in combination with a fungal biocontrol agent on *C. sepium*. *T. pratense* cv. Rutinova was chosen as the green cover plant and *S. convolvuli* strain LA39 as fungal biocontrol agent. Effects of *T. pratense* on *C. sepium* alone and compatibility of *T. pratense* and *S. convolvuli* strain LA39 were assessed in the greenhouse. Field performance of these agents was tested in a maize field naturally infested with bindweed.

**Materials and methods**

**Plants and soil**

Seeds of maize (*Zea mays* L. var LG2253) and *T. pratense* cv. Rutinova were obtained from the seeds department of the Swiss Federal Research Station for Agroecology and Agriculture (FAL, Reckenholz, Switzerland). For the greenhouse experiments, *T. pratense* was sown into a seedbed and transplanted into the experimental pots at the three leaves stage. Seeds of *C. sepium* (collected in parks located in the city of Zürich) were scarified by stirring them in 95% sulphuric acid for one hour and surface-disinfected by stirring them successively in 5% sodium hypochlorite (15 min) and 10% hydrogen peroxide (15 min). They were then germinated on 0.85% water agar for three days in the dark at 24°C before planting. For the field experiments, *Z. mays* and *T. pratense* were sown directly into the chosen field site and an established, natural population of *C. sepium* was used.

The field experiment was performed in Eschikon, Switzerland, at a site with a cambisol (texture: loam, 15% clay, 42% silt, 43% sand, 3.5% organic matter content, pH 7.0). This same soil was used in all greenhouse experiments.
Inoculum preparation

For the greenhouse experiments and the 1996 field experiment, spores of S. convolvuli strain LA39 (Pfirter and Défago, 1998) were grown on V8 agar plates (10% v/v Campbell V8 juice, 30 mM CaCO₃, 1.2% w/v Oxoid agar No. 1). For the 1997 field experiment, however, sterilised cracked wheat was used. This had been autoclaved (121°C, 20 min) twice with a 24 h interval. All cultures were incubated at 20°C under continuous white fluorescent light. At two weeks, the spores were dislodged from the media collected using sterile distilled water, and the suspension was filtered through cheesecloth and adjusted to the desired density using a haemacytometer. The spore suspension was incorporated into an oil emulsion (0.1 ml Tween-80, 10 ml pure rape seed oil, 90 ml sterile distilled water) in a 1:9 (v/v) ratio as described by Lawrie et al. (1997).

Influence of T. pratense on C. sepium in the greenhouse

Plastic pots divided into four compartments with PVC separators (37 cm x 27 cm x 32 cm) were filled with 32 dm³ soil (Figure 1). Three levels of competition (none, competition from shoots or competition from shoots and roots) of T. pratense on C. sepium were created in these pots (Figure 1). The competition by shoots was produced by T. pratense planted at the ratio of 2:1 of T. pratense to C. sepium and the competition by roots a ratio of 1:1.

Competition level:

<table>
<thead>
<tr>
<th>None</th>
<th>Shoots</th>
<th>Shoots and Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>🌾 C. sepium</td>
<td>🌾 T. pratense</td>
<td>🌾 C. sepium harvested</td>
</tr>
</tbody>
</table>

*Figure 1. Experimental setup used to study competition between T. pratense and C. sepium. C. sepium was exposed to three levels of competition from T. pratense: none, competition from shoots or competition from shoots and roots. Each pot (thick lines) comprised four compartments separated by PVC sheets (thin lines). In each pot, the C. sepium plant marked with a circle was evaluated at harvest.*

In the greenhouse, daylight was maintained at a minimum of 390 μmol·m⁻²·s⁻¹ photosynthetic photon flux density (PPFD) with artificial light for 16 h followed by a 8 h dark period. Temperatures were kept between 10°C and 25°C and relative humidity
between 60% and 90%. Three times per day, 200 ml tap water was added to the pots (i.e. soil water content about 15% w/w, 30% saturation soil porosity). After three weeks, fertiliser (Wuxal G, Dr. R. Maag AG, Dielsdorf, Switzerland) corresponding to 27 kg N/ha was added to the pots. The height of the *T. pratense* canopy was kept at 20 cm above soil surface level by cutting. Two months after planting *T. pratense*, *C. sepium* seedlings were added. Four weeks later, the light intensity in the *T. pratense* canopy was measured in each pot with a quantometer (Li-cor, Li-189, LI-COR Inc., Lincoln, USA) every two hours (i.e. 8, 10, 12, 14, 18, 20) during a sunny day at the soil surface level. The percentage light interception was calculated relative to the average light intensity at soil surface level in the treatments with no competition at each sampling time. One *C. sepium* plant in the centre of each pot (Figure 1) was harvested four weeks after planting and the dry biomasses of roots, stems and leaves was determined after drying the plant material at 80°C for 48 h.

Each treatment (competition level) was studied in six pots (i.e. six replications). The statistical design was randomised. Percentage data were transformed to the arcsine of the square root prior to analysis. Data were subjected to analysis of variance and treatments were compared with Bonferroni’s protected test. Systat 5.05 (SPSS Inc., Evanston, USA) was used and all analyses were performed at *P*=0.05. The experiment was repeated once. Statistical analysis within each repeated experiment as well as after pooling all data from both experiments, yielded the same results. Means and standard errors shown in this report were obtained from the first experiment.

**Influence of S. convolvuli strain LA39 and T. pratense on C. sepium in the greenhouse**

Plastic pots (20 cm Ø, 20 cm depth) were filled with 4.8 dm³ soil. Greenhouse conditions were set as above and twice per day 50 ml tap water was added to the pots. Two *T. pratense* per pot were planted into half of the pots and one week later one seedling of *C. sepium* was added to each pot. Three weeks after planting *C. sepium*, the mycoherbicide was added to half the pots by spraying the spores with an aerosol atomizer until run-off from the leaves took place (i.e. $10^7$ spores/ml at about 7 ml per pot). Immediately after spraying, the plants were covered for 24 h with a wet plastic bag to maintain a high relative humidity and ensure homogenous rates of fungal infection. The plants were treated twice with the a 0.04% v/v aqueous solution of the acaricide Apollo SC (Dr. R. Maag AG; containing 42% w/v clofentezine [systematic name: 3,6-Bis(2-chlorophenyl)-1,2,4,5-tetrazine] as active ingredient) to control mites and once with a 0.02% v/v aqueous solution of the insecticide Karate (Dr. R. Maag AG; containing 5.5% w/v cyathrin [systematic name: 3-(2-Chloro-3,3,3-trifluoro-1-
(propenyl)-2,2-dimethylcyclopropanecarboxylic acid cyano(3-phenoxypyphenyl)methyl ester] as active ingredient) to control aphids. These insecticides were applied according to the instructions of the manufacturer. At inoculation and 3, 6, and 19 days afterwards, the number of leaves for each *C. sepium* plant was counted and disease severity was estimated: each inoculated leaf was visually assessed for the necrotic leaf surface (nls) using the following scale: 0: 0% nls; 2.5: (0%<nls≤5%); 15: (5%<nls≤25%); 50: (25%<nls≤75%); 87.5: (75%<nls<100%); 100: 100% nls (leaf dead). At 19 days after inoculation, the shoot of each plant was cut and the plant roots were extracted from the soil. Dryweights of roots, leaves and stems were determined after drying the plant parts for 48 h at 80°C.

Each combination of treatments (mycoherbicide × green cover plant) was studied in eight pots (i.e. eight replications). The experiment was repeated once. Data were analysed statistically as described above.

**Influence of mycoherbicide *S. convolvuli* strain LA39 and a green cover *T. pratense* for biological control of *C. sepium* in the field**

In 1996, the effects of mycoherbicide *S. convolvuli* strain LA39 and of green cover *T. pratense* were studied in a factorial randomised complete block design with 4 blocks and one plot per treatment per block. On May 10, a field in Eschikon was ploughed and twice harrowed. On May 15, the 16 plots of 3 m × 4 m (12 m²) were sown with *Z. mays* with a constant row distance of 75 cm and 14 cm distance between plants within a row. On May 24, the plots were fertilised with 50-45-50 kg/ha N-P-K. On June 6, plots were handweeded and the *T. pratense* was sown in half of the plots on 30 cm bands between the maize rows. Missing maize plants were resown up to the 4 leaves stage of maize. On June 18, half of the plots were inoculated with *S. convolvuli* strain LA39 at a density of 5 × 10⁶ spores/ml at a rate of 600 l/ha (i.e. 3 × 10¹² spores/ha) using a backpack sprayer (M125, Birchmeier AG, Switzerland) with a 1.5 m sprayboom equipped with 3 flatfan nozzles (Teejet 800067, Spraying Systems, Wheaton, IL). The plots were inoculated a second time on July 3. The control plots were treated with benomyl [systematic name: 1-(butylcarbamoyl)-2-benzimidazolecarbamic acid methyl ester] (225 g active ingredient in 450 L of water per ha) every three weeks throughout the duration of the experiment. Plots were evaluated every two weeks starting on the day of the first application (i.e. June 17, July 3, July 15, July 29, August 12 and August 27) for ground coverage and disease severity of *C. sepium*. For each evaluation, a 75 cm × 210 cm grid with 7 rows (30 cm row width) and 11 columns (7.5 cm column width) was applied at the centre of each plot. To assess bindweed density, the presence of the *C. sepium* was scored (presence or absence) at each of the 77 points of the grid and *C.
*sepium* density was calculated as the percentage of grid points where bindweed leaves were present. Disease severity was assessed by rating the necrotic leaf surface (nls) of the leaf found at each grid point (or the closest leaf when no leaf was found) using the same scale as described above.

As a repetition, in 1997 a field experiment similar to the 1996 one was performed at the same site. The design remained unchanged except that the experimental area was doubled and eight blocks of the same size as in 1996 were used. Soil was ploughed on April 23, fertilised on May 9 (60-100-26 kg/ha N-P-K and 2.5 kg/ha Mg) and harrowed twice on May 13. Maize was sown on May 15 and the field was handweeded on June 2. *T. pratense* was sown June 9. Mycoherbicide on treated plots and benomyl on the control plots were applied on June 18 and on July 1. Evaluations were performed every two weeks on June 16, June 30, July 14, July 28, August 4 and August 25.

The treatments (mycoherbicide × green cover) were studied in a factorial randomised complete block design with four (1996) or eight (1997) blocks and one plot per treatment per block. Disease severity and ground coverage data were transformed to the arcsine of the square root prior to analysis. Data within each evaluation date were subjected to an analysis of variance and treatments were compared with Bonferroni’s protected test. Systat 5.05 (SPSS Inc., Evanston, USA) was used and all analyses were performed at \( P=0.05 \).

**Results and Discussion**

**Influence of *T. pratense* competition on *C. sepium***

Growth of *C. sepium* was strongly inhibited in the presence of *T. pratense* regardless of whether shoots or the combination of shoots and roots were responsible for the competition (Figure 2). Compared to the control, the relative reductions of biomass in competition were similar for leaves, stems and roots. Competition by shoots alone reduced *C. sepium* biomass in the same way as competition by roots and shoots. It was therefore concluded that the observed reduction was mainly caused by shoot competition.

Any two plants can compete for resources such as light, water or nutrients (Berkowitz, 1988). In the present study, water and nutrients were supplied to the pots so that these resources were not limiting factors and consequently interference on the level of these resources could be avoided. However, light availability was strongly reduced to
a similar extent in both competition treatments (Figure 2). Therefore it was concluded that the main effect of shoot competition was due to the reduction of light available to \textit{C. sepium}. Indeed, van Ast and van Groenendael (1993) stated that \textit{C. sepium} is a plant sensitive to light competition. Studies on other weeds showed similar reactions to shading by crops (McLachlan \textit{et al.}, 1993) or to artificial light interception (Dall'Armellina and Zimdahl, 1988).

![Figure 2](image-url)

**Figure 2.** Biomass of and light available to \textit{C. sepium} grown in three competition levels with \textit{T. pratense}. The mean of light availability (\%) is shown in relation to the treatment with no competition. Statistical relationship between the treatments is shown for the light interception (Arabic letters) and for total plant biomasses (Greek letters). Means followed by the same letter were not significantly different.

In the experiment presented here, the reduction of light availability caused by the canopy of \textit{T. pratense} was detrimental to \textit{C. sepium}. Consequently, \textit{T. pratense} seems to be useful as a biological control measure for \textit{C. sepium}.

**Influence of \textit{T. pratense} and \textit{S. convolvuli} strain LA39 on \textit{C. sepium} in the greenhouse**

Necrotic lesions of \textit{S. convolvuli} covered up to 65\% of the surface of the inoculated \textit{C. sepium} leaves, indicating favourable conditions for the fungal disease (Figure 3). In the treatments not inoculated with the mycoherbicide, no infected leaves were observed (data not shown). There was no difference in necrotic leaf surface between the treatments with and without \textit{T. pratense}. In contrast, Groves and Williams (1975) reported that a competing plant may promote the disease caused by a fungal pathogen. In another study, alfalfa (\textit{Medicago sativa L.}) lost its resistance to \textit{Verticillium albo-atrum} Rke. et Berth. if light intensity fell below a certain threshold (Pennypacker \textit{et al.}, 1994).

In the current work, the plants grown in presence of \textit{T. pratense} produced fewer leaves compared to those grown without the green cover (Figure 4). The biomass
produced by *C. sepium* 19 days after inoculation, showed a similar trend like the numbers of leaves, in that *T. pratense* strongly reduced the plant biomass whereas the pathogen had no influence (Figure 5). The individual plant parts were reduced by *T. pratense* at similar rates like in the competition experiment, suggesting that the growth conditions were similar in both experiments (Figures 3 and 5).

![Graph showing effect of *T. pratense* on necrotic leaf surface](image)

**Figure 3.** Effect of *T. pratense* (clover) on necrotic leaf surface of *C. sepium* treated with *S. convolvuli* strain LA39 (mycoherbicide). Treatments were compared at each sampling date with Bonferroni's test. Means followed by the same letter were not significantly different.

![Graph showing effect of *S. convolvuli* and *T. pratense* on leaves per plant](image)

**Figure 4.** Effect of *S. convolvuli* strain LA39 (mycoherbicide) and *T. pratense* (clover) on the number of leaves per plant of *C. sepium*. Treatments were compared at each sampling date with Bonferroni's test. Means followed by the same letter were not significantly different.

In conclusion, *T. pratense* appears to be useful as a control measure for *C. sepium*. However, even though *S. convolvuli* caused severe necrosis of the inoculated bindweed leaves, the disease had no effect on *C. sepium* growth. Possibly, this is a
result of the lack of fungal propagation caused by absence of rain splash in the greenhouse which is responsible for fungal spread in the field (Pfirter et al., 1997). As a consequence, new, uninoculated plant leaves remain healthy explaining why S. convolvuli had neither an influence on the total number of leaves nor on the biomass of C. sepium in these experiments.

![Figure 5](image-url)

*Figure 5. Effect of S. convolvuli strain LA39 (mycoherbicide) and T. pratense (clover) on the biomass per plant of C. sepium. Columns represent total plant biomass means. Total plant biomasses were compared at each sampling date with Bonferroni’s test. Columns followed by the same letter were not significantly different.*

Field trials for the evaluation of the influence of green cover T. pratense and mycoherbicide S. convolvuli strain LA39 on C. sepium in a maize field

In the inoculated plots, disease severity was different in 1996 and in 1997 but the development of the epidemics was similar in time (Figure 6). In both years, the disease severity in mycoherbicide treated plots increased for a month after the first mycoherbicide application. It stabilised at six weeks and resumed increasing thereafter. The protection of the control plots treated with benomyl was not complete, because in both years some necrotic leaf surface was observed in these treatments. In 1997, the disease severity in the control plots was higher compared to 1996.
Figure 6. Effect of applying *S. convolvuli* strain LA39 (mycoherbicide) and of underseeding *T. pratense* (clover) on the necrotic leaf surface of *C. sepium* as measured in a maize field in 1996 and 1997 in Eschikon, Switzerland. At each sampling date, treatments were compared with Bonferroni's test. Means followed by the same letter were not different statistically.

Figure 7. Effect of applying *S. convolvuli* strain LA39 (mycoherbicide) and of underseeding *T. pratense* (clover) on the ground coverage of *C. sepium* as measured in a maize field in 1996 and 1997 in Eschikon, Switzerland. At each sampling date, treatments were compared with Bonferroni's test. Means followed by the same letter were not different statistically.
In the field, application of the mycoherbicide caused a strong reduction of the ground coverage of *C. sepium* in comparison to control plots throughout the experiment (Figure 7). The development of the *C. sepium* ground coverage was similar in 1996 and 1997, but levels of ground coverage were higher in 1996. Unlike in greenhouse conditions, in the field a high disease severity led to extensive defoliation of the bindweeds which is documented by the reduction in ground coverage. This supports the hypothesis that the fungus is propagated by rain splash which is present in the field but not in the greenhouse.

In these experiments, *T. pratense* never had an influence on disease severity. This is a similar finding to the greenhouse experiment where the course of the epidemic was not affected by the presence of *T. pratense* (Figure 6). In 1996, *T. pratense* enhanced the control of *C. sepium* in combination with *S. convolvuli* in the beginning of the experiment (July 3 and July 15) but not later (Figure 6). At the last sampling (August 27), *T. pratense* suppressed *C. sepium* ground coverage compared to the control but there was no effect of *T. pratense* and *S. convolvuli* combined compared to *S. convolvuli* alone. In 1997, the presence of *T. pratense* did not influence the ground coverage with *C. sepium* throughout the experiment. Overall, competitiveness of *T. pratense* was not sufficient to suppress *C. sepium* in these experiments. Possibly *C. sepium* could be controlled if another, more competitive green cover plant was used. In vineyards such a plant, *Stellaria media* (L.) Vill., has been described (Stalder *et al.*, 1974).

Contrasting weather conditions in 1996 and 1997 may partially explain the differences observed in the disease severity and ground coverage in the two years (data not shown). In 1996, a wet and cool month of May was followed by a warm period in June, July and August. In 1997, May was drier than the year before, whereas June and July were cooler with more rain. The month of August was warmer in 1997 than in 1996 and less rain was measured. Overall, maize had a better year in 1997 compared to 1996, because of a warm start in May and a good water supply in June and July. In contrast, conditions were not favourable for the development of the underseeded *T. pratense* in 1997 compared to 1996, because sowing was followed by a cool and wet period. Throughout the season, *T. pratense* was not able to grow much (data not shown). This might explain the observation that *T. pratense* had a small influence on *C. sepium* in 1996 and none at all in 1997. In contrast, the cool and wet weather in June and July of 1997 might have helped the mycoherbicide in two ways. Firstly, the infection of bindweed leaves by *S. convolvuli* strain LA39 is more efficient in wet than in dry conditions (Pfirter and Défago, 1998), and secondly the frequent rain might have been helpful in promoting an abundant spread of the fungal spores.
In the present work, the fungal biocontrol agent *S. convolvuli* strain LA39 was used in a maize field underseeded with *T. pratense*. As *S. convolvuli* strain LA39 reduced the bindweed ground coverage, this fungal biocontrol agent could be useful for the control of *C. sepium* in the field. Furthermore, the action of the fungus was not affected by the presence of *T. pratense* underseeded to maize as a living green cover. Living green covers used in modern maize production systems suppress the growth of many weeds, but bindweeds are not controlled sufficiently (Hall and Hartwig, 1990; Garibay et al., 1997; Pfirter et al., 1997). Since in this work the combination of *S. convolvuli* strain LA39 with the green cover plant was possible, a main requirement for successful implementation of biological control agents into integrated pest management systems, like maize with living green covers, was fulfilled (Müller-Schärer and Scheepens, 1997). Further research should elucidate long term effects of the application of *S. convolvuli* on the bindweed population in a field. Investigation of the gross effect of a mycoherbicide application on the reserves stored in the rhizomes of *C. sepium* would clarify the usability of the method, as these reserves determine the emergence of the bindweed in the next growing season.

**Acknowledgements**

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Part 3 *Stagonospora convolvuli* LA39 for biocontrol of field bindweed infesting cotoneaster in a cemetery

**Summary**

Next to arable fields, field bindweed (*Convolvulus arvensis*) infests also roadsides, hedges and parks, and it is difficult to control with herbicides. In this work, the potential of the mycoherbicide *Stagonospora convolvuli* strain LA39 for use as a biological control of field bindweed was tested in a cemetery, where a ground cover of cotoneaster was extensively infested by the weed. Application of *S. convolvuli* resulted in extensive necrosis of field bindweed leaves within 20 days, and necrosis became more extensive in the 20 following days. Bindweed density decreased continuously to about half of that in the uninoculated control plots within 40 days after application. No effect on emergence of field bindweed was observed in the year following the application of *S. convolvuli*. In conclusion, *S. convolvuli* may be useful as a mycoherbicide for the control of field bindweed in amenity areas but it would need to be applied every year.
**Introduction**

Field bindweed (*Convolvulus arvensis* L.) is an important weed on roadsides, in hedges and parks. Chemical herbicides available for bindweed control affect many other plants, including some widely used as groundcovers in parks, where effective control of bindweeds often requires expensive and frequent hand weeding. Therefore, selective bindweed control through alternative strategies would be useful in this type of environment.

The mycoherbicide *Stagonospora convolvuli* Dearness & House strain LA39 (isolated in our laboratory from a leaf of a diseased *C. arvensis* plant) is specifically pathogenic to the bindweeds *C. arvensis* and *Calystegia sepium* (L.) R. Br. and other *Convolvulaceae* (Pfirter and Défago, 1998). Greenhouse and field trials have shown that strain LA39 is a promising biological control agent for bindweeds in agricultural systems (Pfirter *et al.*, 1997). The objective of this study was to assess strain LA39 for biocontrol of *C. arvensis* in a non-agricultural environment.

Cemeteries were chosen as a model because in Switzerland, *Cotoneaster dammeri* C. K. Schneider is often used as a ground cover between graves, where favourable conditions exist for infestation by bindweeds. Graves are organised in rows, and within a row they are surrounded with *C. dammeri*, which covers about half the surface of the cemetery (cover photo). A cemetery located in Zürich, Switzerland, and where *C. dammeri* was heavily infested by *C. arvensis* was selected for the experiment.

**Materials and Methods**

Two treatments (mycoherbicide and control) were studied, using a block design. Six blocks, each with two 2-m² plots, were selected and the mycoherbicide was sprayed on one plot per block. The six control plots were treated with benomyl (1-(butylcarbamoyl)-2-benzimidazolcarbamic acid methyl ester) (225 kg a.i. in 450 L of water per ha). This systemic fungicide, which has no effect on growth of *C. dammeri* or *C. arvensis*, was used to prevent spread of the pathogen into control plots. Treatments were applied at 10:00 HR on 10 Aug. 1996, a day with 10 h of sunny, dry weather, and a mean temperature of 20 °C. During the experiment the average temperature was 15.8 °C, total rainfall was 120 mm and sunshine averaged 5.2 h·d⁻¹.
The fungal inoculum was produced by growing LA39 on sterilised couscous grains (i.e. coarse wheat flour) for 14 d (20 °C, 70% relative humidity, continuous light), as described by Pfirter and Défago (1998). The grains were washed in sterile distilled water to dislodge the conidia and the suspension was filtered through two layers of cheesecloth. The conidial suspension was adjusted to $10^8$ conidia/mL (as determined with a haemacytometer), a concentration effective for infection of *C. arvensis* (Pfirter and Défago, 1998). The conidial suspension was incorporated into an oil emulsion (final concentration $10^7$ conidia/mL), as described by Lawrie *et al.* (1997). The plots were sprayed with the final preparation at a rate of 300 L·ha$^{-1}$ using a backpack sprayer (model M125, Birchmeier AG, Künten, Switzerland).

Each plot was evaluated immediately before spraying, 20 and 40 d after treatment, and at the start of the next growing season in April 1997. At the start of the experiment there were no necrotic lesions on the leaves. A 50 cm × 100 cm grid (10 cm mesh size) was placed at the centre of each plot. To assess bindweed density, presence of the weed was scored (presence or absence) at each of the 66 points of the grid. Bindweed density was calculated as the percentage of grid points where bindweed leaves were present. Disease severity (necrotic leaf surface; NLS) was assessed by visually rating the percentage of leaf surface that was necrotic for the leaf found at each grid point, or the closest leaf when no leaf was found there. The following scale was used: 0 = 0% NLS (no symptoms); 2.5 = 1% to 5% NLS; 15 = 6% to 24% NLS; 50 = 25% to 75% NLS; 87.5 = 76% to 99% NLS; 100 = 100% NLS (entire leaf dead). For each plot, the average NLS was calculated using data obtained with the 66 leaves studied.

Bindweed density and NLS data were arcsine transformed and analysis of variance was used to determine treatment effects. Data were analysed at $P \leq 0.05$ and Systat 5.05 (SPSS Inc., Evanston, IL) was used. For presentation, data were back-transformed to percentages.

**Results and Discussion**

Spraying of bindweed-infested *C. dammeri* with *S. convolvuli* strain LA39 resulted in extensive infection of *C. arvensis*, which displayed 60% NLS as early as 20 d after inoculation (Figure 1). The fungus had no apparent effect on the canopy of *C. dammeri*. In the control plots, necrotic spots were almost never found on leaves 20 d after inoculation, but disease severity reached 20% in these plots at 40 d (Figure 1). The application of benomyl did not prevent spread of the fungus from LA39-treated plots to
control plots throughout the 40-d period after inoculation. Indeed, an important increase in disease severity took place in the control treatment between 20 and 40 d, and this increase was similar to that in the LA39 treatment (Figure 1).

In control plots, bindweed density increased from 32 to 49% during the first 20 d, but did not increase further during the next 20 d (Figure 1). This was expected, as bindweed reaches its highest density in August in Zürich. In plots treated with LA39, bindweed density decreased to 17% during the 40-d period after inoculation.

Figure 1. Percentage necrotic leaf surface (A) of C. arvensis and bindweed density as a percentage of grid points occupied (B), in plots treated with S. convolvuli strain LA39 (triangles) and in untreated control plots (squares). Error bars represent standard deviations. Asterisks indicate a significant effect of treatments ($P < 0.05$).

Plots also were monitored in the spring of 1997 to assess the residual effect of mycoherbicide application on emergence of the bindweed in the next growing season.
In both treatments bindweed density amounted to 14% ± 4% (mean ± SD) and necrotic lesions were not found on *C. arvensis*. This lack of residual effect of the mycoherbicide on bindweed development implies that LA39 needs to be applied every year for bindweed control, probably because bindweeds possess substantial reserves in their rhizomes (Barr, 1945).

A second experiment was performed in the same cemetery and at the same time, using the same experimental design, but with plots that were half the size. Results similar to those from the first experiment were obtained regarding the effect of LA39 on infection of *C. arvensis* and bindweed density (data not shown).

In conclusion, *S. convolvuli* strain LA39 proved to be effective for bindweed control in *C. dammeri* by causing defoliation. This fungus may be useful where bindweed is the main weed to be controlled, especially in amenity areas where groundcovers and bushes provide favourable conditions for its development and where environmentally-friendly control strategies are welcomed. Future research will determine if LA39 can suppress bindweeds consistently, in different years and in different climatic regions.

**Literature Cited**


Leer - Vide - Empty
Chapter V.

Rhizobacteria degrading the calystegine alkaloids
Leer - Vide - Empty
Part 1 Contribution of calystegine catabolic plasmid to colonisation of the rhizosphere of calystegine-producing plants by Sinorhizobium meliloti Rm41

Summary

Calystegines are plant secondary metabolites produced by the roots of a few plant species. The ability to catabolise calystegines is infrequent in rhizosphere bacteria. In Sinorhizobium meliloti Rm41, the endosymbiont of the Legume Medicago sativa, the ability to catabolise calystegines results from the presence of the genes cac (for calystegine catabolism) located on the non-symbiotic plasmid pRme41a. The effect of the cac catabolic plasmid pRme41a on the ability of Rm41 to colonise the rhizosphere of calystegine-positive plants was studied using derivatives of Rm41 with or without cac catabolic plasmid. When these derivatives were inoculated alone, the presence of a cac catabolic plasmid had no effect on their colonisation of the rhizosphere, regardless of whether plants produced calystegines or not. However, a spontaneous rifampicine-resistant mutant of Rm41 containing a cac catabolic plasmid reached population levels in the rhizosphere of calystegine-positive plants that were several orders of magnitude higher than those of the same strain without the plasmid, when each was co-inoculated with a derivative of Rm41 cured of pRme41a. In contrast, the cac catabolic plasmid provided little or no selective advantage in the rhizosphere of calystegine-negative plants. In conclusion, the cac catabolic plasmid pRme41a can contribute to the ability of S. meliloti Rm41 to colonise the rhizosphere of alternative, non-Legume plant hosts producing calystegines.
Introduction

Many species of unicellular symbiotic nitrogen-fixing bacteria (hereafter collectively referred to as rhizobia) possess a large fraction of their genome as plasmids, and the involvement of plasmids in the symbiosis between rhizobia and the root of Legumes is well documented (Martinez et al., 1990). Colonization of the rhizosphere of Legumes by rhizobia can be promoted by their utilisation of specific carbon substrates exuded by the root, e.g. trigonelline (Boivin et al., 1991), flavones (Hartwig et al., 1991) or homoserine (van Egeraat, 1975). Plasmid genes are involved in the catabolism of some of these compounds by rhizobia (Boivin et al., 1991).

However, part of the life cycle of rhizobia takes place in the soil or in the rhizosphere of plants other than Legumes (Schmidt and Robert, 1985). Interestingly, plasmids can contribute also to the saprophytic properties of rhizobia in vitro (Charles and Finan, 1991) or in soil (Moënne-Loccoz and Weaver, 1995). One of three plasmids harbored by Sinorhizobium meliloti strain Rm41 enables the strain to catabolise the calystegines produced by the roots of bindweeds Calystegia sepium (L.) R. Br. and Convolvulus arvensis L. (Tepfer et al., 1988). Calystegines are polyhydroxy nortropane alkaloids (Dräger, 1995) and they are synthesised by only a few plant species (mostly Solanaceae) besides bindweeds, e.g. Atropa belladonna L. (Tepfer et al., 1988), Hyoscyamus niger L. (Asano et al., 1996) or Solanum tuberosum L. (Nash et al., 1993). Legumes do not produce calystegines (Tepfer et al., 1988). Calystegines act as glycosidase inhibitors (Asano et al., 1996; Molyneux et al., 1993), insect deterrents, and can cause neurological disorders in livestock (Nash et al., 1993). In addition, calystegines may serve as allelopathic compounds by inhibiting seed germination (Goldmann et al., 1996).

S. meliloti strain Rm41 was isolated from a nodule of Medicago sativa (Ördögh and Szende, 1961). The cac genes are responsible for calystegine degradation by Rm41 and they are located on the non-symbiotic plasmid pRm41a (Tepfer et al., 1988). Subcloning of this cac catabolic plasmid revealed that the cac genes were spread over a 30 to 40 kb region of the 225 kb plasmid (Boivin et al., 1990). Since only a minority of bacterial isolates from soil are capable of calystegine degradation and most of them were found in the rhizosphere of calystegine-producing plants, it was hypothesised that calystegines could act as nutritional mediators between calystegine-producing plants and calystegine-degrading microorganisms, thereby favouring colonisation of the
rhizosphere of the former by the latter (Tepfer et al., 1988 and 1988a; Boivin et al., 1990; Goldmann et al., 1996).

The objective of this study was to assess the significance of the presence of a cac catabolic plasmid in S. meliloti Rm41 for the adaptation of the bacterium to the rhizosphere of calystegine-producing plants. This was achieved by comparing derivatives of Rm41 with or without cac catabolic plasmid for colonisation of the rhizosphere of calystegine-producing and calystegine-negative plants.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in the study are listed in Table 1. S. meliloti Rm41 was isolated in Hungary from a nodule of M. sativa (Ördögh and Szende, 1961). The strain contains the 225-kb non-symbiotic plasmid pRme41a (i.e. plasmid a), which harbours the cac catabolic genes (Tepfer et al., 1988; Boivin et al., 1990), and the two symbiotic megaplasmids pRme41b (i.e. plasmid b) and pRme41c (i.e. plasmid c), each about 1500 kb in size (Banfalvi et al., 1985). Strain Rm41 containing all three plasmids a, b and c will hereafter be referred to as Rm41(abc). Strain Rm41(bc) is a derivative of Rm41(abc) cured of plasmid a (Huguet et al., 1983). All strains were routinely grown in tryptone-yeast extract (TY) medium (Beringer, 1974). Rifampicin was used at 100 µg/ml, kanamycin at 50 µg/ml and tetracycline at 5 µg/ml.

In this study, strains were studied in natural soil experiments or co-inoculation experiments and resistance to rifampicin was used as a marker to distinguish them from indigenous soil bacteria or co-inoculated strains. Several spontaneous rifampicin-resistant (Rif') mutants of strains Rm41(abc) and Rm41(bc) were isolated on TY medium containing 100 µg/ml rifampicin. The Rif' mutants Rm41R(abc) and Rm41R(bc) were derived from Rm41(abc) and Rm41(bc), respectively, and they were selected based on growth properties identical to those of the corresponding parental strain in M9 medium (Maniatis et al., 1982), containing 0.1 M mannitol as sole carbon source. In addition, strains Rm41R(abc) and Rm41R(bc) displayed the same profiles of carbon substrate utilisation (95 carbon substrates; GN Biolog plates; Biolog Inc., Hayward, CA; Table 1) and the same population levels in the rhizosphere of several plants grown in artificial soil (see legend of Fig. 1) when compared with Rm41(abc) and Rm41(bc), respectively. According to the assay described by Tepfer et al. (1988), strains Rm41(abc) and Rm41R(abc) were able to degrade calystegines (Cac-positive)
but not strains Rm41(bc) or Rm41R(bc) (Cac-negative). In conclusion, the Rif\textsuperscript{r} mutants Rm41R(abc) and Rm41R(bc) were indistinguishable from Rm41(abc) and Rm41(bc), respectively, on the basis of saprophytic properties.

Table 1. Plasmids and bacteria used in the study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Cac Description\textsuperscript{a}</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pRme41a (noted plasmid a)</td>
<td>+ non-pSym, cac catabolic plasmid</td>
<td>Tepfer et al. (1988)</td>
</tr>
<tr>
<td>pRme41a::Tn5 (noted plasmid A)</td>
<td>+ Tn5 derivative of pRme41a; cac, Km\textsuperscript{r}</td>
<td>Huguet et al. (1983)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>- Mob\textsuperscript{r}, Tra\textsuperscript{r}, Tc\textsuperscript{r}, Km\textsuperscript{r}</td>
<td>Figurski and Helinski (1979)</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
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</tr>
<tr>
<td>Sinorhizobium melilot\textsuperscript{b}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rm41(abc)</td>
<td>+ Wildtype, contains plasmids a, b, c</td>
<td>Öröggh and Szende (1961)</td>
</tr>
<tr>
<td>Rm41R(abc)</td>
<td>+ Spontaneous Rif mutant of Rm41(abc)</td>
<td>This work</td>
</tr>
<tr>
<td>Rm41(bc)</td>
<td>- Rm41(abc) cured of pRme41a</td>
<td>Huguet et al. (1983)</td>
</tr>
<tr>
<td>Rm41R(bc)</td>
<td>- Spontaneous Rif mutant of Rm41(bc)</td>
<td>This work</td>
</tr>
<tr>
<td>Rm41R(ABC)</td>
<td>+ Rm41R(bc) complemented with pRme41a::Tn5; Rif; Km\textsuperscript{r}</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Agrobacterium tumefaciens</strong></td>
<td></td>
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<tr>
<td>GMI9019(A)</td>
<td>+ Km\textsuperscript{r}</td>
<td>Huguet et al. (1983)</td>
</tr>
<tr>
<td>HB101(pRK2013)</td>
<td>- Tc\textsuperscript{r}; helper strain (mobilization)</td>
<td>Figurski and Helinski (1979)</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
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</table>

\textsuperscript{a} non-pSym; non-symbiotic Plasmid; Mob\textsuperscript{r}, genes for mobilisation of plasmids; Tra\textsuperscript{r}, genes for conjugative transfer of plasmids; Tc\textsuperscript{r}, resistance to tetracycline; Km\textsuperscript{r}, resistance to kanamycin; Rif\textsuperscript{r}, resistance to rifampicin.

\textsuperscript{b} Utilisation of 95 carbon sources was studied using Biolog GN-Microplates, according to manufacturer’s recommendations, and results (read with an ELISA reader; 570 nm) indicated that the five strains Rm41(abc), Rm41(bc), Rm41R(abc), Rm41R(bc) and Rm41R(ABC) could grow on N-acetylgalactosamine, N-acetyl-D-glucosamine, adonitol, alanitramide, L-arabinose, D-arabitol, bromosuccinate, camitine, D-cellobiose, dextrin, erythritol, formate, D-fructose, L-fucose, D-galactonate lactone, D-galactose, gentiobiose, D-glucurate, D-glucose, L-glutamate, glycerol, hydroxy-L-proline, inosine, inositol, D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, B-methyl-D-glucose, methylsuccinate, L-proline, L-psicose, D-raffinose, L-threitol, D-sorbitol, succinate, sucrose, D-trehalose, turanose and urocanate as sole carbon sources.

None of the five strains could assimilate acetate, cis-aconitate, D-alanine, L-alanine, L-alanylglycine, \(\gamma\)-aminobutyrate, L-asparagine, L-aspartate, citrate, \(\beta\)-hydroxybutyrate, 2,3-butanediol, 2-aminoethanol, \(\alpha\)-cyclodextrin, D-galacturonate, D-glucosamine, glucose-1-phosphate, glucose-6-phosphate, glucuronamide, D-gluconate, DL-\(\alpha\)-glycerol phosphate, glycosgen, glycyL-L-aspartate, glycyL-L-glutamate, L-histidine, \(\alpha\)-hydroxybutyrate, \(\gamma\)-hydroxybutyrate, p-hydroxyphenyl acetate, itaconate, \(\alpha\)-ketobutyrate, \(\alpha\)-ketoglutarate, \(\alpha\)-ketovalerate, DL-lactate, L-leucine, malonate, methyl pyruvate, L-ornithine, L-phenylalanine, phenethylamine, propionate, putrescine, L-pyrogulatamate, quinate, D-saccharate, sebacate, D-serine, L-serine, succinate, L-threonine, thymidine, Tween 40, Tween 80, uridine or xylitol.

Complementation of Rm41R(bc) was performed using plasmid pRme41a::Tn5 (Huguet et al., 1983), hereafter referred to as plasmid A. Plasmid A was introduced into Rm41R(bc) by means of a triparental mating using Agrobacterium tumefaciens strain GMI9019(A) (Huguet et al., 1983) as donor and the helper strain Escherichia coli HB101(pRK2013) (Figurski and Helinski, 1979), as outlined by Voisard et al. (1989). Plasmid A confers resistance to kanamycin (Km\textsuperscript{r}) and Rm41R(ABC) was selected based on Rif\textsuperscript{r} Km\textsuperscript{r} phenotype. As expected, the transconjugant was sensitive to tetracycline. The presence of plasmid A in the strain was confirmed by plasmid profile analysis using a modified Eckhardt gel electrophoresis technique (as described by Hynes and McGregor, 1990). In addition, complementation of Rm41R(bc) with plasmid A restored
the ability to catabolise calystegines, as indicated using the calystegine degradation assay of Tepfer et al. (1988).

**Experimental system used to study rhizosphere colonisation**

Seeds of hedge bindweed (*C. sepium*; collected in the city of Zürich, Switzerland) and field bindweed (*C. arvensis*; obtained from MT Valley Seed Service, Fresno, CA) were scarified in 95% H₂SO₄ for 60 min before surface disinfection. Scarified bindweed seeds and seeds of the belene (*Hyoscyamus niger* L., Solanaceae, calystegine-positive obtained from the Botanical Garden, Zürich), maize (*Zea mays* cv. LG 22.43; obtained from FAL Seed Department, Reckenholz, Switzerland) and alfalfa (*M. sativa*; obtained from Samen Mauser, Winterthur, Switzerland) were surface-disinfected in 5% (w/v) sodium hypochlorite for 15 min and 10% hydrogen superoxide for 15 min, rinsed with sterile distilled water and then germinated for 3 days in the dark on 1/10 strength tryptic soy agar (Oxoid) at 24°C before planting.

Experiments were done with natural soil and with artificial soil. The natural soil was a loam (15% clay, 42% silt, 43% sand; pH 7.0; 3.5% organic matter) collected from the surface horizon of a cambisol located at a fallow site near Eschikon, Switzerland (Natsch et al., 1994). The soil was passed through a 5-mm-mesh screen and stored at 15°C. The soil was mixed with coarse quartz sand (1.5 to 2.2 mm particle size) in a 3:1 ratio shortly before use. The artificial soil was a loamy sand (22% silt, 78% sand; pH 7.0; no organic matter) and corresponded to the artificial sandy loam described by Keel et al. (1989), except that vermiculite was not added. Natural and artificial soil microcosms consisted of 300 g soil in a 1-liter Erlenmeyer flask. Knop solution was added for plant nutrition at the rate of 8 ml per flask. Microcosms containing artificial soil were sterilised by autoclaving (121°C for 30 min). Inoculations were performed into soil and each microcosm was planted immediately with one seedling.

**Soil microcosm experiments**

First, the ability of the Cac-positive strains *S. meliloti* Rm41R(abc) and Rm41R(ABC) and the Cac-negative strain Rm41R(bc) to colonise the rhizosphere of calystegine-positive *C. sepium* and calystegine-negative *M. sativa* and *Z. mays* was investigated in soil microcosms inoculated with a single strain. The strains were grown in TY broth at 27°C with shaking for 36 h. The cells were washed twice with sterile distilled water and the cell suspensions adjusted to 4.7 or 5.7 log colony-forming units (CFU) per ml when preparing inocula for artificial soil (sterile) or natural soil (non-sterile), respectively. Soil was sprayed with a bacterial suspension at the rate of 6 ml per
flask, which represented 3 log CFU per g of artificial soil and 4 log CFU per g of natural soil. A higher inoculation level was chosen for natural soil since those microcosms were not sterile. Water content of soil was about 11% w/w. The flasks were stopped with cotton rolls after planting of seedlings and placed for 21 days in a growth chamber at 70% relative humidity with 16 h light (160 μE / m² / s) at 22°C, followed with 8 h of dark at 18°C.

Second, a co-inoculation experiment was performed to investigate the ability of the Cac-positive strain *S. meliloti* Rm41R(ABC) and the Cac-negative strain Rm41R(bc) to colonise the rhizosphere in competition with the Cac-negative strain Rm41(bc). The objective was to determine the implications of the presence of a cac catabolic plasmid (here plasmid A) for rhizosphere colonisation of a calystegine-positive plant under conditions of competition for root exudates. Artificial soil was used rather than natural soil so that root exudates represented the only source of organic substrates for microorganisms. The experiment was performed with three calystegine-positive plants (*C. sepium, C. arvensis* and *H. niger*) and two calystegine-negative plants (*M. sativa* and *Z. mays*). Bacterial cells were prepared as described above and each microcosm was inoculated with 6 ml of a cell suspension containing 8.7 log CFU of Rm41(bc) and 4.7 log CFU of either Rm41R(bc) or Rm41R(ABC) per ml, i.e. 7 log CFU of Rm41(bc) and 3 log CFU of either Rm41R(bc) or Rm41R(ABC) per g of soil. Seedlings were planted and each microcosm was placed for 21 days in the growth chamber described above.

Third, co-inoculation experiments were done to further investigate the ability of the Cac-positive strain *S. meliloti* Rm41R(ABC) to colonise the rhizosphere of *C. sepium* in competition with the Cac-negative strain Rm41(bc). These experiments were similar to that described above, except that (1) the kinetics of root colonisation was determined by performing several destructive samplings during the 21-day-long experiment or (2) several inoculation levels (i.e. 2, 3 and 4 log CFU per g soil) for Rm41R(ABC) were studied.

**Samplings and determinations**

Plants were harvested at 21 days, except in the experiment where the kinetics of root colonisation was studied. The root systems were washed gently for 10 s in sterile water to remove loose soil. Root systems (and closely-adhering soil) were transferred to 50-ml bottles containing sterile distilled water (root:water ratio of 1:10 w/w) and the bottles were shaken for 20 min at 350 rpm on a rotary shaker to extract rhizosphere bacteria. Small nodules were present on roots of *M. sativa* but the extraction procedure did not rupture them. Ten-fold dilution series were prepared and aliquots were spread-plated onto TY plates containing rifampicine to enumerate culturable cells of Rif² strains.
Rm41R(abc), Rm41R(bc) or Rm41R(Abc). TY plates were used in the co-inoculation experiments, where CFUs of Rm41(bc) were obtained by subtracting counts on TY + rifampicine (i.e. Rm41R(bc) or Rm41R(Abc)) from counts on TY (i.e. total introduced bacteria). Plates were incubated at 27°C and colonies were counted at four days. No colony was found on TY + rifampicine when samples from uninoculated natural soil microcosms were studied.

**Statistical design and analyses**

Each experiment was carried out using a completely-randomised design. In the experiment with single inoculations, each treatment (i.e. each combination of plant x bacterium x soil type) was replicated five times and the experiment was repeated twice. In each co-inoculation experiment, each treatment was replicated three times and the experiment was repeated once.

Colonies counts were log transformed before computing means and standard deviations and performing statistical analyses. An analysis of variance (ANOVA) was done for each experiment. In the co-inoculation experiment comprising several samplings, this procedure was followed at each sampling time. Treatments were then compared (where appropriate) using Bonferroni’s protected test. All analyses were done at a significance level of $P= 0.05$, using the program Systat (version 5.1, SSPS Inc. Evanston, IL).

**Results**

**Rhizosphere colonisation by single strains with or without cac catabolic plasmid**

In natural soil, the Cac-positive strain *S. meliloti* Rm41R(abc) colonised the rhizosphere of *C. sepium*, *M. sativa* and *Z. mays* at approximately 7.4 log CFU/g root at 21 days after soil inoculation (Fig. 1A). The Cac-negative strain Rm41R(bc) was found at population levels that were similar to those of Rm41R(abc), regardless of whether the experiment was done with calystegine-positive (*C. sepium*) or calystegine-negative plants (*M. sativa* and *Z. mays*). Complementation of Rm41R(bc) with plasmid A, which restored the ability to catabolise calystegines, had no influence on colonisation of the rhizosphere of *C. sepium*, *M. sativa* or *Z. mays* grown in natural soil at 21 days after inoculation. The same experiment was also done in artificial soil microcosms (Fig.
1B), under gnotobiotic conditions, and the three strains Rm41R(abc), Rm41R(bc) and Rm41R(ABC) were recovered at similar CFUs in the rhizosphere at 21 days after soil inoculation, regardless of the plant species studied. In conclusion, the presence of a cac catabolic plasmid did not influence rhizosphere colonisation when S. meliloti strains were inoculated alone.

![Figure 1. Colonisation of the rhizosphere of calystegine-positive C. sepium and calystegine-negative M. sativa and Z. mays by Rif derivatives of S. meliloti Rm41(abc) containing a cac catabolic plasmid (Rm41R(abc) and Rm41R(ABC)) or lacking such a plasmid (Rm41R(bc)). Soil was inoculated with either Rm41R(abc), Rm41R(bc) or Rm41R(ABC) prior to planting of seedlings, and sampling was carried out 21 days later. (A) In natural soil, strains were inoculated at 4 log CFU / g soil. (B) In artificial soil, strains were inoculated at 3 log CFU / g soil. Error bars represent standard deviations. In (A) and in (B), the results of a two-way ANOVA (plant species x strain) indicated that there was no statistical difference between treatments (P<0.05). The experiment in artificial soil microcosms was also performed with S. meliloti strains Rm41(abc) and Rm41(bc), which were recovered at respectively 7.3 ± 0.1 and 7.4 ± 0.1 log CFU / g root (C. sepium), 7.0 ± 0.4 and 7.5 ± 0.4 log CFU / g root (M. sativa), and 7.7 ± 0.2 and 7.9 ± 0.4 log CFU / g root (Z. mays), as determined by colony counts on TY plates at 21 days.]

**Rhizosphere colonisation by co-inoculated strains with or without cac catabolic plasmid**

Co-inoculation experiments were carried out in artificial soil microcosms with strain Rm41(bc) inoculated at 7 log CFU / g soil and either Rm41R(bc) or Rm41R(ABC) inoculated at only 3 log CFU / g soil. The Cac-negative strain Rm41(bc) was found at approximately 7.7 log CFU / g root in the rhizosphere of C. sepium, M. sativa and Z. mays at 21 days, regardless of whether it was co-inoculated with Rm41R(bc) or Rm41R(ABC) (Fig. 2A). In the rhizosphere of the calystegine-positive plants C. sepium, C. arvensis or H. niger, the Cac-negative strain Rm41R(bc) was recovered at population levels that were 2.5 (C. sepium) to 3.5 (H. niger) log CFU lower than those of the Cac-positive strain Rm41R(ABC) (Fig. 2B). However, there was little or no difference in rhizosphere colonisation between Rm41R(bc) and Rm41R(ABC) when the same co-
inoculation experiment was performed with the calystegine-negative plants *M. sativa* or *Z. mays*, respectively. In conclusion, the presence of a *cac* catabolic plasmid contributed to rhizosphere colonisation of calystegine-producing plants by *S. meliloti* when in competition with a Cac-negative derivative.

**Figure 2.** Colonisation of the rhizosphere of calystegine-positive (*C. sepium, C. arvensis* and *H. niger*) and calystegine-negative plants (*M. sativa* and *Z. mays*) by *S. meliloti* Rm41R(bc) and Rm41R(Abc) when each was co-inoculated with Rm41(bc). Artificial soil was inoculated with 3 log CFU / g soil of either Rm41R(bc) or Rm41R(Abc) and 7 log CFU / g soil of Rm41(bc) prior to planting of seedlings, and sampling was carried out 21 days later. Colony counts of the two co-inoculated strains in a same treatment are shown one above the other in (A) and (B). Error bars represent standard deviations. (A) Colony counts of Rm41(bc) in the rhizosphere. They were statistically identical in all treatments (P<0.05). (B) Colony counts of Rm41R(bc) and Rm41R(Abc) in the rhizosphere. For each plant species, statistical differences between Rm41R(bc) and Rm41(Abc) are indicated with an asterisk (P<0.05).

**Kinetics of rhizosphere colonisation by co-inoculated strains with or without cac catabolic plasmid and effect of inoculum levels**

The ability of the Cac-positive strain Rm41R(Abc) to colonise the rhizosphere of calystegine-positive *C. sepium* in competition with the Cac-negative strain Rm41(bc) in artificial soil microcosms was further studied. In the kinetics experiment, the population
size of Rm41(bc) stabilised at about 7.4 log CFU / g root as early as 7 days after inoculation, but CFUs of Rm41R(ABc) increased throughout the 21-day experiment, up to about 6 log CFU / g root (Fig. 3A). In the experiment where Rm41(ABC) was co-inoculated at 2, 3 or 4 log CFU / g soil, the population size of Rm41(ABC) in the rhizosphere of C. sepium at 21 days was statistically identical in the three treatments (i.e. about 6 log CFU / g root; Fig. 3B). In conclusion, the Cac-positive strain Rm41R(ABc) reached about 6 log CFU / g root in the rhizosphere of calystegine-positive C. sepium, despite the establishment of the co-inoculated Cac-negative strain Rm41(bc) and regardless of the inoculum level chosen for Rm41R(ABC).

![Figure 3. Colonisation of the rhizosphere of calystegine-positive C. sepium by S. meliloti Rm41R(ABc) in competition with Rm41(bc). Error bars represent standard deviations. (A) Artificial soil was inoculated with 3 log CFU / g soil of Rm41R(ABc) and 7 log CFU / g soil of Rm41(bc) prior to planting of seedlings. The kinetics of rhizosphere colonisation was determined by destructive samplings. Colony counts of Rm41R(ABc) and Rm41(bc) in the rhizosphere were statistically different at each sampling time (P<0.05). (B) Artificial soil was inoculated with 2, 3 or 4 log CFU / g soil of Rm41R(ABc) and 7 log CFU / g soil of Rm41(bc) prior to planting of seedlings, and sampling was carried out 21 days later. Colony counts of Rm41R(ABc) and Rm41(bc) in the rhizosphere were statistically different in each of the three treatments (P<0.05).](image_url)

**Discussion**

Comparison of the population levels *S. meliloti* Rm41R(abc), Rm41R(bc) and Rm41R(ABc) reached in the rhizosphere, when strains were inoculated alone, indicated that the presence of a *cac* catabolic plasmid had no effect on rhizosphere colonisation at 21 days, regardless of whether plants produced calystegines or not (Fig. 1). Roots of *C. sepium* are thought to produce calystegines constitutively (Tepfer *et al.*, 1988), and
indeed the alkaloids can be detected by thin-layer chromatography (as described by Dräger, 1995) shortly after germination of seeds (our unpublished data). This absence of effect of a cac catabolic plasmid on rhizosphere colonisation was observed in microcosms where inoculants either had to compete with indigenous microorganisms (i.e. in natural soil; Fig. 1A) or were favoured for growth (i.e. in artificial soil under gnotobiotic conditions; Fig. 1B). This suggests that organic carbon was not limiting for colonisation of the rhizosphere under these experimental conditions. A similar conclusion was drawn when investigating the capacity of pseudomonads with or without the ability to catabolise opines to colonise the rhizosphere of non-modified, opine-negative tobacco and transgenic, opine-producing tobacco in the absence of indigenous soil microorganisms (Savka and Farrand, 1997). The fact that colonisation of the rhizosphere of the symbiotic host M. sativa was not influenced by the presence of a cac catabolic plasmid is in accordance with previous results indicating that pRm41a did not contribute to the nitrogen-fixing symbiotic interaction of Rm41(abc) with the Legume (Banfalvi et al., 1981).

A second experiment was performed in artificial soil, in which Rm41(bc) was co-inoculated with either Rm41R(bc) or Rm41R(Abc). Under those conditions, the Cac-positive strain Rm41R(Abc) reached population levels that were several orders of magnitude higher than those of Cac-negative Rm41R(bc) in the rhizosphere of calystegine-positive plants (Fig. 2B). Similarly, the selective advantage afforded by the non-symbiotic plasmid pRtrW14-2a for colonisation of the rhizosphere of clover by Rhizobium leguminosarum W14-2 was apparent only when the strain was in competition with a W14-2 derivative cured of the plasmid (Moënne-Loccoz and Weaver, 1995). In the current work, differences in rhizosphere colonisation between Rm41R(bc) and Rm41R(Abc) did not result from a toxic effect of calystegines on Rm41(bc), as Cac-negative strains derived from Rm41(abc) could colonise the rhizosphere of calystegine-positive plants successfully (Fig. 1 and 2A). The magnitude of the selective advantage conferred by the cac catabolic plasmid varied from one calystegine-positive plant to the next, perhaps as a result of differences in calystegine availability in the rhizosphere. In the rhizosphere of calystegine-negative plants, the presence of the cac plasmid A in Rm41R(bc) had no effect (Z. mays) or a moderate effect (symbiotic host M. sativa) on colony counts of the strain (Fig. 2B). The difference between counts of Rm41R(bc) and Rm41R(Abc) in the rhizosphere of M. sativa was much lower than those in the rhizosphere of calystegine-positive plants. It seems possible that other traits located on pRme41a might be responsible for this difference in M. sativa, because the cac genes occupy only a 40 kb region of the 225 kb plasmid pRme41a (Boivin et al., 1990), and many important traits can be plasmid-borne in rhizobia (Charles and Finan, 1991; Baldani et al., 1992; Moënne-Loccoz and Weaver,
Furthermore, all three non-symbiotic plasmids naturally present in *R. leguminosarum* W14-2 contributed positively to the ability of the strain to colonise the rhizosphere of clover (Moenne-Loccoz and Weaver, 1995). Based on this, it is conceivable that such a *cac* catabolic plasmid encodes phenotypes other than calystegine degradation and that may influence the behaviour of host cells in the rhizosphere of *M. sativa*.

Experiments designed to assess further the ability of the *Cac*-positive strain Rm41R(Abc) to colonise the rhizosphere in competition with the *Cac*-negative strain Rm41(bc) in artificial soil microcosms indicated that the carrying capacity of roots of calystegine-positive *C. sepium* for Rm41R(Abc) was about 6 log CFU / g root under those experimental conditions. First, this population level was attained by growth of the *Cac*-positive strain taking place throughout the 21 day experiment, despite the fact that its *Cac*-negative competitor (inoculated at a higher population level) had reached its final (and higher) population level already by day 7 (Fig. 3A). Similar findings were reported on colonisation of the rhizosphere of opine-producing plants by co-inoculated bacteria differing in their capacity to utilise opines for growth (Savka and Farrand, 1997). Furthermore, the population level of about 6 log CFU / g root in the rhizosphere of *C. sepium* at 21 days was attained by Rm41R(Abc) regardless of its inoculation level in soil (Fig. 3B). Likewise, inoculation levels did not influence CFUs achieved on roots of transgenic opine-producing *Lotus corniculatus* by bacteria with the ability to catabolise opines, when co-inoculated with bacteria without this ability (Guyon *et al.*, 1993).

The increased competitiveness of rhizosphere bacteria with the capacity to utilise an unusual carbon substrate produced by the root was shown with transgenic plants that had acquired the ability to synthesize opines (Guyon *et al.*, 1993; Oger *et al.*, 1997; Savka and Farrand, 1997). The opine concept (Tempé *et al.*, 1989) was extended to rhizopines (Murphy *et al.*, 1995), which are secondary metabolites synthesised by rhizobia specifically during the symbiosis (i.e. by bacteroids) and whose utilisation as carbon source by the same rhizobia provide them with enhanced competitiveness for nodulation of Legumes (Murphy *et al.*, 1992). Despite the fact that root-produced rhizopine, opines and calystegines can all provide a selective and specific advantage for rhizosphere colonisation to bacteria capable of catabolising them, a distinct feature of calystegines is their constitutive production by plant roots. Indeed, production of the other two metabolites requires the involvement of bacteria for transfer of Ti-DNA from *A. tumefaciens* to the plant cell in the case of opines (Tempé *et al.*, 1989) or directly for synthesis of the compound in the case of rhizopines (by bacteroids; Murphy *et al.*, 1995). Since the formulation of the opine concept, many authors have hypothesised on the feasibility of engineering plant-bacteria associations (Tepfer *et al.*, 1988a; Oger *et
al., 1997; Savka and Farrand, 1997). Together with opines and rhizopines, calystegines may also be envisioned as target molecules for establishing trophic links between specific plants and rhizobacteria.

In this work, it was shown that a microorganism degrading calystegines was favoured for colonisation of plants producing the alkaloids, which may be plant defence compounds (Nash et al., 1993; Goldmann et al., 1996). Whether this could, in turn, affect bindweed development or its capacity to resist to pathogens is not known. Preliminary results indicated that colonisation of roots of C. sepium by calystegine-degrader S. meliloti Rm41(abc) did not influence the ability of Stagonospora convolvuli strain LA39 to infect and defoliate calystegine-producer C. sepium in the greenhouse (data not shown).

In conclusion, this study indicates that the presence of a cac catabolic plasmid can contribute to the ability of S. meliloti Rm41(abc) to colonise the rhizosphere of calystegine-producing plants in the presence of a bacterial competitor unable to utilise calystegines as a substrate. Non-symbiotic plasmids encoding unusual catabolic traits such as calystegine utilisation may favour the adaptation of S. meliloti to the rhizosphere of alternative, non-leguminous host plants.

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Part 2 Calystegine degradation capacities of microbial rhizosphere communities of Zea mays (calystegine negative) and Calystegia sepium (calystegine positive)

Summary

Calystegines are tropane alkaloids produced by the roots of a few plant species. A bioassay was developed to identify roots with a Microbial rhizosphere community capable of Calystegine Degradation (i.e. MCD roots). In a field survey, the proportion of MCD roots of Zea mays (calystegine-negative) varied from 20 to 80%. In field experiments, the proportions of MCD roots of Z. mays and Calystegia sepium (calystegine-positive) grown in a same plot were similar to each other but variable in time, and overall they were higher than that of Z. mays roots from adjacent plots free of C. sepium. In autoclaved soil, no root of C. sepium or Z. mays plants propagated as seeds were MCD, indicating that calystegine-degrading microorganisms were not seedborne. However, MCD roots were found as early as one day after planting of rhizomes of C. sepium in autoclaved soil or planting of axenic seedlings of either plant in natural soil microcosms. Overall, microorganisms capable of degrading calystegines were harboured not only in the rhizosphere of the calystegine-producing plant but also in that of the calystegine-negative plant and probably in bulk soil.
Introduction

Calystegines are plant secondary metabolites and were first found in the roots of *Calystegia sepium* (L.) R. Br. (i.e. hedge bindweed, morning glory) (Tepfer et al., 1988). Other plant species have also been described to produce these tropane alkaloids e.g. *Atropa belladonna* L. and *Convolvulus arvensis* L. (Tepfer et al., 1988), *Hyoscyamus niger* L. (Asano et al., 1996), *Morus alba* L. (Asano et al., 1994), *Physalis alkekengi* var. *francheti* L. (Asano et al., 1995), *Solanum tuberosum* L. and *Solanum melongena* L. (Nash et al., 1993). Calystegines can be poisonous to arthropods (Nash et al., 1993) and mammals (Todd et al., 1995), presumably as a result of glycosidase inhibition (Asano et al., 1996; Molyneux et al., 1993). Calystegines may also act as allelochemicals in the interactions between plants (Goldmann et al., 1996).

*Sinorhizobium meliloti* strain Rm41 (Tepfer et al., 1988) and bacteria from a few other genera including *Pseudomonas* (Tepfer et al., 1988a; Goldmann et al., 1996) have been found to degrade calystegines. In strain Rm41, the genes responsible for calystegine catabolism (*cac*) are harboured on the 225–kb self–transmissible, non–symbiotic plasmid pRme41a (Tepfer et al., 1988). Subcloning revealed that the *cac* locus is spread over a region 30 to 40 kb in length (Boivin et al., 1990). Since *S. meliloti* Rm41 can use calystegines as a source of carbon and nitrogen for growth (Tepfer et al., 1988a), it is thought that the genes coding for the catabolism of these alkaloids can contribute to strain survival during its saprophytic life, i.e. in the absence of the host plant alfalfa (Boivin et al., 1990). In accordance with this concept, Tepfer et al. (1988a) found calystegine–degrading bacteria in the rhizosphere of calystegine–producing plants but not in that of calystegine–negative plants, suggesting a role for calystegines as nutritional mediators in plant–microbe interactions.

However, the general understanding of the role of calystegines in the rhizosphere is based exclusively on the results of calystegine degradation tests performed with only few isolates of rhizosphere bacteria only (Tepfer et al., 1988a; Goldmann et al., 1996). The microbial community of the rhizosphere is highly diverse and it is unlikely that determinations carried out with individual microorganisms could provide a full account on the role of calystegines in plant–microbe interactions. In this study, the relationship between the ability of plants to produce calystegines and that of rhizosphere microorganisms to degrade calystegines was investigated at microbial community level. For this purpose, a new assay was developed to score the capacity of the rhizosphere microbiota to degrade calystegines purified from *C. sepium*. The assay requires the
presence of calystegine-degrading microorganisms at significant population levels for a positive response.

**Materials and methods**

**Plants**

Seeds of *C. sepium* were collected in parks located in the city of Zürich (Switzerland), whereas those of *Z. mays* cv. Silex 170 were obtained from the Seeds Department of the Swiss Federal Research Station for Agroecology and Agriculture (FAL) at Zürich–Reckenholz. A culture of *C. sepium* roots transformed by *Agrobacterium rhizogenes* (Jung and Tepfer, 1987) was used for the production of calystegines because the introduced T–DNA resulted in increased root biomass and alkaloid content of roots. The transformed roots were cultivated in 65 ml of Gamborg B5 medium (Sigma, Buchs, Switzerland) in 300 ml Erlenmeyer flasks with continuous shaking (120 rpm) at 24°C in the dark for three weeks.

**Purification of calystegines**

The transformed roots were removed from liquid cultures, blotted dry and stored at −20°C prior to use. For extraction of calystegines, portions of about 5 g of roots were frozen in liquid N2 and homogenised using a mortar and pestle. The samples were transferred into 1.5 ml Eppendorf tubes and centrifuged at 13,000 × g for 40 min. To remove tannins and phenols, the supernatants (which contained about one third of the initial amount of plant material) were treated with 45% w/v neutral aluminium oxide 90 (E. Merck AG, Dietikon, Switzerland). The samples were mixed with acetonitrile in a 1:9 v/v ratio and passed through a LC–NH2 solid phase extraction column (Supelco, Buchs, Switzerland) conditioned with 6 ml of acetonitrile solution (i.e. water:acetonitrile in a 1:9 ratio), with the objective to remove salts and ions. The column was washed with 3 ml of acetonitrile solution and elution was performed with 3 ml of 0.001 M HCl. The eluates were then passed through an ion exchange LC–SCX column (Supelco) conditioned with 6 ml of 0.001 M HCl, with the aim to separate sugars. After washing the column with 3 ml of 0.001 M HCl, the column was rinsed with 2 M NH3. The eluates were evaporated to dryness in vacuo and redissolved in 250 µl of sterile distilled water.
Analysis of calystegines

The method of Dräger (1995) for calystegine analysis was slightly modified with the objective of facilitating identification of the alkaloids. Samples (2 µl) were applied to a thin-layer chromatography plate (Kieselgel 60, Merck). The plate was developed in a solvent system consisting of CH$_3$OH, 0.6 M NH$_4$Cl and CH$_3$Cl in a 6:2:1 ratio, until the solvent front was 8 cm from the base line. The chromatogram was dried and immersed into a solution prepared with 0.1 ml saturated aqueous AgNO$_3$ and 20 ml acetone for 60 s, before being transferred to a solution of ethanol containing 0.5 M NaOH until brown spots (calystegines) appeared on the white chromatogram. Finally, the chromatogram was fixed in a bath of photographic fixator G 345 (Agfa-Gevaert AG, Dübendorf, Switzerland). After each bath, the chromatogram was dried using a hair dryer. Purity of each extract was checked by comparison with a calystegine standard obtained by D. Tepfer (INRA Versailles, France) according to published protocols (Tepfer et al., 1988). Calystegine concentration in the extracts was adjusted to 2 µg µl$^{-1}$ prior to use in degradation assays.

Biological analysis of extracted calystegines was carried out using S. meliloti strain Rm41(abc) and its derivative Rm41(bc) cured of the cac plasmid pRme41a and unable to catabolise calystegines (Tepfer et al., 1988). The strains were grown in liquid tryptone–yeast extract (TY; Beringer, 1974) at 27°C with shaking (120 rpm) until cultures reached an optical density of 1 at 600 nm (i.e. approximately 7 × 10$^8$ colony–forming units (CFU) ml$^{-1}$; late exponential phase). The cells were washed and resuspended in fresh M9 (Maniatis et al., 1982) without carbon source. Calystegine degradation was assayed in reaction mixtures (final volume 208 µl) consisting of 200 µl of M9 medium without carbon source, 8 µl of calystegine extract or calystegine standard (final concentration: 77 µg ml$^{-1}$) and the test bacterium (cell density of 7 × 10$^7$ CFU ml$^{-1}$). After incubation for 24 h at 27°C with shaking (120 rpm), the samples were centrifuged (10 min, 13,000 × g) and the supernatant evaporated to dryness in vacuo. The residues were taken up in 8 µl sterile distilled water. A 2 µl aliquot was tested for the presence of calystegines by thin–layer chromatography, as described above (Figure 1). For each strain, seven assays were performed with extracted calystegines and seven assays with calystegine standard.

Bioassay for calystegine degradation by rhizosphere microbiota

Samples of about 0.25 g of root (and closely–adhering soil) were transferred each into a 50–ml Erlenmeyer flask containing sterile distilled water (sample:water ratio of 1:100 w/v) and the flasks were shaken for 15 min at 350 rpm. Purified calystegines (8
µl aliquots) were added to 200 µl of extract (final concentration 77 µg ml⁻¹) and the samples were incubated at 27°C with shaking (120 rpm) for 24 h. They were then centrifuged at 13,000 × g for 10 min and the supernatant was evaporated to dryness in vacuo. The residue was taken up in 8 µl of sterile distilled water and analysed for the presence of calystegines by thin-layer chromatography. The absence of calystegines indicated that the rhizosphere sample studied harboured microorganisms with the capacity to degrade these alkaloids and such roots were scored as being positive for microbial calystegine degradation (i.e. MCD roots).

To demonstrate that the absence of calystegines in samples could not have resulted from physico-chemical interactions (adsorption) between the alkaloid and soil particles, the three soils used in microcosm experiments in this study (Eschikon, Reckenholz and Mürren; Table 1) were each suspended in sterile distilled water (1, 3, 30 and 300 mg soil ml⁻¹). Purified calystegines (8 µl aliquots) were added to 200 µl of each suspension. The samples were vigorously shaken at 1000 rpm for 1.5 h and checked for the presence of calystegines, as described above. Three replications were studied per treatment. Calystegines were clearly detected in all samples from each soil, even when soil was used at a rate as high as 300 mg ml⁻¹ (data not shown). Therefore, the presence of soil particles in rhizosphere extracts could not have resulted in false positive results of the bioassay.

Table 1. Main properties of the soils used in the microcosm experiments, which were cambisols.

<table>
<thead>
<tr>
<th>Property</th>
<th>Eschikon</th>
<th>Reckenholz</th>
<th>Mürren</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crop at sampling</td>
<td>maize</td>
<td>maize</td>
<td>potato</td>
</tr>
<tr>
<td>Texture</td>
<td>loam</td>
<td>loam</td>
<td>sandy clay loam</td>
</tr>
<tr>
<td>Clay (%)</td>
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<td>21</td>
<td>27</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>42</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>Sand (%)</td>
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<td>47</td>
<td>50</td>
</tr>
<tr>
<td>pH&lt;sub&gt;H2O&lt;/sub&gt;</td>
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<td>7.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Cation-exchange capacity (cmol kg⁻¹)</td>
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<td>18.8</td>
<td>49.0</td>
</tr>
<tr>
<td>CaCO₃ (%)</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Organic matter (%)</td>
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<td>3.0</td>
<td>12.8</td>
</tr>
<tr>
<td>N (%)</td>
<td>0.16</td>
<td>0.27</td>
<td>0.90</td>
</tr>
<tr>
<td>Altitude (m) above sea level</td>
<td>530</td>
<td>442</td>
<td>1590</td>
</tr>
</tbody>
</table>

* All soils were sampled from the surface horizon of the cropped cambisols.

For validation of the bioassay, S. meliloti strain Rm41(abc) and its derivative Rm41(bc) were used. The strains were prepared as described above, except that cells were washed and resuspended in sterile distilled water. The cell suspensions were diluted in sterile distilled water, as needed. Part of rhizosphere extracts obtained from C. sepium plants sampled at Eschikon (described in section 2.6) were sterilised by autoclaving. The bioassay was as described above, except that 190 µl of extract was
used (instead of 200 µl). Inoculation was performed with 10 µl of cell suspension of strains Rm41(abc) or Rm41(bc), and final cell densities were $5 \times 10^2$ to $5 \times 10^7$ CFU ml$^{-1}$ for strain Rm41(abc) and $5 \times 10^6$ or $5 \times 10^7$ CFU ml$^{-1}$ for strain Rm41(bc). The assay was then carried out as described above. Calystegine degradation was studied for non-sterile rhizosphere extract, as well as sterilised rhizosphere extract inoculated with strains Rm41(abc) or Rm41(bc), and seven replications were used per treatment.

An experiment was done to determine changes in bacterial populations during the 24–h calystegine degradation bioassay. First, the bioassay was performed using non-sterile rhizosphere extracts obtained from Z. mays (15 samples) or C. sepium (15 samples) grown in Eschikon soil. Total numbers of culturable aerobic bacteria were determined by colony counts on 10% tryptic soy agar. Second, the bioassay was carried out with sterilised rhizosphere extract of Z. mays (5 samples) or C. sepium (5 samples) inoculated with S. meliloti Rm41(abc) at $7.5 \times 10^6$ CFU ml$^{-1}$. Culturable cells of the strain were enumerated by colony counts on TY agar. All plates were incubated at 27°C for 3 days and the colonies counted.

Survey of the proportion of MCD Z. mays roots in fields

Five Z. mays fields were chosen at random in a 6 km × 8 km region East of Zürich (Figure 2) in July 1996. In each field, a 5 m$^2$ area free of C. sepium was selected and ten Z. mays plants were sampled. Root segments were collected from the surface horizon, at a depth of approximately 10 cm below the soil surface (one sample of about 0.25 g of root + closely-adhering soil per plant). Each sample was assayed for calystegine degradation, as described above. For each field, the percentage of MCD roots was calculated based on results obtained with the ten samples.

Proportion of MCD Z. mays roots in plots with and without C. sepium at two field locations

Two Z. mays fields with a patchy C. sepium infestation were chosen near Zürich. The two soils correspond to cambisols (Table 1). One is located at Eschikon (Défago et al., 1987) and the other at Reckenholz (Nievergelt, 1991). At each location, one plot infested with C. sepium and one plot not infested by the bindweed were chosen. Each plot was 5 m$^2$ in surface. On July 9, 1996, ten Z. mays plants were chosen at random from each plot and root segments assayed for calystegine degradation, as described above. The same procedure was followed to study also ten root segments of C. sepium from each of the two plots infested by C. sepium. The whole experiment was repeated on July 24, 1996 using the same plots sampled two weeks before.
Origin of calystegine–degrading rhizosphere microorganisms

The proportion of MCD roots of *Z. mays* and *C. sepium* grown in autoclaved soil microcosms was investigated to determine whether calystegine–degrading microorganisms are seed–borne. Surface soil from Eschikon plots infested by *C. sepium* (see section 2.6) was passed through a 5 mm mesh screen and transferred into 300-ml Erlenmeyer flasks (65 g soil flask⁻¹). The flasks were autoclaved for 20 min at 121°C on two occasions that were two days apart. Seeds of *C. sepium* were collected aseptically from intact fruit capsules and scarified with a sterile scalpel in a sterile hood. Likewise, seeds of *Z. mays* were used without surface disinfection. All seeds were germinated on 0.85% water agar at 24°C in the dark for three days before planting (one seedling per microcosm). The experiment was also performed using *C. sepium* rhizome instead of seed since the species can also propagate vegetatively. Rhizome pieces corresponding to two nodes (about 4 cm in length) were washed with sterile distilled water to remove soil particles prior to planting. The flasks were put in a growth chamber with 16 h of light (22°C) and 8 h of dark (18°C). The water content of the soil was approximately 20% w/w throughout the experiment. Destructive sampling was carried out at 1, 7 and 21 days after planting and the proportion of MCD root systems was determined. The experiment followed a fully randomised design and five replicates (i.e. five flasks) of each treatment were studied at each sampling time. The experiment was repeated on three independent occasions.

Proportion of MCD roots of *Z. mays* and *C. sepium* in microcosms prepared with soil from fields where calystegine–producing plants are present

To gain some insight into the ability of calystegine–degrading microorganisms to colonise the rhizosphere of calystegine–negative (*Z. mays*) and calystegine–positive plants (*C. sepium*), microcosm experiments were performed using natural soil from field sites infested by *C. sepium* (i.e. Eschikon) or free of *C. sepium* but grown with the calystegine–positive plant *S. tuberosum* (syn. potato) (i.e. cambisol from Mürren, Bern canton; Table 1). The microcosms were prepared as described above, except that soil was not sterilised. The seeds were surface–disinfected to remove any possibility that calystegine–degrading microorganisms associated with the seeds could have colonised the rhizosphere. This was achieved by immersing seeds of *C. sepium* (after scarification in 95% *H₂SO₄* for 60 min) and *Z. mays* successively in 5% *NaOCl* for 15 min and 10% *H₂O₂* for 15 min. Seeds were germinated on 0.85% water agar at 24°C in the dark for three days prior to planting (one seedling per microcosm). The experiment was not performed with rhizomes of *C. sepium* because an effective method for
surface disinfection of rhizome is not available. The water content of the soil was approximately 20% w/w throughout the experiment. Growth chamber conditions, sampling characteristics and determinations were as described above. As in the experiment performed with autoclaved microcosms, a randomised design was used and each treatment was studied in five replicates at each sampling time. The experiment was repeated three times. Similar results were obtained in each of the three repeated experiments (as indicated by non-parametric Kruskal–Wallis statistics), therefore all data were pooled for statistical analyses.

Table 2. Colony counts \(^a\) of microorganisms in rhizosphere extracts from C. sepium or Z. mays grown in Eschikon soil, at the beginning and the end of the 24-h calystegine-degradation bioassay.

<table>
<thead>
<tr>
<th>Microorganisms present</th>
<th>Plant species</th>
<th>Colony counts (log CFU ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Resident microorganisms</td>
<td>C. sepium</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Z. mays</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>S. meliloti strain Rm41(abc)</td>
<td>C. sepium</td>
<td>5.8(^b)</td>
</tr>
<tr>
<td></td>
<td>Z. mays</td>
<td>5.8</td>
</tr>
</tbody>
</table>

\(^a\) Means are shown ± standard deviations. They were calculated after pooling all data from three independent experiments. The plant species had no influence on colony counts of the resident microorganisms (at each sampling), or on colony counts of strain Rm41(abc) at 24 h.

\(^b\) Strain Rm41(abc) was added to sterilised rhizosphere extracts at the start of the experiment.

Data analysis

The field survey was not replicated and therefore the observations were not analysed statistically. In the field experiment, non-parametric Kruskal–Wallis statistics were used to test the effects of sampling time (July 9, 1996 and July 24, 1996), field site (Eschikon and Reckenholz), and plants (C. sepium, Z. mays with and without C. sepium). Mann–Whitney U tests were used for pairwise comparisons between the three plant treatments (i.e. C. sepium, Z. mays in plots with C. sepium, Z. mays in C. sepium–free plots). Data obtained in non-sterile soil microcosms were studied by Kruskal–Wallis statistics to determine the effects of soil origin (Eschikon and Mürren) and plant species (Z. mays and C. sepium). Mann–Whitney U tests were used for pairwise comparisons between the four treatments (Z. mays in Eschikon soil and in Mürren soil, and C. sepium in Eschikon soil and in Mürren soil). All analyses were performed at \(P = 0.05\) and Systat 5.05 (SPSS Inc., Evanston, IL) was used.
Results and Discussion

Calystegine extraction

Several methods have been proposed to extract calystegines from the roots of *C. sepium* or other plant species (Tepfer *et al.*, 1988; Todd *et al.*, 1995; Dräger, 1995; Asano *et al.*, 1996). In the current work, solid phase extraction columns were exploited to achieve rapid (one h) extraction of calystegines from *C. sepium* roots. One extraction carried out using 5 g of plant material yielded between 400 and 500 µg of purified calystegines. The efficacy of the extraction was similar to that achieved by Dräger (1995), who obtained about 100 µg of calystegines per g of fresh root.

![Thin-layer chromatogram for determination of calystegines in the calystegine-degradation bioassay](image)

**Figure 1.** Thin-layer chromatogram for determination of calystegines in the calystegine-degradation bioassay. Calystegines (Cal. A and Cal. B) are seen in the calystegine control and in the bioassay carried out with root samples 2, 3 (*Z. mays*) and 6, 9, 10 (*C. sepium*). As control, the bioassay was done also without the addition of calystegines ('Water'). Root samples 1, 4, 5 (*Z. mays*) and 7, 8 (*C. sepium*) were scored as MCD as no calystegines were seen.

The purity of the calystegine extracts obtained here was similar to that of the calystegine standard produced as described by Tepfer *et al.* (1988), as indicated by thin-layer chromatography (data not shown). *S. meliloti* Rm41(abc) degraded the purified calystegines obtained in the current work as well as the calystegine standard, whereas its cac– derivative Rm41(bc) did not catabolise purified calystegines or the calystegine standard.
Effectiveness of the bioassay proposed to study calystegine degradation by rhizosphere microbiota

A majority of rhizosphere extracts from rhizomes of *C. sepium* sampled at Eschikon degraded calystegines in the bioassay proposed here. In contrast, calystegine degradation did not take place when the bioassay was performed with rhizosphere extracts sterilised by autoclaving. These results indicate that calystegine degradation in the bioassay was mediated by microorganisms.

When sterilised rhizosphere extracts were inoculated with a cell suspension of *S. meliloti* Rm41(abc) prior to conducting the bioassay, calystegine degradation was observed in treatments where the strain was introduced at $5 \times 10^6$ or $5 \times 10^7$ CFU ml$^{-1}$, but not when strain Rm41(abc) was inoculated at population levels of $5 \times 10^5$ CFU ml$^{-1}$ or lower. However, full calystegine degradation took place in all treatments with strain Rm41(abc) when assays were incubated for four days instead of 24 h, regardless of inoculation levels. Calystegine degradation did not take place when sterilised extracts were inoculated with the *cac~* strain Rm41(bc), even when the duration of incubation was increased to four days. These results suggest that complete calystegine degradation requires the presence of calystegine-degrading microorganisms at high cell numbers, and that these calystegine-degrading microorganisms could grow during the bioassay. Indeed, the population size of culturable cells of strain Rm41(abc) increased by two orders of magnitude during the bioassay, regardless of whether rhizosphere extracts were obtained from *C. sepium* or *Z. mays* (Table 2). It is possible that the efficiency of calystegine degradation by strain Rm41(abc) and the resident soil microorganisms differs. However, the finding that full calystegine degradation by *S. meliloti* Rm41(abc) in the 24-h bioassay took place only when the strain was added at levels of $5 \times 10^8$ CFU (g root)$^{-1}$ or higher suggests that the calystegine-degradation bioassay may be useful to identify rhizospheres comprising a significant microbial community displaying this catabolic capacity.

Bacterial growth took place also when the bioassay was performed using non-sterile rhizosphere extracts of either plant, as indicated by the increase in colony counts of the resident aerobic bacteria during the bioassay (Table 2). Those counts were lower than CFUs of strain Rm41(abc), probably because nutrient availability was higher in autoclaved soil. Similar numbers of resident culturable aerobic bacteria were obtained whether the samples scored MCD or not, suggesting that bacterial growth did not depend on calystegine degradation in this bioassay.
Table 3. Proportion (%) of roots of C. sepium and Z. mays with a microbial rhizosphere community capable of calystegine degradation (i.e. MCD roots) in natural soil microcosms prepared with Eschikon soil (site infested by C. sepium) or Mürren soil (site cropped with S. tuberosum).

| Soil origin | Plant species  | Sampling time \n| | | Day 1 | Day 7 | Day 21 |
|---|---|---|---|---|
| Eschikon | C. sepium | 33 ± 12 a | 47 ± 12 ab | 58 ± 8 a |
| | Z. mays | 40 ± 0 a | 13 ± 12 a | 18 ± 17 b |
| Mürren | C. sepium | 53 ± 12 a | 60 ± 20 b | 58 ± 22 a |
| | Z. mays | 53 ± 42 a | 67 ± 31 b | 43 ± 12 ab |

<table>
<thead>
<tr>
<th>Statistical effects</th>
<th>Soil origin</th>
<th>Plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS d</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

a Means are shown ± standard deviations.
b Seeds of C. sepium were scarified with H2SO4 before surface-disinfection. Seeds of Z. mays and C. sepium were surface-disinfected with 5% NaOCl and 10% H2O2.
c At each sampling time, the statistical relationships between the four treatments is shown with the letters a and b (P=0.05).
d NS, not significant; *, significant at P=0.05.

Occurrence of MCD roots of a calystegine-negative plant (Z. mays) in the field

The calystegine-degradation bioassay was used to study Z. mays plants sampled at five field sites (Figures 1 and 2). MCD Z. mays roots were found at each location, and their proportion was comprised between 20% (Pfäffikon) and 80% (Uster, Seegräben and Auslikon). Overall, calystegine-degrading microorganisms at levels sufficient to achieve full degradation of the alkaloids in the bioassay were present in 32 of the 50 rhizosphere samples studied. C. sepium, which is the main calystegine-producing plant known in this agrosystem, was not found at any of the five sites.

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Figure 2. Locations in the region of Zürich, Switzerland where the proportion of MCD Z. mays roots was sampled. The proportion of MCD Z. mays roots is given as size of the black circle relative to the dotted circle. A plot free of C. sepium was chosen at random at each location and one root sample was assayed for each of 10 plants collected at random in each plot. Overall, the proportion of MCD Z. mays roots in the survey was 64%.
These results were not expected as Z. mays does not produce calystegines. Tepfer et al. (1988a) did not find any bacterial isolate capable of degrading calystegines in the rhizosphere of plants other than calystegine producers. Similarly, Goldmann et al. (1996) observed that 11 of 51 bacterial isolates obtained from the rhizosphere of C. sepium or C. arvensis could degrade calystegines, whereas no calystegine-degrading bacterium was obtained from the rhizosphere of calystegine-negative plants. However, the number of strains investigated in these two studies was limited, because each bacterial isolate had to be tested individually for calystegine degradation. Indeed, the model strain for calystegine degradation is S. meliloti Rm41(abc) (Tepfer et al., 1988), which was isolated from a nodule of Medicago sativa L. and not from C. sepium (Ördögh and Szende, 1961). The fact that the proportion of MCD Z. mays roots was high (Figure 2) suggests that microorganisms capable of calystegine degradation are widespread in soil. Based on results obtained with strain Rm41(abc), it can be estimated that calystegine-degrading microorganisms may represent as much as a few percents of all culturable rhizosphere bacteria for roots scored as MCD. From these figures, it is conceivable that calystegine-degrading isolates could have been missed in previous studies.

Influence of the presence of C. sepium on the proportion of MCD roots of Z. mays in the field

The effect of C. sepium infestation on the proportion of MCD Z. mays roots was assessed in field experiments where adjacent plots free of C. sepium or infested by the bindweed were available. Whereas data obtained at Eschikon were similar to those from Reckenholz, sampling time had a strong influence on the proportions of MCD roots (Figure 3). Apparently, calystegine-degrading microorganisms became favoured for colonisation of the rhizosphere of Z. mays by the second sampling, perhaps as a consequence of specific climatic conditions or changes in exudation patterns of Z. mays roots linked to plant development between the two samplings. Since Z. mays is calystegine-negative, this effect appears to be independent of the presence of calystegines in the rhizosphere. Overall, the proportion of MCD Z. mays roots was statistically higher in plots infested by the bindweed than in those without C. sepium (Figure 3), obviously a result contributed by data from the first sampling. In bindweed infested plots, C. sepium and Z. mays displayed a same proportion of MCD roots.

The presence of C. sepium plants in these plots implies that calystegines could be released into soil as part of root exudates and/or in decaying roots of C. sepium. The presence of calystegines in soil organic matter may sustain the presence of calystegine-degrading microorganisms at significant population levels in
non-rhizosphere soil and contribute to their subsequent colonisation of the rhizosphere of a calystegine-negative plant like *Z. mays*. Unfortunately, no information is available on the persistence of calystegines released into soil after the death of roots. Other types of alkaloids have been shown to persist in soil and become adsorbed to soil particles (Starr *et al*., 1995). The ability of bacteria to catabolise a specific compound present in root exudates e.g. opine, rhizopine or flavonoids is thought to improve their growth (Hartwig *et al*., 1990; Guyon *et al*., 1993) or competitiveness in the rhizosphere (Murphy *et al*., 1995; Oger *et al*., 1997; Savka and Farrand, 1997). Calystegines may have a wider significance by favouring calystegine-degrading microorganisms not only in the rhizosphere of calystegine-producing plants but perhaps also in bulk soil containing residual calystegine.

A

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C. sepium

Z. mays in plots infested with *C. sepium*

Z. mays in plots free of *C. sepium*
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![Figure 3. Proportion (%) of MCD roots of *Z. mays* and *C. sepium* in field experiments carried out at Eschikon and at Reckenholz (A). *Z. mays* was sampled from plots infested with *C. sepium* and from plots without bindweed. The statistical effect of the experimental factors were studied by Kruskal–Wallis and Mann–Whitney *U* statistics (*)](image)

### B

<table>
<thead>
<tr>
<th>Statistical Effects</th>
<th>Probability</th>
<th>Significance at <em>P</em>=0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling time</td>
<td>0.004</td>
<td>*</td>
</tr>
<tr>
<td>Field site</td>
<td>0.502</td>
<td></td>
</tr>
<tr>
<td>Plants</td>
<td>0.004</td>
<td>*</td>
</tr>
<tr>
<td><em>C. sepium</em> versus <em>Z. mays</em> within <em>C. sepium</em>–infested plots</td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td><em>Z. mays</em> in <em>C. sepium</em>–infested versus <em>C. sepium</em>–free plots</td>
<td>0.047</td>
<td>*</td>
</tr>
</tbody>
</table>

**Figure 3**. Proportion (%) of MCD roots of *Z. mays* and *C. sepium* in field experiments carried out at Eschikon and at Reckenholz (A). *Z. mays* was sampled from plots infested with *C. sepium* and from plots without bindweed. The statistical effect of the experimental factors were studied by Kruskal–Wallis and Mann–Whitney *U* statistics (*).

**Propagation of calystegine–degrading rhizosphere microorganisms**

No root of *C. sepium* or *Z. mays* was MCD when autoclaved Eschikon soil was sown with seeds. Since a majority of roots of either plant species were MCD at the
Eschikon field site (Figure 3), this result indicates that the rhizosphere microorganisms responsible for the ability of rhizosphere extracts to degrade calystegines do not belong to the microbiota naturally associated with the seed. Indeed, a significant proportion of *C. sepium* samples were MCD when autoclaved Eschikon soil was planted with washed rhizomes of the plant (i.e. 25 ±3%, 50 ±3% and 35 ±17% at 1, 7 and 21 days after planting, respectively; results pooled from three independent experiments). Furthermore, this indicates that calystegine-degrading microorganisms can be present in close association with the surface of the *C. sepium* rhizome.

**Proportion of MCD roots of *Z. mays* and *C. sepium* grown in microcosms prepared with soil from fields where calystegine-producing plants are present**

In natural soil microcosms, a significant proportion of seedlings were already MCD at one day after planting of axenic seedlings in Eschikon or Mürren soil, regardless of whether *C. sepium* or *Z. mays* was studied (Figure 1, Table 3). This result suggests that calystegine-degrading microorganisms were present at significant numbers in soil before the introduction of the plants, a hypothesis already formulated in the case of soils from Eschikon and Reckenholz (see section 3.4). The presence of microorganisms capable of calystegine degradation in *C. sepium*-free soil from Mürren (1600 m above sea level) cannot be explained by earlier *C. sepium* infestation since this plant species does not grow at this altitude in Switzerland. However, the Mürren soil has been cropped continuously with *S. tuberosum*, another calystegine-producing plant species (Nash et al., 1993).

In Mürren soil, the proportion of MCD roots remained similar for *C. sepium* and *Z. mays* at the other two samplings carried out (Table 3). In contrast, the proportion of MCD *Z. mays* roots in Eschikon soil significantly decreased by day 7 and did not change from days 7 to 21, whereas the proportion of MCD roots of *C. sepium* increased somewhat in time. Consequently, MCD roots were three times more frequent for *C. sepium* than for *Z. mays* at 21 days after sowing. Obviously, calystegine-degrading microorganisms were more competitive for colonisation of *C. sepium* than *Z. mays* in this soil. The reason why this did not take place in Mürren soil remains to be determined. Perhaps the amount of calystegines in a soil under continuous *S. tuberosum* cultivation (Mürren) was higher than that resulting from natural *C. sepium* infestation (Eschikon), so that in the rhizosphere the calystegine status of the plants was more influential in Eschikon soil than in Mürren soil.
Significance of calystegines and calystegine degradation capacity for soil microorganisms

Microorganisms with the capacity to degrade organic substrates specific of certain plant roots are considered to be favoured for colonisation of the rhizosphere of these plants (Hartwig et al., 1990; Guyon et al., 1993; Murphy et al., 1995; Oger et al., 1997; Savka and Farrand, 1997). This concept has been evoked repeatedly in the case of rhizosphere bacteria with the ability to catabolise the calystegines produced by the roots of plants like C. sepium (Tepfer et al., 1988a; Boivin et al., 1990; Goldmann et al., 1996). However, MCD roots were commonly found in the case of Z. mays, a calystegine–negative plant. Apparently, calystegine–degrading microorganisms were neither restricted to the rhizosphere of calystegine–producing plants nor impaired for colonisation of the rhizosphere of calystegine–negative plants. So far, only rhizobia and pseudomonads have been identified among rhizosphere isolates capable of calystegine degradation (Tepfer et al., 1988a; Goldmann et al., 1996). Calystegine degradation by bacteria other than S. meliloti Rm41(abc) does not involve the genes cac as in the latter strain (Tepfer et al., 1988a; Goldmann et al., 1996), raising the possibility that the rhizosphere microbiota involved in calystegine degradation is highly diverse both in terms of microbial taxonomy and catabolic pathways.

The occurrence of MCD roots of Z. mays and C. sepium shortly (one day) after introduction of axenic seedlings into natural soil suggests that calystegine–degrading microorganisms were present at significant population levels in soil, perhaps because of the presence of calystegines in soil organic matter following the death of roots containing these alkaloids. If this were the case, it could provide an explanation for the higher proportion of MCD Z. mays roots in field plots infested by C. sepium compared with plots not infested. In this context, the ability of symbiotic bacteria such as S. meliloti Rm41(abc) to catabolise calystegines would constitute not only an adaptation to alternative, non–symbiotic host plants but probably also a selective advantage for survival in soil in absence of roots.

Acknowledgements

We are indebted to D. Tepfer (INRA Versailles, France) for kindly providing calystegine standard, root culture of transformed C. sepium and strains Rm41(abc) and Rm41(bc). We thank J. von Allmen for providing soil from Mürren, H.P. Pfirter for discussions and technical assistance, and U. Schenk and M. Hildman for technical
assistance. This work was supported in part by the COST Action 816 (European co-operation in the field of scientific and technical research).

**Literature cited**


Leer - Vide - Empty
Chapter VI.

General Discussion

There are several reasons why *Calystegia sepium* and *Convolvulus arvensis* are very successful weeds in agricultural fields and in amenity areas. These perennial weeds with their deep reaching rhizomes are able to escape many of the methods used for weed control. The abundant reserves stored in these rhizomes enable the plants to survive repeated defoliations. Fragmentation and dispersal of rhizomes leads to vegetative propagation, because each rhizome piece with a bud develops into a new plant.

This study aimed at measuring the efficacy of the mycoherbicide *Stagonospora convolvuli* Dearness & House strain LA39 (Pfirter and Défago, 1998) for bindweed biocontrol in the field and determining its impact on bindweed growth, biomass allocation and carbohydrate reserves. Another objective consisted in improving bindweed biocontrol by combining the mycoherbicide and plant competitors in the greenhouse and the field. Lastly the potential of the specific microbial degradation of calystegines, secondary metabolites of the bindweeds (Tepfer et al., 1988), for bindweed biocontrol was studied.

**Effects of the mycoherbicide on bindweed growth**

Biocontrol agent *Stagonospora convolvuli* strain LA39 was chosen for this work, because (1) it had been effective for bindweed biocontrol in greenhouse experiments, (2) efficient formulation and application methods have been found and (3) a cheap and reliable mass production medium was known (Pfirter and Défago, 1998; Pfirter and Guntli, unpublished data).

In Chapter III Part 1 of this thesis, the effects of *S. convolvuli* strain LA39 on *C. sepium* were studied in pots placed outside to mimic field conditions. The pots were used in order to be able to observe the entire root systems of the plants and to exclude interference with other plants (Klimeš and Klimešová, 1994). A disadvantage of this
approach is the disturbance of the development of the underground plant parts in the pots.

Plants were grown from seeds or vegetatively propagated from rhizomes to represent both modes of propagation of the bindweeds. Both are likely to occur in agricultural fields in that the transmittable seeds cause new infestations, while rhizomes are responsible for bindweed multiplication in an infested field. Another factor included in this experiment were two levels of nutrient availability simulating different fertilisation levels known to interfere with the growth of *C. sepium* (Klimeš and Klimešová, 1994) and likely to be encountered in different fields.

In this experiment, LA39 infected the bindweeds. LA39-treated plants had fewer leaves, their climbing stems were higher, more nodes and flowers were produced and the carbohydrate reserves content of the rhizomes was reduced. However, total plant biomass was not affected by LA39 in this experiment.

These results indicate that the effects of LA39 were partly compensated by increased leaf production. Necrosis of leaves and defoliation caused by LA39 probably leads to a reduced photosynthesis rate of the bindweeds. The production of new leaf surface is a known reaction of the bindweeds to reduced photosynthesis rates (Bakke and Gaessler, 1945).

However, the reduction in carbohydrate reserves of the rhizomes in the present work suggests that the perennial *C. sepium* was considerably affected by LA39 and that not all of these effects could be compensated for. Possibly, the new leaf surface was produced at the cost of rhizome carbohydrate reserves. The amount of carbohydrate reserves stored in the underground rhizomes largely influences the winter survival and the emergence of the weeds in the next growing season (Frazier, 1943; van Ast and van Groenendael, 1993). Therefore, the mycoherbicide *S. convoluli* LA39 seems to be able to control the bindweeds on the long run by depleting the carbohydrate reserves stored in the rhizomes of *C. sepium*.

Next to reducing the photosynthetic leaf surface, fungal pathogens often affect their host plants with toxic compounds called mycotoxins (Durbin, 1983). Several mycotoxins are produced by LA39 and these seem to affect bindweed development (Florence Marquis and Blaise Nicolet, personal communication).
Combination of the mycoherbicide with plant competitors for bindweed biocontrol

In the experiment reported in Chapter IV Part 1, the two green cover plants, *Trifolium pratense* and *Lolium multiflorum*, each suppressed the growth of the bindweeds *C. sepium* or *C. arvensis*, respectively, in the greenhouse. There was evidence that this suppression was due to competition for light between the weed and the green cover plant. LA39 caused severe necrosis and subsequent death of infected leaves regardless of the presence or the absence of the green cover plants. However, the fungus did not affect plant biomass production.

Parts 2 and 3 of Chapter VI describe two field trials conducted in maize underseeded with *T. pratense* and in an amenity area grown with *Cotoneaster dammeri*, respectively. These two situations represent one agricultural and one non-agricultural environment, and in both an established bindweed population was the major component of the weed flora. The maize field was infested with *C. sepium*, and *C. arvensis* was the weed in the amenity area.

In field trials, standard spraying equipment was used which is a realistic methodology for a future application of mycoherbicide LA39. Measurements were performed in two-week intervals to ensure a close pursuit of the development of the epidemic. Disease severity was measured with the help of a visual rating scale, as it is normally used in field experiments (Gomez and Gomez, 1984). Bindweed ground coverage was estimated with the method of Daget–Poissonet using an estimated precision of ±12% (Daget and Poissonet, 1971) which was found sufficient for describing the effects of the epidemic.

In both field trials, the fungus effectively infected the bindweeds and ground coverage was reduced starting at two weeks after application. LA39 caused severe epidemics during the respective observation periods. The disease severity increased throughout the observation periods. In the maize field trials, humid and cool weather conditions caused more severe disease and the bindweed ground coverage was more reduced in 1997 compared to 1996. In both trials, LA39 reached its target regardless of whether bindweed were growing in a dense canopy of cotoneaster or in maize underseeded with red clover.

These results indicate that LA39 is promising as a bindweed mycoherbicide in the field. LA39 could be used where there are no methods available for bindweed control, as encountered in organic farming in Switzerland. A biocontrol agent could also be an alternative in amenity areas where bindweeds are hand weeded every three to four weeks in some circumstances.
Little seems achieved if in the practical application of a mycoherbicide specific for bindweeds, the place of the bindweeds is taken over by another weed species. Therefore it is necessary, that the bindweeds are the main or only component of the weed flora. Such situations occur in the so called 'maize meadows'. In this maize production system, meadows or living green cover plants can be directly band-seeded with maize (Hall and Hartwig, 1990; Burgos and Talbert, 1996; Garibay, 1996; Garibay et al., 1997). The main advantage of a maize meadow in comparison to underseeding is the established green cover at the time of bindweed emergence. This integrated pest management (IPM) is suppressive to most weed species, but unfortunately it does not suppress the bindweeds sufficiently (Pfirter et al., 1997). However, the combination of a maize meadow with a bindweed biocontrol agent seems a promising strategy for IPM in maize.

In this thesis, *T. pratense* and *L. multiflorum* were chosen as plant competitors, because they are widely used as catch crop plants or as green cover plants. In the greenhouse, these competitor plants displayed good activity for bindweed control and they reduced biomass and the number of leaves of bindweeds (Chapter IV Parts 1 and 2). In the field trials, *T. pratense* underseeded in maize had no apparent effect on the bindweed ground cover (see Chapter VI Part 2). In comparison with bindweed growing outside the maize field, maize seemed to reduce bindweed growth as soon as rows were closed and shaded by the maize (personal observation). In amenity areas, where it is possible to choose the green cover plant, *C. dammeri* was not able to suppress *C. arvensis* in the experiment presented in this work (Chapter IV Part 3). However, the dense canopy of this plant competitor might have forwarded a favourable humid microclimate causing the strong infection by the fungal inoculum.

As plant competitors do not seem sufficient for bindweed control, they were combined with the mycoherbicide LA39 for improved activity. Greenhouse results indicate that the effects of the mycoherbicide LA39 and plant competitors on bindweeds do not interfere (Chapter IV Parts 1 and 2). In some cases the effects of green cover plants and mycoherbicide were additive. In the field, the effect of *T. pratense* on bindweed growth was much smaller than in the greenhouse environment (Chapter VI Parts 1 and 2), probably because in the field *T. pratense* was poorly established at the time of bindweed emergence compared to the greenhouse (D. Guntli, personal observation).

Apparently, the competitiveness of *T. pratense* demonstrated in the greenhouse experiment (Chapter VI Part 1) could not be exploited in the field. Underseeded *T. pratense* is sown only after the maize and usually emerges in phase with or after the bindweeds. Consequently, underseeding plant competitors seems not active for bindweed biocontrol in the field. Possibly, in alternative production systems plant
competitors could be grown at bindweed emergence, and thereby enhance the biocontrol activity of the plant competitors. However, the insufficient suppression of bindweeds in one of these systems (i.e. the 'maize meadows') indicates that there is need to search for plants more competitive towards the bindweeds.

Green cover plants are mainly used for soil erosion control in integrated production (IP). The experiments reported here showed that green covers may also have bindweed biocontrol activity as plant competitors. Furthermore, the activities of mycoherbicide LA39 and plant competitors did not interfere negatively in bindweed control. Therefore, LA39 could be implemented in integrated production. This is an essential requirement for biocontrol agents according to Müller-Schärer and Scheepens (1997).

In the pot experiment described in Chapter III Part 1, the disease severity was lower compared to the field trials described above (Chapter VI Parts 2 and 3). Different microclimates in the field and in the pot experiments could be responsible for this difference. In the field, the canopy of maize or of cotoneaster is less dried out by wind and retains moisture (e.g. dew) better compared to single pots placed apart from each other. The drier climate in the pots is hindering fungal growth and may be the reason for the lower disease severity.

Furthermore in the field experiments, the maize crop or the perennial cotoneaster green cover are plants in competition for resources with the bindweeds. Such a competition was absent in the pot experiment. The effect of the crop (maize or cotoneaster) itself may therefore also partially explain the higher disease severity observed in the field compared to the pot experiment.

The interaction of the bindweeds with microbes capable of degrading calystegines, a type of bindweed alkaloids

According to Fellows et al. (1992), calystegines have antiviral and insect deterring properties and therefore it seems likely that they are plant defence compounds. In Chapter V of this thesis, microbial degradation of the calystegines was studied. These tropane alkaloids are produced by C. sepium and C. arvensis and few other plants species (Tepfer et al., 1988; Fellows et al., 1992; Dräger, 1995). Microorganisms studied were Sinorhizobium meliloti strain Rm41 (Tepfer et al., 1988) as well as those present in natural microbial rhizosphere communities.
Calystegines can be used as carbon source by strain Rm41, and in Part 1 of Chapter V, the plasmid for calystegine degradation provided a specific advantage to S. meliloti strain Rm41 for root colonisation of calystegine-producing plants. This is the first proof of the ecological significance of calystegines for soil microbes, and it strengthens the hypotheses of Tepfer et al. (1988 and 1988a) and Goldmann et al. (1996). Similarly, plants producing opines and rhizopines have been shown to specifically favour microorganisms capable of using these compounds as carbon sources (Guyon et al., 1993; Hartwig et al., 1990; Murphy et al., 1995; Oger et al., 1997; Savka and Farrand, 1997). It has been proposed by Boivin et al. (1990) that the calystegine degradation genes found in S. meliloti strain Rm41 may be important to ensure its survival in absence of its legume host M. sativa.

In Part 2 of Chapter V, microbial communities of plant roots were examined for their capacity to degrade calystegines. A new biotest identified roots whose microbial communities had the potential for calystegine degradation (i.e. MCD roots). In the field, the frequency of MCD maize roots varied greatly, but the proportion of MCD maize roots was higher in presence than in absence of C. sepium. Calystegine-degrading microorganisms were not seed-borne but colonised the rhizosphere of seedlings from soil. Vegetatively-propagated C. sepium carried calystegine degrading microorganisms on their rhizomes.

Surprisingly, microbes capable of calystegine degradation could represent as many as a few percents of all the microbes living in the rhizosphere of plants not producing calystegines. In previous studies, calystegine degrading microorganisms have only been found associated with calystegine producing plants (Tepfer et al., 1988; Goldmann et al., 1996). However, the number of investigated single strains was limited in these studies and possibly some calystegine degrading microbes were missed. Furthermore, the calystegine degrading S. meliloti strain Rm41 was isolated from the nodule of M. sativa, which does not produce calystegines (Ördögh and Szende, 1961).

Besides their influence on rhizosphere microbes, calystegines have also been reported to be allelopathic substances (Tepfer et al., 1988a; Goldmann et al., 1996). Goldmann et al. (1996) observed that calystegines inhibit the germination and root elongation of M. sativa. Based on this observation, it can be proposed that calystegine degradation by S. meliloti Rm41 plays two roles (i) to promote rhizosphere colonisation by Rm41 of calystegine-producing plants like bindweeds and (ii) to assist the establishment of the calystegine-susceptible symbiotic host M. sativa in bindweed-infested soil by detoxifying calystegines (Goldmann et al., 1996). Perhaps this hypothesis can be extended to non-symbiotic rhizobacteria colonising the rhizosphere of M. sativa and/or other calystegine-susceptible plants. From a practical point of view, the ability to harbour calystegine-degrading microbes in the rhizosphere may be a useful
trait to look for when screening for potential plant competitors for biocontrol of bindweeds.

Whether colonisation of the rhizosphere of calystegine-positive plants by calystegine-degrading microbes can have in turn an effect on the colonised plant is not known. Tepfer et al. (1988a) and Goldmann et al. (1996) found a calystegine-degrading *Pseudomonas fluorescens* in the rhizosphere of *C. sepium*. Since many pseudomonads can promote the growth of plants, they suggested that by enriching beneficial bacteria selectively, calystegine-producing plants might create favourable conditions for their own growth. However, since calystegines are likely to represent plant defence compounds, another possibility is that calystegine-degrading microbes could have a negative impact on bindweeds by degrading the alkaloids. In this study, colonisation of the bindweed rhizosphere by calystegine-degrading *S. meliloti* strain Rm41 had no effect on the interaction between the foliar mycoherbicide *S. convolvuli* LA39 and the plant, but this was perhaps due to the fact that LA39 alone was very effective. This issue deserves further attention and needs to be considered in the case of less virulent pathogens.

The microbial composition of the rhizosphere of *C. sepium* could be altered with inoculation of bacteria. In the context of biological control, deleterious bacteria which degrade calystegines probably would be enriched in the rhizosphere of the calystegine producer *C. sepium*. We propose to include calystegine degradation as a useful trait in screening programmes designed to identify rhizobacteria potentially deleterious to bindweeds. Alternatively, this trait could be inserted genetically in bacteria deleterious to bindweeds. In this work, attempts to mobilise pRme41a into other bacterial species failed, but other genetic strategies could be considered since the *cac* genes have been cloned (Boivin et al., 1990).

Calystegines are not only produced in belowground structures of bindweeds, but also in stems and leaves (Dräger, pers. com.). Therefore, interactions between bindweeds and microorganisms in the phyllosphere might be similar to those in the rhizosphere (as seen in Chapter V). Whether microorganisms present in the phyllosphere of bindweeds (or capable of infecting leaves, like LA39) display calystegine degradation is unknown. From the viewpoint of biocontrol however, this trait is probably of less interest in the phyllosphere, where at least one effective microbial biocontrol agent (i.e. LA39) is already available.
Conclusions

The following conclusions were reached in the present thesis:

1) The pathogen *Stagonospora convolvuli* strain LA39 can contribute to biological bindweed control in the field. The mycoherbicide caused necrosis of bindweed leaves and it reduced the bindweed rhizome carbohydrate reserves, thereby affecting the vegetative propagation of the bindweeds.

2) Greenhouse experiments showed that *Lolium multiflorum* or *Trifolium pratense*, which are widely used for soil erosion control, have also a potential as competitors for the biological control of bindweeds. This effect is largely due to a superior competitiveness for light.

3) If the pathogen LA39 and plant competitors were combined, they did not negatively interfere and their effects on bindweed were additive. Therefore LA39 could be used in Integrated Production (IP) systems comprising such plants as green covers.

4) Although the plant competitors tested were successful in bindweed control in the greenhouse, they failed to control bindweed in the field experiments. There is a need to search for more competitive plants for bindweed control. This search could focus on plants competitive for light since bindweeds are poor competitors for light.

5) Like green cover plants, crops also can compete with the bindweeds. Apparently, a dense canopy of *Cotoneaster dammeri* is more suppressive to the bindweeds than *Zea mays*, which emerges after the bindweed starts to grow in the spring and closes rows late in the season. Possibly, crop rotations suppressive to bindweeds could be designed for infested fields.

6) Calystegine-degrading rhizobacteria were present in the rhizosphere of calystegine-negative plants and probably in soil. However, calystegine degrading rhizobacteria were selectively favoured for the colonisation of the rhizosphere of calystegine-producing plants like bindweeds. Therefore, the capacity to degrade calystegines could be a trait to consider when screening for microbial control agents against bindweed.
7) The calystegine-degrading *Sinorhizobium meliloti* strain Rm41 did not have any effect on the growth of the bindweeds but the bacterium is not a pathogen. Attempts to mobilise the calystegine degradation plasmid pRme41a into other bacteria failed. However, the transfer of the *cac* genes to Cac- bindweed pathogens could lead to improvement in bindweed control.

8) As calystegines might be defence compounds of the bindweeds, a factor to consider in the selection of new plant competitors for bindweed control could be their relationship to the calystegines. This search could concentrate on plants tolerant to the allelopathic compounds calystegines or harbouring calystegine degraders in their rhizosphere (obstructing the calystegine-mediated defence of the bindweeds).

9) In this thesis, a mycoherbicide, plant competitors and microbial calystegine degraders were tested for their potential for bindweed control. The results of the work suggest that combinations of different methods are much more likely to solve the bindweed problem compared to using one method alone.

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Curriculum vitae

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Leer - Vide - Empty
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