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Plant pathogens as biocontrol agents of *Cirsium arvense* – an overestimated approach?

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Abstract

Cirsium arvense is one of the worst weeds in agriculture. As herbicides are not very effective and not accepted by organic farming and special habitats, possible biocontrol agents have been investigated since many decades. In particular plant pathogens of *C. arvense* have received considerable interest and have been promoted as "mycoherbicides" or "bioherbicides". A total of 10 fungi and one bacterium have been proposed and tested as biocontrol agents against *C. arvense*. A variety of experiments analysed the noxious influence of spores or other parts of living fungi or bacteria on plants while others used fungal or bacterial products, usually toxins. Also combinations of spores with herbicides and combinations of several pathogens were tested. All approaches turned out to be inappropriate with regard to target plant specificity, effectiveness and application possibilities. As yet, none of the tested species or substances has achieved marketability, despite two patents on the use of *Septoria cirsii* and *Phomopsis cirsii*. We conclude that the potential of pathogens for biocontrol of C. arvense has largely been overestimated.

Keywords

Cirsium arvense, bioherbicide, biological control, fungi, bacteria

Introduction

Cirsium arvense (L.) Scop. (Canada thistle) is a perennial root-budding geophyte capable of sprouting from creeping roots that make it a vigorous pioneer in open, disturbed habitats especially on nutrient-rich deep soils (Tiley 2010). Likely to be native of Europe, Western Asia and North Africa (Kazinczi et al. 2001), it has spread worldwide

(Figure 1), to become one of the most noxious weeds on agricultural land (Skinner et al. 2000). The most severe problems are caused in cereal fields and pastures, especially in Europe (Guillerm and Maillet 1982, Franzini 1982, Dietl 1982, Niemeth 2001, Purgar and Hulona 2008, Macak et al. 2008, Privalov et al. 2008), North America (Alex 1966) and New Zealand (Rahman 1982). Canada thistle was introduced to North America probably in the 17th century from Eurasia (Moore 1975, Tiley 2010). There it has become an invasive weed that aggressively suppresses crops on cultivated land and native plants on fallow land (Moore 1975, Stachion and Zimdahl 1980).

C. arvense reproduces sexually with seeds and vegetatively with an expanding system of root buds. While seeds aid long distance dispersal, the clonal propagation via the root system is considered to be most important for the effective colonization of a given location. New shoots develop out of root buds and build up dense patches of thistle shoots over the whole growth period. The formation of 106 shoots per square metre supported by a root system measuring 399 m in total length was observed by Stach (1996). With respect to the effect of Canada thistle on agriculture it is noteworthy that new shoots can develop out of very short root parts if the latter bear at least one root bud. At the end of the growing season only the above ground green parts of the plants die, while the root system overwinters.

The density of shoots and the long root system suppress the growth of most other plants. In the case of arable crops this causes a suppression of the cultivated plants. Yield losses of up to 60% have been reported depending on the kind of crop and on the weed density. In cereal crops for example, densities of 6 to 20 Canada thistle shoots per square metre cause up to 30% loss in grain yield. The overall global annual losses have been estimated at 320 million US$ (Bailey et al. 2000).

In conventional farming, herbicides are commonly applied to control Canada thistle. However, herbicides can damage non-target plant species (Matarczyk et al. 2002, Rodwell and Sheffield 2005), other trophic levels (Bunemann et al. 2006) and adjacent ecosystems (Hayes et al. 2002, Relyea 2005, Perez et al. 2007). Additionally, in the case of *C. arvense* herbicides mostly affect the aboveground plant parts and not the root system. Therefore, they need to be applied several times a year and every year anew, making this procedure ineffective and expensive. In New Zealand, for example, the annual costs for herbicides, mowing and vaccination of grazing animals wounded by the thistle's spines (Gourlay 2004) amount to NZ$ 27 million just for the pastoral industry in two regions of New Zealand.

In organic farming, where herbicides are not accepted, several other methods for thistle growth control are used. Hoeing and mowing, for example, are used as mechanical control methods (Hurrell and Bourdot 1996, Bacher et al. 1997, Kluth et al. 2003, Graglia et al. 2006, Lukashyk et al. 2008). Both of them do not harm the thistle substantially as they do not destroy the root system. On the contrary, hoeing can even support the clonal spread of thistles, because they are able to form new shoots out of very short root cuttings. Mowing may even have a positive effect on the performance of the thistle as it can reduce the competitiveness of associated plant species (Edwards et al. 2000).

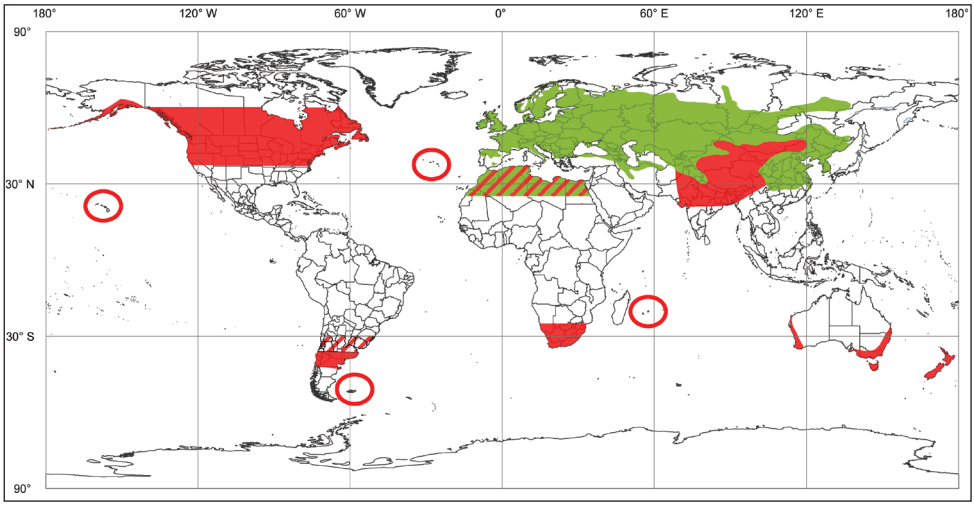

Figure 1. Distribution area of *Cirsium arvense*. The green area represents the native area, red indicates the invaded area. Circles indicate major invaded island groups. For northern Africa it seems not to be clear if this is part of the native area or already invaded. In South America the hatched area indicates that the invaded area could be larger but references seem to be scarce. According to Meusel and Jäger (1992), Weber (2003), Tiley (2010), ISSG (2011).

Another possibility to curtail weeds is biological control with the help of biocontrol agents, usually insects, fungi, bacteria or viruses (McFadyen 1998). In the case of Canada thistle, useful control agents have been sought especially among competing plant species, herbivorous insects and fungus species. Experiments were performed with different competing clover species (Lukashyk et al. 2008) and grass/clover mixtures (Graglia et al. 2006) which, together with mowing, resulted in a reduction of *C. arvense* shoot density of up to 90%. Additionally, a strongly decreased above ground biomass was achieved, presumably by suppressing the regrowth of thistle shoots after mowing. Ang et al. (1994) showed for arable crops that increased interspecific competition from non-crop plants can reduce the abundance of *C. arvense*. Though Edwards et al. (2000) found similar results in a permanent grassland community, this technique has been classified as too intense and costly to be accepted among organic growers (Graglia et al. 2006). This technique is also not applicable in ruderal sites or habitats of conservational value.

Additionally, numerous studies about herbivores as potential biocontrol agents of *C. arvense* were performed. In a recent review, Cripps et al. (2011) reviewed five insect species that have been released in North America and New Zealand, however without any indications of successful control. Neither the coleopterans *Altica carduorum* Guérin-Méneville, *Lema cyanella* (L.) (Chrysomelidae), *Hadroplontus litura* (F.) (= *Ceutorhynchus litura),* and *Rhinocyllus conicus* (Frölich) (Curculionidae), nor the dipteran *Urophora cardui* (L.) (Tephritidae) could be established at all locations, where they were released. Additionally, none of the species had a significant influence on the Canada

thistle populations (Cripps et al. 2011, Julien and Griffith 1999). Some other herbivorous beetles, like the chrysomelid *Cassida rubiginosa* Müller (e.g. Ang et al. 1995, Bacher and Schwab 2000, Clough et al. 2007) or the curculionid *Larinus planus* (F.) were accidentally introduced to North America. They became established at several locations, but also had little or no impact on Canada thistle (Julien and Griffiths 1999). The curculionid *Cleonus piger* Scop. (Watson and Keogh 1980) showed a considerable impact on *C. arvense* but has never been released as a biocontrol agent. A reason for this is certainly that the host range of *Cleonus piger* includes the artichoke and it could therefore not be considered as a suitable biocontrol agent (Cripps et al. 2011).

In their review Cripps et al. (2011) concluded that none of the herbivorous arthropod species had a significant influence on Canada thistles. Since plant pathogens are often cited as second major group of biocontrol agents (e.g., Charudattan and Dinoor 2000), we analysed the existing studies on plant pathogens, mainly fungi and bacteria, as biocontrol agents to close this review gap. There are plenty of studies on this topic adopting a range of taxonomically diverse organisms and approaches. With the present paper we aim at summarizing the results of these works and at presenting a comprehensive review on the application of fungi and bacteria for biocontrol of Canada thistle, *C. arvense*.

Biological control is usually defined as the usage of living organisms to control other organisms. The current praxis, however, ranges from whole organism applications to the use of reproductive stages such as spores, parts of organisms and purified compounds. Such secondary metabolites may be included or excluded when defining biological control, see Ash (2010).The wide usage of the term "mycoherbicide" also plays with the obvious similarity between organisms, isolated compounds and synthetic herbicides, when applied as an aerial spray. Therefore, we decided to include also fungal and bacterial products into this review, especially since six out of eleven biocontrol agents as listed below served as compound source and since they were specifically targeted against *C. arvense*.

Fungi as biocontrol agents

A total of 10 fungal species have been tested as biocontrol agents of *C. arvense* (Table 1). Some experiments tested the performance of the living fungi while others used fungal products, such as toxins.

Puccinia punctiformis

Most work was done on the biotrophic rust fungus *Puccinia punctiformis* (syn. *P. obtegens* (Link) Tul. and C. Tul. and *P. suaveolens* (Pers.) Rostr.), which is considered to have the highest potential as a mycoherbicide (French and Lightfield 1990). The big advantage of *P. punctiformis* for a use as a biocontrol agent is its species specificity to

Table 1. Pathogens of *Cirsium arvense,* proposed for biocontrol. For further details, compare text. Effectivity is subdivided into high (ability to kill the plant) and limited (not able to kill the plant). Specificity is subdivided into very high (specific to one species), high (specific to a few species of one family), low (many species of one family), very low (many species of different families).

Systematics	Pathogen	Affected plant part	Effectivity	Specificity	Main references
Basidiomycota	*Puccinia punctiformis*	leaves, shoots	limited, local	very high	Frantzen (1994), French et al. (1988), French and Lightfield (1990), Kluth et al. (2003)
Ascomycota	*Phomopsis cirsii*	dead stems and leaves, roots	high	high	Leth and Andreasen (1999), Leth and Andreasen (2000), Leth et al. (2008)
	Sclerotinia sclerotiorum	dead and decaying stems and leaves	limited, local	very low	Brosten and Sands (1986), Bourdot et al. (1993, 1995)
	Alternaria cirsinoxia	leaves	limited	low	Berestetskii et al. (2010), Green and Bailey (2000 a, b), Green et al. (2001a)
	Phoma destructiva	dead and living plant material	high	unclear	Guske et al. (1996), Guske (2002), Kruess (2002)
	Phoma exigua	leaves	inconsistent	very low	Bithell and Steward (2001), Waipara (2003), Bilder and Berestetsky (2006), Scott et al. (1975)
	Stagonospora cirsii	leaves	high, with restrictions	low	Gasich and Berestetskiy (2006), Mitina et al. (2005), Yuzikhin et al. (2007)
	Septoria cirsii	leaves	high	very high	Leth (1985, 1990)
	Phyllosticta cirsii	unknown, only extracted phytotoxins tested	unknown	unknown	Berestetskiy et al. (2005), Evidente et al. (2007, 2008a)
	Fusarium spec.	seeds, seedlings, leaves, roots	inconsistent	low	Bailey BA et al. (1997 b, 2000), Bailey KL et al. (2000), Gronwald et al. (2004)
Bacteria	*Pseudomonas syringae* pv. *tagetis*	leaves, shoots	high	low	Bailey KL et al. (2000), Johnson and Wyse (1991), Johnson et al. (1996), Lukens and Durbin (1985), Rhodehamel and Durbin (1985), Tichich and Doll (2006)

C. arvense. However, single reports of *P. punctiformis* on other *Cirsium* species and Asteraceae genera (Tykhonenko and Minter 2002, Berner et al. 2002) require further investigation. Research on *P. punctiformis* in biocontrol started almost 100 years ago when Olive (1913) studied how *C. arvense* became infected by the rust fungus and pro-

duced systemically infected shoots. The importance of these observations for a possible control of *C. arvense* was recognized by Cockayne (1915) and Ferdinandsen (1923). Later studies were carried out attempting to stimulate spore germination (French et al. 1988, French 1990, French and Lightfield 1990, Frantzen 1994, French et al. 1994), to artificially spread spores in order to obtain higher infection rates (Thomas et al. 1994, Guske et al. 2003, Kluth et al. 2003, Demers et al. 2006, Wandeler and Bacher 2006, Müller et al. 2011) and studying interactions between *P. punctiformis* and insects (Friedli and Bacher 2001a, Kluth et al. 2001, Kluth et al. 2002, Cripps et al 2009).

Puccinia punctiformis causes two different kinds of infections, local and systemic infections. While local infections cause only small lesions on thistle leaves and influence the plant's performance only marginally (Kluth et al. 2005), systemic infections usually kill the infected shoots within a few months, mostly before flowering (French and Lightfield 1990). Most studies were unable to reach higher rates of systemic infection than 20 to 50% by artificial inoculation (e.g., Van den Ende et al. 1987, French et al. 1988, Frantzen 1994, Wandeler and Bacher 2006, Müller et al. 2011). This is considered inadequate for a successful suppression of *C. arvense* (Van den Ende et al. 1987, Van Leest and Scheepens 1994).

Wandeler and Bacher (2006) observed that the weevil *Ceratapion* (= *Apion*) *onopordi* Kirby (Coleoptera: Curculionidae) acts as a vector of *P. punctiformis* and that *C. arvense* becomes systemically infected after spore transmission. Only females were found to cause systemic infection (Friedli and Bacher 2001 a, b) suggesting that egg-laying, not feeding on the host plant is likely to be the underlying mechanism. Unfortunately, spore transmission by female *C. onopordi* did not result in an adequate infection and control level, either. The highest infection rate reached in this semi-field study was about 42%, whereas a rate of more than 80% or 90% would be necessary for effective control. Moreover, Cripps et al. (2009) found that rust infection rates were similar in areas with or without the weevil, indicating that its presence does not enhance systemic rust infection.

One can conclude that *P. punctiformis* as a potential biocontrol agent against *C. arvense* presently has the serious handicap that there are no suitable methods to cultivate this biotrophic rust fungus, to produce sufficient amounts of infectious spores, and to applicate spores in an effective and economic manner to obtain the necessary infection rate. The most difficult step in this chain of argumentation is obviously the lack of understanding of the process by which a systemic infection is initiated.

Sclerotinia sclerotiorum

Sclerotinia sclerotiorum (Lib.) de By. is able to attack shoots and roots and can kill Canada thistles (Brosten and Sands 1986). Under natural conditions this fungus leads to localised patches of dead thistle shoots, ranging from one to several dead shoots. The extent of the destruction is possibly limited by *Sclerotina*'s slow rate of expansion (Brosten and Sands 1986). All studies based on artificial infection showed mortality of vegetative shoots and a reduction in the root biomass (Bourdot et al. 1993, 1995, Bourdot and Harvey 1996).

Higher infection rates were achieved with plants that were experimentally wounded before the treatment (Bourdot et al. 2004). The potential of *S. sclerotiorum* as control agent seems to be limited, as thistle shoots need to be re-infected in the next growing season because the fungus seems to be unable to hibernate in the root system of the thistle (Bourdot et al. 2006). A further limitation is the high variability of the impact of the fungus on the host population, leading to a reduction ranging between 20 and 95%. This depends on site, fungal strains and resistence of the *C. arvense* clones. Additionally, *S. sclerotiorum* needs a minimum of free water like rain or dew for a successful infection. This also limits the use as a biocontrol agent to some climate regions where free water is available (Brosten and Sands 1986). The major objection against the use of *S. sclerotiorum*, however, is its lacking host specificity and occurrence on several hundreds of known host plants. Whereas its virulence on *C. vulgare, Carduus nutans* and many more wildflower species (Bourdot and Harvey 1996) may not pose a problem, the virulence on canola and many vegetable species Pennycook 1989) limits its use as a biocontrol agent. As *S. sclerotiorum* is not virulent on grasses and *Trifolium* ssp. Hurrell and Bourdot (1993) proposed using this pathogen on pastures. Since *S. sclerotiorum* can survive for a long time in the ground (Bourdot et al. 2000) and as its spores are spread easily, its use on pastures may cause hazards after changes of land use and for adjacent areas, even if a safety zone is allowed (De Jong et al. 2002)

Alternaria cirsinoxia

Another fungus widely discussed as a biocontrol agent is *Alternaria cirsinoxia* E.G. Simmons and K. Mort., firstly isolated from *C. arvense* in Canada in 1993 (Simmons and Mortensen 1997). Though it causes severe foliar necrosis (Green and Bailey 2000 a, b, Green et al. 2001a) its usefulness as a biocontrol agent is limited by a number of shortcomings. First, the fungus is not species-specific. Green et al. (2001a) tested several plant species from different families. With the exception of leafy spurge (*Euphorbia esula*, Euphorbiaceae) only Asteraceae were infected, but among these crops like sunflower (*Helianthus annuus*) and safflower (*Carthamus tinctorius*) could be found. Secondly, climatic conditions must be appropriate for the formation of appressoria and penetration of the leaf epidermis by the pathogen (Green et al. 2001a). Climatic conditions are also a limiting factor for the performance of the mycelium. The mycelium survives at temperatures around 0°C and can also overwinter; temperatures above 40°C kill it, thus it could only be used in temperate climates. The growth optimum is reached at 20 to 25 °C (Green and Bailey 2000 b, Green et al. 2001b). Also humidity conditions are limiting for a survival of the fungus, as high air humidity or even free water is necessary for the germination of the conidia (Green and Bailey 2000b). *Alternaria cirsinoxia* is primarily pathogenic on older, senescing leaves of *C. arvense* and infected plants can recover by developing new, healthy leaves (Green and Bailey 2000a, Gannibal and Berestetsky 2008) which additionally limits the fungus' potential as a bioherbicide (Green and Bailey 2000a). Berestetskii et al. (2010) identified zinniol as one of the

phytotoxic substances in *A. cirsinoxia*. However, the use of zinniol as natural herbicide is apparently limited by its non-specific phytotoxic activity and its cytotoxicity.

A combined treatment of *A. cirsinoxia* and the herbicide glyphosate on *C. arvense* was also tested. In a controlled environment, the combination of herbicide and the fungus caused more severe damage to Canada thistle than glyphosate alone, but did not reach a sufficient level of control. Moreover, the effects of *A. cirsinoxia* and glyphosate were not consistent in repeated field trials (Green and Bailey 2001). In conclusion, *A. cirsinoxia* is not suitable for the biological control of Canada thistle due to its low host specificity, unspecific toxicity and limited infection power.

Phomopsis cirsii

Phomopsis cirsii Grove, a necrotrophic fungus, was found on dead stems and leaves of *C. arvense* and *C. eriophorum* in Great Britain (Grove 1935) and later on those of *C. palustre* in Norway (Jørstad 1965) and Denmark (Leth 1985). In 2008, Leth et al. also found the fungus on seeds of *C. arvense*. Early season symptoms are black leaf veins and small limited necrotic lesions on stems, dying back of young shoots and wilting of shoots. Late season symptoms are black necrotised peduncles and bracts, black veins and black or brown necrotic lesions on the mature stems, often containing yellow patches with sporulating pycnidia (Leth et al. 2008). It can overwinter in dead stems and forms conidia that are spread by rain splash or invertebrates. The fungus can be cultivated on artificial substrates, and several experiments showed that it is possible to infect shoots of *C. arvense* by spreading the fungal mycelium (Leth and Andreasen 1999, Leth and Andreasen 2000, Leth et al. 2008). Precondition is that conidia and mycelial fragments are in contact with free water at least for 18 h to cause infection. This time period can be shortened to 6 h by the addition of alginate (Leth and Andreasen 2000). Spraying the mycelium on two-year old thistle shoots resulted in a 50% reduction of fresh weight of the shoots (Leth and Andreasen 1999). In other experiments, isolates killed 100% of the inoculated plants (Leth et al. 2008), indicating a different virulence of different fungal strains. Leth et al. (2008) suggested that it may be possible to increase the pathogen's virulence against a broad range of genotypes of *C. arvense* by optimising the cultivation practices. It remains to be investigated whether this fungus is really restricted to *Cirsium* species and whether it is able to kill whole thistle clones. If this turns out to be the case, the pathogen could become a promising candidate for the biocontrol of Canada thistle. Some applications of *Ph. cirsii* were covered by a patent (Leth 1985), for more details see below.

Phoma species

Phoma destructiva Plowr. was first mentioned in 1915 by Jamieson as the cause of a fruit rot in tomatoes. Later it was also mentioned to cause leaf blight in tomato (Ebben and Critchle 1972) but the host spectrum is uncertain as Guske et al. (1996) and

Guske (2002) claim specificity of the fungus for C. *arvense*. This contradiction may be accounted for by the presence of different varieties or special forms within *Ph. destructiva* (Aulakh et al. 1969). Guske et al. (1996) were the first to mention this fungus as a biocontrol option against *C. arvense*. Germinating conidia cause systemic infections which influence the C/N ratio negatively and therefore reduce the plant growth (Huber 1998), leading to chlorosis of the above-ground plant parts, a reduction in the number of flower heads and seeds and a reduced biomass (Kruess 2002). It is possible to inoculate thistle shoots (Kruess 2002) with this perthotrophic (Guske 2002) fungus. Perthotrophic means that the fungus lives on dead plant material, killed before by the fungus itself. This reduced plant quality was mentioned as a contraindication against a combination of the fungal pathogen with the herbivorous beetle *Cassida rubiginosa.* Infected plants were less attractive as hosts and larval performance and survival of the beetle were reduced, so that synergistic effects were excluded (Kruess 2002) or perhaps masked through decreased attractiveness of thistles to this beetle.

Better results were reached by a combination of *Ph. destructiva* with other plant pathogens. The application of a mixture of four pathogens, *Ph. destructiva, Ph. hedericola* (Durieu and Mont.) Boerema, *Ph. nebulosa* (Pers.) Mont. and a *Mycelium sterilum* significantly reduced the reproduction of the plants and also affected their roots, shown by a loss of dry root weight of 32% (Guske et al. 2004). A combination of *Ph. destructiva* with *P. punctiformis* reduced the shoot density (Kluth et al. 2005) but not all tested combinations of pathogens enhanced the control effect. A combination of *Ph. hedericola* and *P. punctiformis* was less effective than *Ph. hedericola* alone. The single application of *Ph. hedericola* or *Ph. nebulosa* was less harmful to thistles than the combination of both. Application of *Ph. nebulosa* alone caused death of all main shoots. This fungus is nevertheless inappropriate as a biocontrol agent, as more secondary shoots arose after the primary ones died (Guske et al. 2004).

Another *Phoma* species found on *C. arvense* is *Ph. exigua* Desm. The weak leaf spot pathogen (Waipara et al. 1997), preliminarily identified as *Ascochyta sonchi* (Mel'nik 2000) and later reclassified to *Ph. exigua* (van der Aa et al. 2000, Boerema et al. 2004), parasitizes more than 300 plant species and is discussed as a biocontrol agent against *Taraxacum officinale* (Stewart-Wade and Boand 2004) and *Gaultheria shallon* (Zhao and Shamoun 2006). The Canada thistle was originally not identified as a host of *Ph. exigua* (van der Aa 2000, Boerema et al. 2004) but could later be confirmed as such (Bithell and Steward 2001, Waipara 2003, Bilder and Berestetsky 2006). Inoculation experiments showed that an artificial infection with the fungus is possible, but with inconsistent results between different isolates. The disease development was much faster on detached than on attached leaves, but the short-term experiment described by Bithell and Stewart (2001) does not allow further conclusions on the progress of this infection. Scott et al. (1975) identified several phytotoxins in *Ph. exigua* which they recommended for biocontrol. However, among these phytotoxins unspecific phyto- and cytotoxic cytochalasins are common and cytochalasin A and B even cause potato gangrene (Scott et al. 1975). Moreover, the main toxin ascosonchine is not virulent (Evidente et al. 2006), so that *Ph. exigua* cannot be recommended for biocontrol (Cimmino et al. 2008).

Stagonospora cirsii

Stagonospora cirsii Davis is a causal agent of brown foliar lesions on *C. arvense*. If sprayed on seedlings during a dew period, it can kill nearly 100 % of the treated plants. The fungus can also be dusted as mycelium powder onto the soil surface which led to the death of 60% of treated seedlings in one study. Older plants are also affected but not killed. The fungus is able to survive over long periods, at least in sterile soil and remains viable on organic substrate after a cold winter period, but an infection of the thistle roots seemed to be impossible (Gasich and Berestetskiy 2006), which restricts its potential as a mycoherbicide.

S. cirsii also produces phytotoxins, demonstrated by the phytotoxic activity of culture filtrates to leaves and roots of *C. arvense* (Mitina et al. 2005). Yuzikhin et al. (2007) isolated a new phytotoxin, a nonenolid named stagonolide, from the fungus. The phytotoxin was shown to be unspecific in general but more selective against Asteraceae including sunflower (*Helianthus annuus*). Other crops, such as pepper (*Capsicum annuum*), tomato (*Lycopersicon esculentum*), wheat (*Triticum aestivum*), pea (*Pisum sativum*) and radish (*Raphanus sativus*) were also affected and displayed leaf necrosis. Stagonolide was most harmful to leaves and acts as a strong inhibitor of root growth in seedlings of *C. arvense* (30% decreased root length) and other Asteraceae. Other isolated nonenolides, stagonolide B-F, showed no toxicity against *C. arvense* (Evidente et al. 2008 b). Later, another four nonenolides were isolated by Evidente and coworkers (Evidente et al. 2008 c). Three were new compounds, named stagonolides G, H, and I, the fourth was identified as modiolide A, known from the fungus *Paraphaeosphaeria* sp., living on the horse mussel *Modiolus auriculatus* (Tsuda et al. 2003). Stagonolide G showed no toxic activity, whereas stagonolide H was most toxic to *C. arvense* leaves, causing necrotic lesions. Also other plant species tested showed necrotic lesions after inoculation with stagonolide H, but were less sensitive. The authors concluded that this phytotoxin is highly phytotoxic and selective and recommend it as a potential natural herbicide. However, as the fungus is highly infectious on seedlings of various plants and its extracted toxins are not specific and also not that selective as mentioned by the authors, we question the potential of *S. cirsii* as a biocontrol agent of *C. arvense*.

Septoria cirsii

Septoria cirsii Niessl causes leaf spot on Canada thistle. Because of its host specificity and effective control of Canada thistle in the field, it had been proposed as a biocontrol agent (Leth 1985). Cultures of *S. cirsii* produce copious amounts of a phytotoxin which was identified as beta-nitropropionic acid. The toxin inhibits seed germination, root elongation and causes chlorosis and necrosis of the leaves of Canada thistle (Hershenhorn et al. 1993). *S. cirsii* is considered to be specific to the genus *Cirsium*, though infections were also found on artichoke (*Cynara scolymus*), another Asteraceae. According to susceptibility tests, no signs of infection were found in plants outside the tribe

Cardueae of Asteraceae (Leth 1985). Active components of the fungus were suggested as a mycoherbicide and their application seemed to be rather promising.

The application of *Septoria cirsii* and *Phomopsis cirsii* as mycoherbicide had been covered by the patent of Leth (1985, 1990). This patent looked interesting but so far never reached the market. At that time, Leth worked for Novo Industri A/S, Denmark. In the 1990's, Novo Industri sold its plant protection division to Abbott including most of the patent rights, but not the *Phomopsis* patent. However, around 1999 Novo Industri abandoned the case due to lack of interest and eventually, all patents on bioherbicides were abandoned. If no other party showed interest in the meantime, the patents would have expired in 2004-2005 (personal communication Bo Hammer Jensen).

Phyllosticta cirsii

The fungus *Phyllosticta cirsii* Desm. has been evaluated as another possible biocontrol agent of Canada thistle (Berestetskiy et al. 2005). Since the genus *Phyllosticta* is known to produce bioactive metabolites, studies concentrated on the isolation of different phytotoxins. Evidente et al. (2007) identified the four phyllostictines A to D, and later isolated phyllostoxin and phyllostin as further compounds (Evidente et al. 2008 a), with phyllostoxin being highly phytotoxic and phyllostin not being toxic. Phyllostoxin was proposed as a potential natural herbicide but its toxicity against other plant species was not tested and thus its specificity is unknown. Evidente et al. (2008 a) also investigated potential side-effects of this substance and concluded that antimicrobial or zootoxic activities were lacking. However, these results base on only limited tests with three bacteria species, one fungus species and one crustacean species and cannot be generalised. Until further data become available phyllostoxin or *P. cirsii* itself cannot be regarded as suitable biocontrol agents of Canada thistle.

Fusarium species

The genus *Fusarium* includes many species that are pathogenic to *C. arvense,* for example *F. equiseti* (Corda) Sacc. (Gasich and Berestetskiy 2007). Species that occur on seeds can cause the death of the seedlings, e.g. *F. solani* (Mart.) Sacc. and *F. oxysporum* E.F. Sm. and Swingle (Fischl et al. 2004). Isolates of different *Fusarium* species reduced the emergence of new shoots by 45-70% and shortened root growth by 25-52% when applied as a suspension on the surface of root cuts (Bailey et al. 2000). Nep 1, an extracellular protein produced by *F. oxysporum* f. sp. *erythroxyli* (Bailey 1995, Bailey et al. 1997 a), can cause necrosis of leaves of dicotyledonous plants after foliar application (Bailey et al. 1997b, 2000a, 2000b, Jennings et al. 2000). Gronwald et al. (2004) showed rapid desiccation and necrosis of leaves. The greatest effect was observed in recent, fully expanded leaves, with 60 to 80% of the leaves being necrotic after a few hours of foliar application. Two weeks after application the dry

weight of the shoots was reduced by 30 to 41%. Similar results were obtained by a foliar application of Nep 1 in combination with the bacterium *Pseudomonas syringae* pv. *tagetis*. However, as neither the *Fusarium* spp. nor the extracted protein Nep1 are species specific, they cannot be regarded as biocontrol agents.

Bacteria as biocontrol agents

The bacterium *Pseudomonas syringae* pv. *tagetis* (Pst), first found on *Tagetes erecta* (Hellmers 1955), is able to cause leaf spot and apical chlorosis on a number of Asteraceae, including *C. arvense* (Johnson and Wyse 1991, Johnson et al. 1996, Rhodehamel and Durbin 1985, Styer and Durbin 1982). The apical chlorosis is due to the production of the unspecific compound tagetitoxin (Lukens and Durbin 1985, Durbin 1990). This toxin causes decreased vigour, inhibition of flowering and increased winter mortality (Johnson et al. 1996) and it led to study Pst as a potential biological weed control agent. Bacteria have many advantages compared to fungi: they grow very fast in liquid culture, can be stored frozen or dried and are suited for genetic manipulation and selection (Johnson et al. 1996). Nevertheless, they were ignored for a long time as possible biocontrol agents mainly because of their inability to penetrate intact plants (Templeton 1982). Field studies with a spray application of Pst and a surfactant resulted in 100% disease incidence and greater severity of disease symptoms than observed in natural infections. This led to a mortality of 57% of the plants meaning a significant reduction of the thistle population (Johnson et al. 1996). Another field study by Hoeft et al. (2001) showed similar results.

Application of Pst resulted in reduced survival of *C. arvense*, less height growth and seed production. Less seed production leads to a reduced soil seed bank and less regrowth of the thistle. Gronwald et al. (2002) tested different application methods and effects of repeated applications. The authors found apical chlorosis in 67% of the plants, resulting in a 31% reduction of plant height; they counted 81% fewer flower heads and a survival rate reduced by 20% after two applications. Tichich and Doll (2006) also found repeated applications to be more effective than a single one, as a single application causes chlorosis but no loss of dry weight (Bailey 2000). In a growth chamber experiment with foliar application of Pst, Gronwald et al. (2002) showed a loss of dry weight of 52% and a loss of chlorophyll content of emerging leaves of 92%. Tagetitoxin inhibits plastidic RNA polymerase III, thus preventing chloroplast biogenesis, so that infected plants produce new cells without chloroplasts and incapable of photosynthesis (Lukens and Durbin 1985, Lukens et al. 1987, Mathews and Durbin 1990, Steinberg et al. 1990). To target the photosynthetic activity of aboveground plant parts appears to be a much better strategy than to try to deplete the roots' reserves, followed by mechanical methods such as mowing (Tichich and Doll 2006).

However, also the repeated foliar application of the sap from naturally infected thistles led only to a 50% incidence of disease, still not sufficient to effectively suppress thistle growth (Tichich and Doll 2006). Further possibilities to increase the effectiveness of Pst as a biocontrol agent include a strict selection for humid application periods to ameliorate the

initial conditions for the plant pathogen (Tichich and Doll 2006, Tichich et al. 2006), selecting strains that produce more toxin (Gronwald et al. 2002, Tichich and Doll 2006), or increase toxin production by optimal environmental and nutritional conditions (Bender et al. 1999, Li et al. 1998), especially a high nitrogen supply during cultivation (Styer 1982).

These studies succeeded due to the combined application of Pst with Silwet L-77 or a similar organosilicone surfactant that facilitated the entry of bacteria into leaves (Zidack et al. 1992, Zidack and Backman 1996) via the stomata and hydathodes, because of their property to lower surface tension (Neumann and Prinz 1974, Field and Bishop 1988, Stevens et al. 1991). A combination of Pst with a chemical herbicide such as glyphosate further increased disease symptoms and reduction of fresh and dry weight significantly (Bailey et al. 2000). This suggests synergistic effects between the bacterial agent and the herbicide (Christy et al. 1993).

Host specificity tests showed that tagetitoxin acts on a variety of Asteraceae (Johnson and Wyse 1991, Johnson et al. 1996, Rhodehamel and Durbin 1985, Styer and Durbin 1982). Durban et al. (1989) described that wheat seedlings, after a first contact with tagetitoxin, completely lacked chlorophyll and Durbin (1990) designated tagetitoxin a "non-host selective" compound. Obviously this substance is suitable as a non-selective herbicide but not as a highly selective biocontrol agent.

Conclusion

Mycoherbicides have been praised since decades to solve problems of weeds in a variety of habitats and as an upcoming strategy in organic farming but today results are still disappointing: only eleven products seem to have made it to the market worldwide (Charudattan and Dinoor 2000, Khetan 2001, Ash 2010). A recent search among patents yielded 71 citations (Ash 2010) but this does not necessarily indicate a huge product pipeline but rather underlines that most of them never will be realised. On a global level, the reasons for this situation are multiple and heterogeneous but may be similar to those outlined for *C. arvense* and its pathogens. The primary reason for the failure of most of the tested plant pathogens against *C. arvense* is the missing host specificity (among the here presented pathogens, this refers, e.g., to *Alternaria cirsinoxia, Sclerotinia sclerotiorum, Phoma exigua,* and *Pseudomonas syringae*). A useful and safe biocontrol agent has to be as specific as possible. Species-specificity would be ideal but is obviously very difficult to find. Genus specificity may be acceptable quite often but has to be tested very carefully. Less pronounced specificity, e.g. on family level, usually cannot be accepted. Also the varying and low virulence of the pathogens pose a problem (e.g., *Alternaria cirsinoxia, Sclerotinia sclerotiorum, Phomopsis cirsii*) as constant levels of virulence must be ensured for a successful inhibition of the growth of the target weed. None of the proposed fungi is able to kill a thistle clone, thus confirming the conclusion in Charudattan's (2005) review that weeds with a robust capacity for vegetative regeneration are more difficult to control with pathogens. Another restriction en-

countered is the obligate biotrophic nature of the rust *Puccinia punctiformis* which poses the problem that this fungus cannot be cultivated in the laboratory to produce the necessary amount of inoculum.

This review shows for *C. arvense*, one of the single most important weeds of the world, that despite nearly 100 years of research it was so far not possible to use fungi and other pathogens as biocontrol agents. While it is generally undoubted that pathogens are important regulators of plant populations (e.g., Mitchell and Power 2003), the specific situation in a highly disturbed agricultural landscape is different since natural regulation mechanisms are not strongly developed against *C. arvense*. At least for Canada thistles, one could conclude that the potential of fungi as biocontrol agents has been overestimated even if Charudattan (2005) would state that this approach is still underdeveloped. There is always a chance to find new and suitable biocontrol agents when increasing the search effort. Nevertheless, for us it is today very difficult, to advice on suitable and promising future research approaches for a biological control of Canada thistles.

The current regulatory situation where microbial products need to go through the same registration procedure as conventional pesticides certainly represents a huge barrier for potential applicants. This may explain the considerable number of dead patents. Size and diversity of a research consortium and the financial power of the industrial partners may be further decisive parameters (Ash 2010, Bailey et al. 2010). Another problem is target selectivity. Good biocontrol praxis demands an as high target specificity as possible. Economically speaking, however, such a small application basis is not interesting at all. Therefore one could propose to accept agents of only medium target selectivity since most applications would only occur in monocultures. While this even may be correct for *C. arvense*, further candidate habitats would certainly include more diverse landscapes and even natural habitats of conservational value. Since Canada thistles are invasive in most parts of the world, related, endemic thistle species, though protected and non-targets, suddenly could be affected by such an agent of low specificity.

In the case of *C. arvense* the research development of the last years, however, points into the direction of applying secondary plant compounds. Such substances quite often are structurally modified and can be produced synthetically. By this, unspecific but powerful herbicides may come up. Though sometimes the term "bioherbicide" is still used to indicate the biotic origin of such compounds they are as good or bad as chemical herbicides with the classic problems of effectivity, selectivity, degradability and potential side effects.

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