

## MINIREVIEW

# The role of plant–microbiome interactions in weed establishment and control

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\*Corresponding author: Bioresources Unit, Department of Health and Environment, AIT Austrian Institute of Technology GmbH, 3430 Tulln, Austria. Tel: +43-664-8251172; E-mail: [friederike.trognitz@ait.ac.at](mailto:friederike.trognitz@ait.ac.at)**One sentence summary:** In this review, we highlight the importance of soil and plant microbiomes for the establishment and control of weeds and invasive plants. We believe that our paper provides a composite overview of current knowledge on the role of weeds/invasive plants in natural ecosystems.

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## ABSTRACT

The soil microbiome plays an important role in the establishment of weeds and invasive plants. They associate with microorganisms supporting their growth and health. Weed management strategies, like tillage and herbicide treatments, to control weeds generally alter soil structure going alongside with changes in the microbial community. Once a weed population establishes in the field, the plants build up a close relationship with the available microorganisms. Seeds or vegetative organs overwinter in soil and select early in the season their own microbiome before crop plants start to vegetate. Weed and crop plants compete for light, nutrition and water, but may differently interact with soil microorganisms. The development of new sequencing technologies for analyzing soil microbiomes has opened up the possibility for in depth analysis of the interaction between ‘undesired’ plants and crop plants under different management systems. These findings will help us to understand the functions of microorganisms involved in crop productivity and plant health, weed establishment and weed prevention. Exploitation of the knowledge offers the possibility to search for new biocontrol methods against weeds based on soil and plant-associated microorganisms. This review discusses the recent advances in understanding the functions of microbial communities for weed/invasive plant establishment and shows new ways to use plant-associated microorganisms to control weeds and invasive plants in different land management systems.

**Keywords:** weeds; invasive plants; microbiome; biological control

## INTRODUCTION

Weeds cause severe losses in agriculture due to competition between the crop and weeds for nutrition, light and humidity, with a maximum estimated yield loss of 34%. The crop loss caused by weeds is estimated at more than \$100 billion US dollars per year (Swanton, Nkoa and Blackshaw 2015). There are several ways of weed management, including weed prevention through crop rotation, crop competition and cultivation. Direct management strategies involve mechanical weeding or herbicide treatment.

Weeds and weed problems are anthropocentric terms applied to various species and plant populations (Buhler 2003). No

universal definition is shared between scientists but a common perception is that weeds grow on undesirable places. Here in this review, we focus on weeds, which are difficult to manage, cause yield losses and/or can be harmful to mammals. Invasive plants are non-native species whose introduction does or is likely to cause economic or environmental harm or harm to human, animal or plant health (Beck et al. 2008). Overall, all invasive plants are in fact highly problematic weeds. Most weeds are well adapted to unfavorable conditions, and disturbed land provides space, nutrients and light for weeds to develop. Agricultural practices like tillage and monoculture favor weed

establishment. Seeds of weeds can stay in the soil for several years until conditions are favorable for germination. After germination, the weed plants grow fast, rapidly establish populations and then soon reach the flowering phase. They produce numerous seeds, which are easily dispersed over long distances. Some weeds produce vegetative reproduction organs that help them to survive in soils. Weeds overwinter as seeds or vegetative organs in the soil and therefore are in close contact with native soil microorganisms.

The plant depends on the rhizosphere microbiome as a product of natural selection (Pérez-Jaramillo, Mendes and Raaijmakers 2015). It has been postulated that modern cultivars have lost the traits needed to recruit host-specific root microbiota as compared to their wild relatives (Bulgarelli et al. 2013). This is illustrated by the fact that significantly more plant growth-promoting bacteria were found in some weed species than in potato plants collected from a potato field (Sturz et al. 2001). Associated microorganisms may influence plant traits including disease resistance (van Wees, van der Ent and Pieterse 2008), growth, abiotic stress tolerance (Marasco et al. 2012), nutrient acquisition (Jones, Nguyen and Finlay 2009), flowering time and biomass production (Panke-Buisse et al. 2015).

The composition of plant microbiota depends on several factors such as the environment, climate, plant genotype and developmental stage of the host plant (Bakker et al. 2012; Hardoim et al. 2015). Every plant species seems to select its own microbiome, and this influences plant competitiveness, health and productivity (Berg et al. 2014). Plants are able to recruit their own microorganisms from the soil for the benefit of the plant, but it is largely unknown which mechanisms are responsible for the selection (Agler et al. 2016). Keeping plant-microbiome interactions intact is of particular importance for weeds and invasive plants, because these plants most often occur in disturbed ecosystems. In such late successional ecosystems, nutrient cycles are often more tightly closed and nutrient availability is reduced for any individual plant (van der Putten, Klironomos and Wardle 2007). Therefore, plants strongly depend on symbiotic microorganisms, and invading plants in natural ecosystems that are not able to establish mutual interactions with soil microorganisms are less likely to get well established (van der Putten, Klironomos and Wardle 2007).

Rhizospheres host highly complex microbial communities (Schlaeppli and Bulgarelli 2015), which are affected by agricultural management practices (Kennedy 1999; Carbonetto et al. 2014; Lehman et al. 2015). Besides causing changes in the microbial community structure, agricultural management may also affect microbiome functions. Changing the crop management system towards reduced tillage, maintenance of high soil organic matter and limited input of agrochemicals resulted in an increased prevalence of deleterious rhizobacteria (DRB) associated with weed seedlings (Li and Kremer 2006). In addition, higher activities of specific soil enzymes that apparently contribute to natural weed suppression were encountered.

Beside fungicides, herbicides are the main chemical compounds used in agriculture, and more than 40% of the pesticides used worldwide are herbicides. Resistances to herbicides are increasingly building up in weeds across all major modes of action and against all chemicals, threatening the efficacy of weed management in agriculture. At present, 450 herbicide-resistant weed biotypes are listed worldwide (Heap 2015). Therefore, novel types of herbicides are needed. However, the last herbicide with a new mode of action was introduced more than 25 years ago (Heap 2015). Only few bioherbicides have been registered between 1977 and 2010 in the USA as compared to multiple

biopesticides against fungi, bacteria and insects (Duke, Owens and Dayan 2014). This may be at least partly due to the still high efficacy of synthetic herbicides and their low cost. However, in organic agriculture, weed management is costly (McErlach and Boydston 2014), and products that are available on the market for organic farmers often are not effective enough and have high application costs.

## WEED-ASSOCIATED MICROBIOTA

### The soil-crop-weed microbiome

Most agricultural weeds are early successional plant species and, as such, are particularly apt to establish and thrive well in disturbed habitats like those created on agricultural land (Smith 2015). Evidence is accumulating that the endophytic and otherwise associated microbiota have a role in the weeds' ability to strive in suboptimal environments through contributing stress resistance and other plant strengthening functions. During plant domestication and agricultural intensification, the domesticated plants may have lost traits linked to recruit host-specific root microbiota (Pérez-Jaramillo, Mendes and Raaijmakers 2015). On the other hand, weeds have more positive feedback interactions with soil microorganisms and seem to have a greater dependence on these associations than crops (Massenssini et al. 2014). Additionally, plant-associated microorganisms may be transmitted from weeds to other plants also through different routes, e.g. by insects. Overall, this implies that microbial endophytes and other associated microorganisms of weeds are of enormous value as resources of plant-beneficial functions such as pathogen antagonism, plant growth promotion or inference of plant stress resistance (Fig. 1). In this context, management practices that still maintain weed populations to a certain extent in the field warrant that plant beneficial microbiota are conserved and can support plant growth and health, at least under specific conditions.

### Endophyte-weed associations

Among the best studied endophyte associations of weedy plants are symbioses of grasses with endophytic *Neotyphodium* fungi and their *Epichloë* teleomorphs, also referred to as systemic, clavicipitaceous, type 1, or *epichloë* endophytes, which appear to be exclusive to a small number of grass species such as tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis*) or perennial ryegrass (*Lolium perenne*). About 20%–30% of all grass species are colonized by *Neotyphodium* endophytes and their sexual relatives *Epichloë* (Leuchtman 1997). These endophytes are mutualistic colonizers of leaves and stems and are vertically transmitted by seeds, contributing to their successful dissemination to the next generation (Sánchez Márquez et al. 2012) and loss of the fungal partner can be associated with the loss of important traits (Saikkonen et al. 2004). Initially considered a serious problem in agriculture due to their toxicity to livestock, these fungi later became recognized for possessing advantageous traits such as the production of insect feeding deterrents (e.g. Schardl et al. 2013). Evidently, they may also infer increased resistance of the colonized grasses against serious fungal grass pathogens such as *Drechslera siccans* or *Fusarium* spp. (Wiewióra, Żurek and Żurek 2015). Saikkonen et al. (2013) showed that endophyte colonization of meadow fescue (*Scherodonus pratensis* ex. *Lolium pratense* and *F. pratensis*) clearly promoted competitive dominance in experimental monocultures and was able to retard weed invasion. Also a large

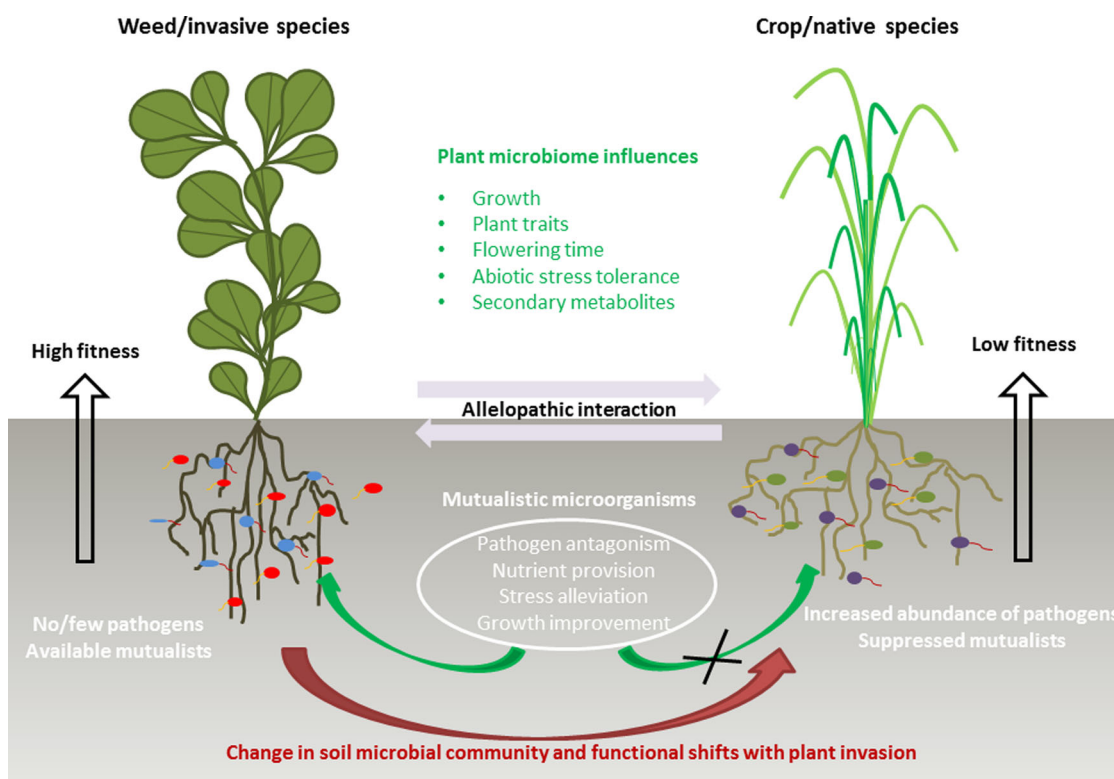


Figure 1. Schematic overview of microbial interactions between weeds/invasive plants and crop/native plants.

number of other fungal endophytic species have been reported in wild und cultivated cereals and grasses (Sánchez Márquez et al. 2012), such as non-clavicipitaceous endophytes that systemically infect their host like *Fusarium verticillioides* (Bacon and Hinton 1996), as well as fungal species that appear to behave as non-systemic, not vertically transmitted endophytes in grasses (including entomopathogenic fungi). Fungal endophytes appear to be highly diverse also in herbaceous plants, and they were thought to be commonly transmitted horizontally, infecting the plants through air, water or soil-borne spores (Wearn et al. 2012).

It has to be further elaborated to which extent fungal endophytes are essential for weed establishment and also to which extent they outcompete other beneficial or pathogenic microorganisms or to which extent they are shared with other (non-weed) plants. Few studies suggested antagonism or competition between fungal endophytes and mycorrhizae (Sánchez Márquez et al. 2012; Wearn et al. 2012). Generally, fungal endophytes have been reported to be plant specific and to be transmitted via seeds; however, fungal endophytes were also found on and within pollen grains (Hodgson et al. 2014), suggesting that endophyte transmission can be within and between plant generations.

For bacterial endophytes, actually, colonization from seeds is a common and important trait that secures their establishment in the next generation and favors mutualism (Truyens et al. 2015). Several studies indicate vertical transmission of endophytic bacteria, for instance, in rice, Hardoim et al. (2012) observed a high number of species in several seed generations. Also in the maize ancestor teosinte and in various modern maize races it appears that certain taxa within seed-borne endophyte communities (e.g. *Clostridium*, *Paenibacillus*) are vertically transmitted (Johnston-Monje and Raizada 2011). Likewise, some bacterial endophytes in small plants such as *Chenopodium album*

and *Stellaria media* seem to be vertically transmitted over generations via seeds (van Overbeek et al. 2011). Few seed taxa are likely to interact with those in the bulk soil and may act as colonizers of the surrounding soil and with co-inhabiting plants. Thus, the contribution of the seed microbiome to the overall plant–soil microbial communities might be significant in terms of potential effects of the respective microorganisms on soil functioning and crop growth (van Overbeek et al. 2011). This aspect merits further research to determine to which extent seed-derived endophytes contribute to the establishment of soil microbiota and to which extent they interact or associate with other plants.

Communities of endophytic bacteria have been extensively studied in staple crop plants such as rice, wheat, maize and millet (e.g. Senthilkumar et al. 2011; Montañez et al. 2012; Sessitsch et al. 2012; Gupta, Panwar and Jha 2013), where they are being increasingly acknowledged for their functions in plant growth promotion, nutrient scavenging, nitrogen fixation and pathogen antagonism (Gond et al. 2015). Increasingly, also weeds including wild crop relatives and other indigenous plants are targeted in inventories of plant beneficial endophytes that may be applied on crops as inoculants and biofertilizers (Pérez-Jaramillo, Mendes and Raaijmakers 2015). For instance, diazotrophic endophytes belonging to the genera *Klebsiella*, *Enterobacter*, *Bradyrhizobium*, *Alcaligenes*, *Azospirillum*, *Herbaspirillum*, *Ideonella*, *Acetobacter* and *Acinetobacter*, which are able to supply nitrogen to their host plants, have been isolated from wild rice (*Oryza alta*) plants (You and Zhou 1989; Baldani, Baldani and Döbereiner 2000; Elbeltagy et al. 2001; Chaudhary et al. 2012).

A diverse community of bacterial endophytes was found in switchgrass (*Panicum virgatum* L.) native to the tallgrass prairies in North America. Important biochemical properties of switchgrass endophytes include abilities to solubilize inorganic

phosphorus and to fix atmospheric nitrogen and to produce cellulases, toxins and phytohormones, implicating on their potential application as biofertilizers in low-input and sustainable feedstock production (Gagne-Bourgue and Aliferis et al. 2013; Bahulikar et al. 2014). Similarly, a rich culturable diazotrophic bacterial community was seen colonizing the fast-growing elephant grass (*Pennisetum purpureum* Schum.) in the tropics, which included isolates able to produce indole acetic acid and/or solubilize phosphate, rendering them good candidates for biofertilizer applications (Videira et al. 2012). Endophytes derived from weedy grasses or wild relatives of crop species may represent an interesting resource of microorganisms exhibiting a range of functions supporting (crop) plant growth, health and stress resistance, which merit further exploration.

## WEED-MICROBIOTA INTERACTIONS AFFECTING PLANT INVASIVENESS AND WEED ESTABLISHMENT

### Weed-microbiota interactions leading to alterations in below-ground microbial communities and nutrient cycling

Below-ground microbial communities play important roles in soil nutrient cycling and in the provision of essential plant nutrients. Plant-associated microbial communities in the rhizosphere are to a great extent shaped by the host plant because the plant provides nutrients in the form of exudates and mucilage-derived substances via the roots (Fig. 1). There are a few studies showing that soil microbial community structures change with plant invasions (reviewed by Wolfe and Klironomos 2005), and such changes implicate also functional shifts. Kourtev, Ehrenfeld and Häggblom (2002) identified major differences in microbial community composition, enzymatic activities and substrate-induced respiration rates in the rhizospheres of two exotic plant species, Japanese barberry (*Berberis thunbergii*) and Japanese stilt grass (*Microstegium vimineum*), as compared to the co-occurring native blueberry (*Vaccinium* spp.) species. Similarly, Rodrigues et al. (2015) identified major soil microbial community shifts brought about by three different invasive plant species, including a grass (*M. vimineum*), a shrub (*Rhamnus davurica*) and a tree (*Ailanthus altissima*), which were investigated at three independent locations in the USA. For comparison, non-invaded reference areas were investigated. Interestingly, all plant invasions shifted microbial communities in a similar way, resulting in increased abundances of several specific bacterial and fungal taxa (belonging to *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Ascomycota*). The study demonstrated an increased abundance of N-cycling taxa as well as N-cycling activity in the invaded areas. Similarly, Hawkes et al. (2005) reported an increased abundance and altered composition of ammonia-oxidizing bacteria and higher nitrification rates in the rhizospheres of invasive (*Avena barbata*, *Bromus hordeaceus*) versus native (*Nassella pulchra*) grass species and a native dicot (*Lupinus bicolor*). Such changes in nutrient cycling are likely to lead to increased N availability for the weed and, thereby, to greater fitness.

Symbiosis between N<sub>2</sub>-fixing rhizobia and legumes is known to play a key role in terrestrial ecosystem functioning, and the efficiency of this symbiosis is of high importance for covering the N demand of (unfertilized) legumes. Introduced legumes may suffer from the lack of appropriate rhizobia, but native legumes may also provide compatible symbionts to introduced legumes (Parker, Malek and Parker 2006). It has been shown that legumes that are promiscuous regarding their microsymbionts are more

likely to become invasive (Parker, Wurtz and Paynter 2007; Callaway et al. 2011). Nodules containing rhizobia have been reported to contain also non-rhizobial bacteria (Aserse et al. 2013; Mora et al. 2014), although the role of these non-rhizobial symbionts is yet unknown. Busby et al. (2016) investigated the symbiont composition of nodules obtained from an invasive legume in North America, *Lespedeza cuneata*, and from native *Lespedeza* species. Nodule bacterial composition differed greatly between native host and invasive *L. cuneata*, and the invasive plant contained a higher number of non-rhizobial taxa. Generally, the functioning of individual microbiome members is often yet unknown, particularly in regard to nutrient mobilization and cycling. Here, more research is required to understand functional consequences, e.g. of microbiome shifts due to invasive plants. This could be achieved by advanced molecular tools such as metagenomic analysis or also analyzing the response of plant microbiota to root exudates of native and invasive plants by stable isotope probing.

Arbuscular mycorrhizal fungi (AMF) are symbionts that colonize roots of most terrestrial plants and extend the root system beyond the reach of host plant roots, thereby supporting nutrient availability, primarily of P and N. Weed plants may substantially alter the availability of nutrients by altering or reducing the abundance and diversity of AMF. In North America, it was shown that the dominance of the garlic mustard weed led to a decline of AMF (Roberts and Anderson 2001). Kourtev, Ehrenfeld and Häggblom (2002) reported a higher abundance of AMF associated with invasive plant species (Japanese barberry and Japanese stilt grass) as compared to the co-occurring native blueberry plant. It seems that invasive plants are able to alter the soil microflora to their own benefit, e.g. by stimulating their own association with AMF (Callaway et al. 2004). Further, it was reported that spotted knapweed builds AMF hyphal connections to neighboring plants and thereby gets access to additional nutrients (Marler, Zabinski and Callaway 1999). The role of multitrophic interactions, e.g. between AMF hyphal networks, other microbiota and plants in plant establishment and particularly in plant nutrition needs to be further investigated. Understanding these networks will also reveal how weeds or invasive plants interact with and modulate such interactions to benefit.

### Weed-endophyte interactions in invasive weeds

Several studies indicate that endophytic bacteria and fungi may enhance the invasion success of exotic grasses in novel habitats. For instance, bacteria isolated from the invasive *Sorghum halepense* grass, which included nitrogen fixers and other plant growth promoters, were attributed a role in plant establishment and persistence. The ability of *S. halepense* to grow on exceptionally nitrogen-poor tallgrass prairie soils, where it changes soil chemical properties and creates monocultures in once diverse communities, was explained by plant-soil feedback systems driven by a consortium of bacteria associated with the grass (Rout and Chrzanowski 2009). Similarly, *Phragmites australis* spp. *australis*, an invasive species non-native to North America that shows aggressive proliferation through seed dispersal, stolons and rhizomes, hosts diverse endophytic fungal and bacterial communities as well as epiphytic bacterial, archaeal and fungal communities and associated oomycetes (Kowalski et al. 2015). While it is known that *Phragmites* rhizosphere bacteria and fungi may enhance plant growth, the specific roles of the plant-associated microorganisms have not yet been evaluated. *Phragmites australis* spp. *australis* is highly stress resistant and it was suggested that fungal endophytes could confer stress



resistance to their host (Fischer and Rodriguez 2013). As elimination of fungal endophytes to weaken competitiveness of the invasive plant could be an approach to controlling invasions, the fungi were tested for their susceptibility to various fungicides. Response to fungicide treatment varied among fungal isolates, and fungicide-resistant phenotypes were encountered (Fischer and Rodriguez 2013). This approach has potential to be taken further, either by applying specific fungicides or preferably by the application of microorganisms outcompeting or antagonizing certain fungal endophytes or chemical molecules interfering with the growth of these fungi. Also additional studies report that fungal endophytes, in particular members of the genus *Neotyphodium*, play an important role in plant establishment and fitness (Rudgers, Mattingly and Koslow 2005; Uchitel, Omacini and Chaneton 2011) supporting the potential of developing a strategy based on eliminating such fungal endophytes. Nevertheless, it has to be considered that plant–endophyte interactions in invasive grasses appear to be multifactorial. This was seen in tall fescue (*Schedonorus arundinaceus*), which is non-native to North America, where infection with the *Neotyphodium coenophialum* endophyte enhanced competitiveness in less productive plant communities but did not seem advantageous in all community types (Yurkonis et al. 2014). Besides the plant genotype, it seems that also the symbiont genotype has an influence on the persistence of the *Neotyphodium* symbiosis with tall fescue and, hence, on vegetation dynamics and plant community composition (Rudgers, Fischer and Clay 2010). Gundel et al. (2010) proposed a model that explains the outcomes of the *Neotyphodium* symbiosis by linking the genetic compatibility of the endophyte and its host grass, mutualism effectiveness and endophyte transmission efficiency.

It has been stated that endophyte effects in invasion ecology are context dependent (Vandegrift et al. 2015). This was based on the observation that the invasive grass *Brachypodium sylvaticum* in its native European range is nearly always infected by *Epichloë sylvatica* (the *Neotyphodium* teleomorph), which reduces the growth range and competitive abilities of the grass but infers increased germination rates and potential protection from seed herbivores and pathogens. In the invaded range in the USA, however, *B. sylvaticum* has lost the endophyte (Vandegrift et al. 2015).

Endophytes have recently been implicated to play a role also in herbicide tolerance of plants (reviewed by Tétard-Jones and Edwards 2016). Several bacterial endophytes have been reported to degrade various herbicides. The endophyte and rhizosphere bacterium *Pseudomonas putida* strain POPHV6, which was originally isolated from stems of poplar trees, showed degradation of 2,4-D and led to lower herbicide accumulation in aerial tissues (Shaw and Burns 2004; Germaine et al. 2006). Similarly, plant-associated bacteria have been identified that were able to degrade and thereby detoxify the atrazine or glyphosate herbicides (Kuklinsky-Sobral et al. 2005; Ngigi et al. 2012). Genetically engineered endophytes, e.g. containing a gene from *Burkholderia xenovorans* encoding a glutathione transferase-related enzyme involved in dechlorination reactions, were shown to mediate detoxification of herbicides (McGuinness et al. 2007). As endophytes have been shown to induce innate stress tolerance mechanisms based on signaling mechanisms involving jasmonic acid, oxylipins and salicylic acid, it has been suggested that endophytes could influence tolerance to herbicides (Tétard-Jones and Edwards 2016). For example, annual ryegrass (*Lolium rigidum*) colonized by the fungal endophyte *N. occultans* showed resistance towards the herbicide diclofop-methyl as a result of higher stress tolerance (Vila-Aiub, Martinez-Ghersa and Ghersa

2003). Many fungal endophytes of grasses exhibit reactive oxygen species scavenging activity and enhanced antioxidant content (Cummins, Cole and Edwards 1999), which might be important for protecting plants from downstream toxicity induced by herbicides (Edwards et al. 2005).

### Weed–microbiota interactions differ in their native versus invaded ranges

Various mechanisms are in place for invasive plants to become more competitive in their invaded versus their native ranges (Broennimann et al. 2007), some of which are based on altered interspecific interactions. The ‘Enemy Release Hypothesis’ (Klironomos, 2002), most prominently, posits that invading species may escape their herbivores and pathogens during the invasion process. According to the ‘Accumulation of Local Pathogens Hypothesis’ (Eppinga et al. 2006), invasive plant species can enhance their performance in the non-native area by increasing the abundance of local pathogens that are adverse to native plants. Similar to interactions involving pathogens, relationships of invading plants with mutualists may also be critical for invasion success. Here, the plants can either benefit from mutualists in the introduced range, as expressed in the ‘Enhanced Mutualism Hypothesis’ (Marler, Zabinski and Callaway 1999), or they may suppress mutualists of other plant species as postulated in the ‘Mutualism Disruption Hypothesis’ (Callaway et al. 2008). Recent studies have highlighted the importance of plant interactions with endophytic microorganisms beside other mechanisms. It appears that interactions with endophytes may significantly contribute to the plants’ greater competitiveness in the invaded versus native ranges via effects on plant growth and resource allocation (Rout et al. 2013).

The impact of fungal endophytes on invasion success has been addressed in several studies of *Centaurea stoebe* (or spotted knapweed), an aggressive invasive plant of western North American grasslands that is native to Eurasia. Endophytic fungal communities of *C. stoebe* were found to be different in its native versus its invaded range. Experimental evidence has led to the conclusion that fungal endophytes strongly influence the ecology and invasiveness of *C. stoebe* in all growth stages, through positive and negative effects on fitness that are probably mediated via chemical factors (Newcombe et al. 2009). It has been argued that enhanced competitiveness of invaders may be brought about either through the cointroduction of endophytes from the native into the invaded range as ‘novel weapons’ or by acquiring a new ‘machinery’ of endophytes in the invaded range through host jumping. Apparently, both processes have shaped the endophytic fungal community of *C. stoebe* plants during invasion (Shipunov et al. 2008).

Recently, it was demonstrated that fungal endophytes enhance the competitive advantage of the *C. stoebe* host by affecting allelopathic interactions (Aschehoug et al. 2014). Intriguingly, the enhanced effects of endophytic fungi in *C. stoebe* were observed only against North American native grass species and not against European natives (Aschehoug et al. 2012). In alignment with these observations, it becomes apparent that plants interact in the invasive place with microorganisms in the root zone in ways that are qualitatively or quantitatively different from interactions in the native place (Fig. 1). This provides evidence that specific geographical patterns exist in the ecology of invaders and soil microorganisms, indicating that regarding soil–plant microbial interactions ‘everything might not be everywhere’ (Rout and Callaway 2012).

Overall, a better knowledge of the role of microbiota associated with invasive plants in the establishment is needed as well as an in-depth understanding on the biology of such endophytes.

## MECHANISMS OF MICROBIAL WEED CONTROL

### Biocontrol strategies based on fungal weed pathogens

Biological control methods most commonly rely on (micro)organisms or natural compounds to reduce the population density of the target (pathogenic, deleterious) organism. Three different major types of biological control have been described. The conservation of natural enemies is the most important and most readily available biological control practice for pest management, but it is not applicable for all pests and weeds. The classical biological or inoculative approach involves the introduction of a natural enemy from its native range to a new area where the weed or pest poses a problem. The biocontrol agent is released once into the new environment and with time, the biocontrol organism builds up a population size that is able to reduce the pest or weed. The introduced population is maintained over very long periods of time (Bale, van Lenteren and Bigler 2008). This type of biological control has been most successful with perennial crops (fruit plantations and forests), where the long-term nature of the ecosystem enables the interactions between pest and natural enemy to become fully established over a period of time (Bale, van Lenteren and Bigler 2008). In classical biological control of weeds, fungi have been favored over bacteria, viral or other biocontrol agents (Morin, Evans and Sheppard 2006). One of the most successful microbial biocontrol agents for weed control is the introduction of the rust fungus (*Puccinia chodrilina*) in Australia to control the rush skeleton weed (*Condrilla juncea* L.). The weed originated from the Mediterranean region (Charudattan and Dinoor 2000) and caused severe problems in cereal crops in Australia. In the Mediterranean area, *P. chodrilina* was found to attack the narrow leaf form. Experiments showed that *P. chodrilina* is effective in a wide range of environments (Supkoff, Joley and Marois 1988), and it was released in 1971 together with two insects in Australia. The fungus got rapidly established by 1975, allowing an annual saving of \$18 million to the Australian farmers (Cullen 1985). While the narrow leaf form of the rush skeleton weed has disappeared in many regions, meanwhile two other forms (mainly the broad leaf form) have become an increasing problem (Charudattan and Dinoor 2000).

Another example of microbial control of weeds is the introduction of the gall-forming fungus *Uromycladium tepperianum* to control the invasive tree *Acacia saligna* in the Cape Floristic region in South Africa. Due to biological control, the weed density declined between 87% and 98% during the years 1991–2005 (Wood and Morris 2007). Most promising for a successful classical biological agent is the introduction of an agent from the dynamically balanced situation found in the center of origin of the weed into the area recently invaded by the species (Charudattan and Dinoor 2000). Plants in the center of origin may not be considered as a weed but when introduced to a new environment free from natural enemies like plant pathogens or other plant competitors they may become severe weeds (Alexander 2010).

One drawback of the classical biocontrol approach of weeds is the development of resistant weed genotypes. By almost killing the weed, the plant tries to escape the pathogen pressure and develops resistance against the pathogen, which makes the control agent ineffective. Another drawback might be host

specificity as the pathogen may find alternative hosts, e.g. when the original host disappears.

The third strategy called augmentation or inundative control refers to all forms of biological control in which natural enemies are applied periodically in high concentrations at the time when the pest or weed causes the problem, analogous to the use of a pesticide. In this approach, the biological agent is not expected to be self-sustaining (Boyetchko 1997) and the control is usually transient, and sometimes re-releases are required more than once per year (Bale, van Lenteren and Bigler 2008). A biological control agent can be used as bioherbicide if the following requirements are fulfilled: (1) the microorganism must be produced in high amounts and should be stable in a formulation, (2) the microorganism should specifically target the weed and have no negative impact on human or environmental health, (3) the microorganism must be genetically stable and (4) able to infect and kill a significant percentage of the weed population under different environmental conditions (Boyetchko 1997).

Several bioherbicides have been developed and registered since the early 1980s (reviewed in Harding and Raizada 2015). Most of the bioherbicides are based on fungal species with two main genera, *Colletotrichum* and *Phoma*. Many *Colletotrichum* species are plant pathogens and cause economic losses and postharvest damage in a wide range of crops (Münch et al. 2008). *Colletotrichum gloeosporioides* is a pathogenic fungus that causes anthracnoses on fruits, leaves and stems of a wide range of plants. Some strains produce phytotoxins, which are able to induce necrosis and reduce the growth of host plants *in vitro* (Jayasankar et al. 1999). The analysis of the secretomes of *C. orbiculare* and *C. gloeosporioides* revealed known effectors from other phytopathogens. During host colonization, *Colletotrichum* pathogens express small secreted proteins and secondary metabolite synthesis genes, proteases and carbohydrate-degrading enzymes (Gan et al. 2013), which are important for their pathogenicity according to the stage of infection. An example of a weed biocontrol application based on *Colletotrichum* is *C. gloeosporioides* f. sp. *malvae*, a hemibiotrophic pathogen of round-leave mallow (Mortensen 1988). This weed pathogen showed to be host specific, it can be easily grown on artificial media and the foliar application was very effective. It was registered as BioMal in Canada (Boyetchko, Bailey and De Clerck-Floate 2009). *Colletotrichum gloeosporioides* f. sp. *aeschynomene* is another example of a bioherbicide based on the genus *Colletotrichum* to control northern jointvetch (*Aeschynomene virginica*) (TeBeest 1982). The bioherbicide was registered as COLLEGO in the USA in 1997, re-approved in 2006 and then sold as LockDown (Bailey 2014). The fungus produces the phytotoxic metabolite ferricrocin, a kind of siderophore whose action mechanism has some relation with chelating activity (Ohra et al. 1995).

More than 2000 species of the genus *Phoma* exist worldwide, some of which are plant pathogens like *Phoma medicaginis*, a pathogen of alfalfa, and *Phoma lingan* and *Leptosphaeria biglosa*, which are important pathogens of oilseed rape and cause the black leg disease. Other species are saprophytes that colonize on decaying plant tissues and live in the soil (Graupner et al. 2003). Several of the species produce phytotoxic metabolites like phomalairdenone, nonenolides, epoxydonesters and putaminoxin (Graupner et al. 2003). The bioherbicide based on *Phoma macrostoma*, which is used to control broadleaf weeds in turfgrass, causes bleaching and chlorotic symptoms in infected plants (Zhou, Bailey and Derby 2004). The pathogen produces the phytotoxic metabolite macrocidins A and B, a new family of cyclic tetramic acids (Graupner et al. 2003). To control

*Chenopodium album*, the species *Ph. chenopodocola* was proposed for biological control. The fungus produces several phytotoxins in liquid culture (Cimmino et al. 2013; Evidente et al. 2015). When the toxins chenopodolin D and chenopodolin B are applied to leaf disks of non-host weeds, a fast development of necrosis was observed, whereas cheniscoumarin and the 9-O-acetyl had no effects on leaf disks (Evidente et al. 2015).

Beside *Colletotrichum* and *Phoma* also other genera were tested as mycoherbicides like *Sclerotinia*, *Chondrostereum*, *Puccinia*, *Alternaria* and *Phytophthora*. Strains were registered in the USA or Canada, but none of these products is currently commercially available (Harding and Raizada 2015). Overall, in addition to pathogens of weeds more sophisticated approaches may be developed based on the use of fungi or their metabolites to interfere with specific microorganisms such as endophytic fungi mediating high stress tolerance to their host. Such an approach might consider fungi, which have not yet been considered to be used for weed control.

In the last year, a *Tobacco mild green mosaic tobamovirus* strain U2 (TMGMV U2) was approved by the US EPA to control the tropical soda apple in pasture and wooded areas.

### Biocontrol strategies based on weed-associated bacteria

Apart from research on fungal pathogens to be used as bioherbicides, bacteria have also been explored for weed control. To date, there is only one bacterial bioherbicide (based on *Xanthomonas campestris* pv. *poae*) that has been registered and commercialized as CAMPERICO® in Japan (Imaizumi et al. 1997) to control annual bluegrass (*Poa annua* L.) in golf courses. The strain was isolated in Japan (JT-P482) and is applied during mowing (Imaizumi, Honda and Fujimori 1999). When the persistence of the bioherbicide strain in the field was investigated, it was found that after a certain period the bacterial control agent in the plants declined to initial levels (Imaizumi, Honda and Fujimori 1999). Although fungal pathogens have been the primary target in biocontrol applications to reduce weed populations, suitable bacterial pathogens are also known. A virulent strain of *X. campestris* (LVA987) was shown to control common cocklebur (*Xanthium strumarium* L.) (Boyette and Hoagland 2013), which is an important weed in soybean, cotton and peanut production. Because of the economic importance of the weed and the fact that resistant biotypes can be found against several herbicides, the weed is a good candidate for biological control. *Xanthomonas campestris* LVA987 was isolated from diseased common cocklebur. By spraying the isolate in combination with Silvet L-77 on seedlings in a dew chamber, a rapid increase in the disease severity was observed (Boyette and Hoagland 2013). In a host range assay, it was shown that plants from the Asteraceae family are affected to some degree by this pathogen but no other plant families (Boyette and Hoagland 2013). Since strain LVA987 was highly effective against plants from the Astereaceae family, it was also tested on horseweed (*Conyza canadensis* L. Cronq.). This weed presents a problem in the production of cotton, sorghum, corn and soybean. Horseweed is a host plant of the tarnished plant bug and of a mycoplasma disease (aster yellow) transmitted by the aster leaf hop (Boyette and Hoagland 2015). Several biotypes of *Co. canadensis* are resistant to certain herbicides, and as herbicides are generally not able to control this weed, biological control would be an alternative. Strain LVA987 caused mortality of *Co. canadensis* of rosette and bolting plants after a dewing period of 8–24 h at 25°C. If the dewing period was shorter than 4 h, no disease of the plants was observed. The experiment was conducted on glyphosate-resistant and glyphosate-susceptible

plants, and no differences between both biotypes were observed (Boyette and Hoagland 2015)

Also *Pseudomonas* spp. have been investigated for their use as bioherbicides. In contrast to *Xanthomonas* ssp. not all *Pseudomonas* ssp. are phytopathogens. Beside pathogenic representatives of *Pseudomonas syringae*, plant growth-promoting and biocontrol strains have also been described in this genus. *Pseudomonas chlororaphis* and *Ps. fluorescens* strains have been used as biocontrol agents, while several strains of *Ps. aeruginosa* and *Ps. stutzeri* show strong plant growth-promoting activities (Shen et al. 2013).

Many *Pseudomonas* strains are characterized as deleterious rhizobacteria (DRB). DRB are a group of saprophytic, non-parasitic pathogens, which excrete exopolysaccharides and allelochemicals in the form of cyanide, phytohormones, siderophores and phytotoxins that can negatively affect the metabolism of plants (Li and Kremer 2006). For example, the biocontrol strain *Pseudomonas* sp. BRG-100 produces extracellular secondary metabolites named as pseudophomins A and B, which are cyclic lipopeptides. This strain can reduce the root growth in green foxtail by 73% to 79%, and is able to colonize root hairs and the root except the root cap of green foxtail (Caldwell et al. 2011). Furthermore, strain *Ps. fluorescens* WH6 produces a compound that inhibits the germination of a large number of graminaceous plants but not of broadleaf weeds (Banowetz et al. 2008). Seed germination was arrested immediately after emergence of coleorhiza and plumule due to the presence of this strain (Banowetz et al. 2009). The compound responsible for the germination arrest was found to be 4-formylaminoxy-1-vinylglycine belonging to the oxyvinylglycines (McPhail et al. 2010). Oxyvinylglycines are known to block reactions catalyzed by enzymes dependent upon pyridoxal phosphate as cofactor. These enzymes include aminotransferases that perform vital functions in nitrogen metabolism, as well as the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which catalyzes a critical step in the biosynthesis of the plant hormone ethylene (Berkowitz et al. 2006).

Several *Ps. putida* strains were used to control velvetleaf and *Striga hermonthica* (Del.), *Ps. fluorescens* strains to control broomrape, wild radish and *S. hermonthica* (Del.) (Stubbs and Kennedy 2012). The *Ps. fluorescens* strain D7, which was isolated from roots of winter wheat, showed a reduction of downy brome (*Bromus tectorum* L.) biomass production of 18%–54% in the field when the strain was applied to the soil (Ibekwe, Kennedy and Stubbs 2010). This strain produces a complex of chromopeptides, peptides, fatty acids and a lipopolysaccharide matrix, but the genetic basis of the phytotoxin production by strain D7 is unknown. Some studies have shown that several genes are involved in the production of a phytotoxin, which are located on several loci (Ibekwe, Kennedy and Stubbs 2010). Strain D7 was registered by EPA in August 2014 and will be commercialized for use against downy brome in wheat.

Besides *Pseudomonas* also strains belonging to various other genera were found to be deleterious, including *Burkholderia*, *Aeromonas*, *Chryseomonas*, *Agrobacterium* and *Vibrio* spp., which were tested for potential use as bioherbicides (see Li and Kremer 2006).

By using rhizobacteria to control weeds, it was shown that the deleterious effect was host specific. Host range tests using several isolates to suppress downy brome demonstrated that the effects of the rhizobacteria and their secondary metabolites are host specific and the amount of reduction in root growth was found to be concentration dependent (Boyetchko 1997). Tests on non-target hosts like spring and winter cereal crops showed



little or no detrimental effects to root and shoot growth. In some cases, the same strains improved plant growth depending on the crop cultivar and microbial strain (Kennedy et al. 1991; Boyetchko 1997). Fungal pathogens are well known for their capacity to produce host-selective toxins, which attack the host, whereas non-host plants are tolerant against the toxin (Livingston and Scheffer 1984). Also soil bacteria or endophytes may produce host-specific phytotoxic secondary metabolites (Carvalho et al. 2007). Plants as well as bacteria produce indole acetic acid (IAA), which acts in low concentrations as a plant hormone stimulating growth and development processes, but in high concentrations it induces phytotoxicity (Grossmann 2010). Beside the concentration, also the plant tissue, physiological stage and plant species determine the sensitivity to auxins (Grossmann 2010). The first reaction to elevated auxin includes the activation of metabolic processes such as the stimulation of ethylene biosynthesis through induction of ACC synthase activity in shoots and an accumulation of abscisic acid. Later the plant reacts to elevated auxin with inhibition of root and shoot growth, decreased internode elongation and leaf growth, and intensified green leaf pigmentation, accompanied by stomatal closure and an increase of reactive oxygen species (Grossmann 2010). Bacterial pathogens like *Agrobacterium* spp. and *Ps. savastanoi* pv. *savastanoi* produce auxins, which cause tumor and gall formation (Spaepen and Vanderleyden 2011). It seems that auxin is a key factor in bacterial phytopathogenesis by regulating auxin signaling in plants. In addition, application of auxin promotes the susceptibility of the plant to bacterial pathogens and increases disease symptoms (Spaepen and Vanderleyden 2011). As an example, *Enterobacter* sp. strain I-3 produces IAA and seedlings of lettuce and radish inoculated with this strain showed reduced biomass production. In *in vitro* studies, it was observed that due to the addition of tryptophan, which serves as a precursor of IAA synthesis, to the culture exudate the root length, leaf width, leaf length and the lateral roots were significantly reduced (Park et al. 2015).

Due to the stimulation of ethylene biosynthesis caused by IAA, cyanide is formed as a coproduct (Grossmann 2010). Cyanide is a potential inhibitor of enzymes involved in major plant metabolic processes including respiration, CO<sub>2</sub> and nitrate assimilation, and carbohydrate metabolism. Cyanide also interacts with the protein plastocyanin, which inhibits the photosynthetic electron transport (Kremer and Soussi 2001). Hydrogen cyanide (HCN) is produced by a wide range of plants, bacteria and algae but also by car exhaust, cigarette smoke and during incomplete burning of fire. HCN production is a common trait of many rhizosphere *Pseudomonas* spp. *Pseudomonas aeruginosa* KC1 was tested for the ability to reduce the growth of *Amaranthus spinosus* and *Portulac oleraca* and proved to produce HCN, which was responsible for the growth reduction in the weeds (Lakshmi et al. 2015). Seedlings of wheat were less affected by HCN compared to weeds. Duke and Dayan (2011) reviewed microbial phytotoxins that share the same target sites in plants like synthetic herbicides. Many bacterial phytotoxins have unique target sites, potentially providing new mode of actions against weeds.

### Microbial contribution to reducing the allelopathic effects of weeds

Plants may release allelopathic compounds or phytotoxins as volatiles, root exudates or by decomposition of plant material (Schreiber and Williams 1967). Allelopathy confers high competitiveness to weeds and has facilitated the invasion of several plant species, including invasive *Centaurea* species (Roché

and Roché 1991), *Cirsium arvense* (Stachon and Zimdahl 1980), *Euphorbia esula* (Steenhagen and Zimdahl 1979), *Setaria faberii* (Bell and Koeppel 1972) and *Sorghum halepense* (Johnson 1975). Furthermore, allelopathic weeds have been reported to affect crop plants (Kohli and Batish 1994). Root exudation of weeds may influence also the structure and functioning of soil microbial communities (Eppinga et al. 2006; Mummey and Rillig 2006), potentially promoting or inhibiting pathogens (Nijjer, Rogers and Siemann 2007) or mutualists (Richardson et al. 2000, b, Weir 2007). For diffuse knapweed (*Centaurea diffusa*), it was shown that this species causes shifts in soil microbial communities (Callaway et al. 2004), and its roots release 8-hydroxyquinoline with antimicrobial activities (Vivanco et al. 2004). Invasive Brassicaceae weeds such as garlic mustard (*Alliaria petiolata*) produce glucosinolates, which seem to be involved in declining the abundance and function of AMF (Roberts and Anderson 2001). In the case of *Solidago canadensis* L. (goldenrods), which originates from North America and is highly invasive in China, growth of local plant species is inhibited due to the production of allelochemicals (Sun, Chen and Zhou 2006). Zhang et al. (2007) showed that the allelochemicals produced inhibited AMF and decreased mycorrhizal colonization of local plant species. Furthermore, allelopathic effects on common soil-borne pathogens, *Phytophthora ultimum* and *Rhizoctonia solani*, were observed (Zhang et al. 2009).

Soil and plant-associated microorganisms may play a role in allelopathy by either detoxifying allelopathic compounds by metabolization, degradation or transformation (reviewed by Mishra, Upadhyay and Nautiyal 2013). After release into the soil, allelochemicals are subject to adsorption on soil particles or organic matter as well as microbial transformation processes, which determine availability and toxicity of allelochemicals (Inderjit 2001). *Parthenium hysterophorus* L. is an invasive plant in countries like India, and allelochemicals including parthenin and phenolic acids are responsible for eliminating native plants (de la Fuente et al. 2000). The bacterial strain *Ps. putida* NBRIC19 showed to reduce the allelopathic effect of *Pa. hysterophorus* and several functions such as biofilm formation and colonization of *Pa. hysterophorus* and modulation of antioxidant enzymes as well as the degradation of the toxic allelochemicals were shown to be involved (Mishra and Nautiyal 2012; Mishra et al. 2012a,b). Also rhizosphere microorganisms may produce phytotoxic secondary metabolites that inhibit weeds (Mishra, Upadhyay and Nautiyal 2013). Furthermore, microorganisms may directly interfere with plant physiology by stimulating or inducing the production of secondary metabolites including allelochemicals.

### SCREENING AND APPLICATION APPROACHES FOR POTENTIAL BIOHERBICIDES

Several sampling strategies exist for obtaining appropriate strain collections for further screening (see Fig. 2). The best way of finding microbes showing allelopathy is to look for sites with suppressed vegetation (Barazani and Friedman 2001). A weed-suppressive soil is defined as a soil in which certain weeds do not establish or persist, or establish and grow with the crop but cause little yield loss due to the reduced vitality caused by allelopathic bacteria (Kremer 2006). Screening for *Pseudomonas* rhizobacteria in weed suppressive soil resulted in 15 *Pseudomonas fluorescens* / *Ps. putida* strains that were able to significantly reduce the germination of *Striga hermonthica* (Del.) Benth (Ahonsi et al. 2002).

Isolation of microorganisms from diseased weeds could lead directly to host-specific pathogens for potential use as bioherbicides (Boyette and Hoagland 2013). Most of the plant pathogens



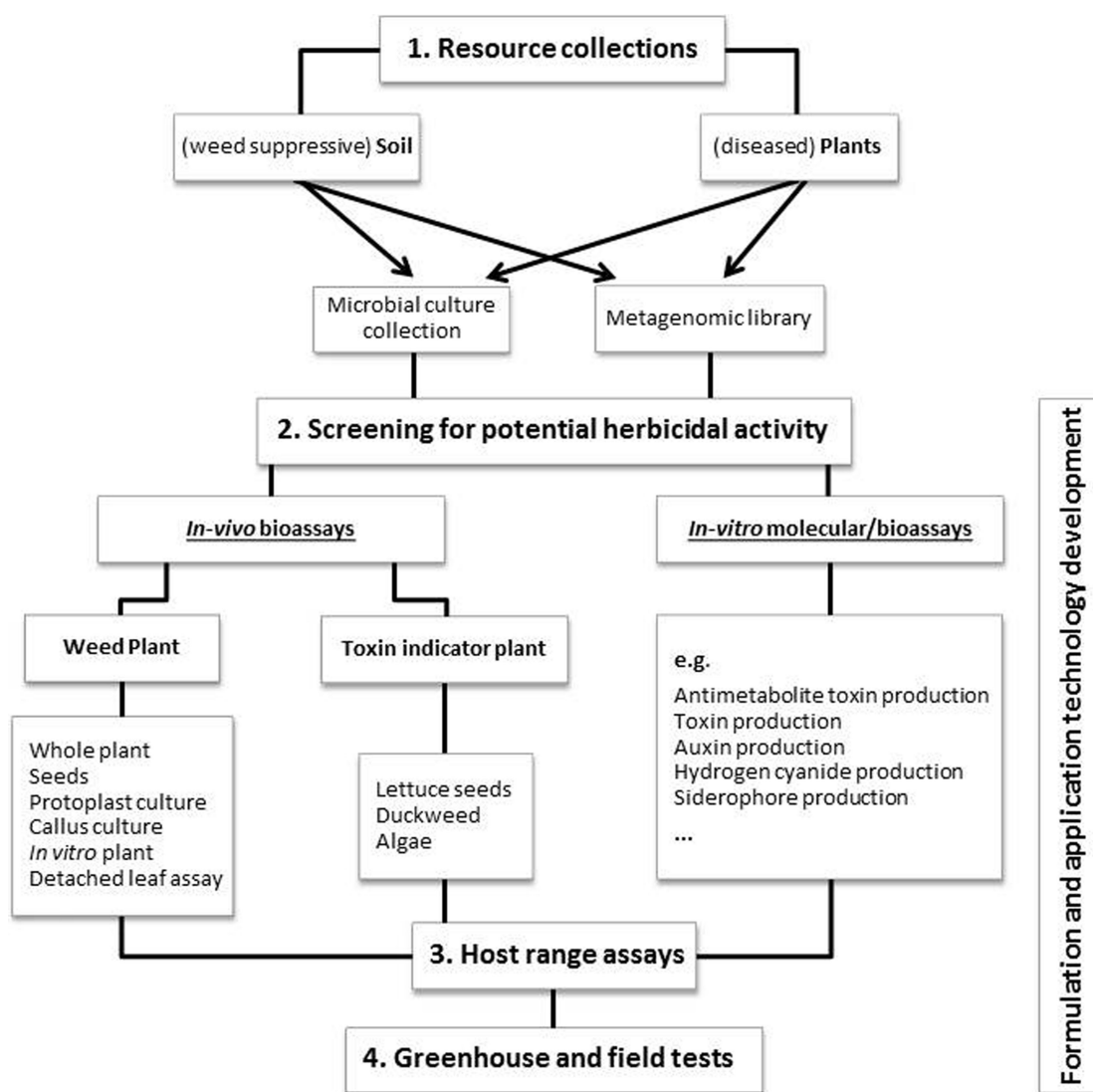


Figure 2. Overview screening approaches for the development of bioherbicides based on microorganism.

are host specific and would be good candidates for selective herbicides, and all currently available fungal bioherbicides are plant pathogens with a narrow host range. In addition, also bacterial pathogens like *Xanthomonas* ssp. have been tested as bioherbicides (Imaizumi et al. 1997; Boyette and Hoagland 2013). The use of endophytic bacteria as bioherbicides is still under-exploited. Kloepper et al. (2013) found endophytes in Leatherleaf fern (*Rumohra adiantiformis*), which are responsible for the deformation of the leaves. The responsible fluorescent pseudomonads are present as latent endophytes also in healthy plants, but if they exceed a certain threshold, symptoms of leaf distortion appear. This example shows that certain endophytes may have different effects depending on their quorum. Many detrimental allelopathic microbes cause deleterious effects when applied artificially in higher concentrations compared to their natural occurrence. Screening isolates from already established strain collections could be another approach, keeping in mind that many endophytic microorganisms could be pathogenic in a non-host plant.

Several methods for the screening for phytotoxic activity are available (Fig. 2). Stubbs and Kennedy (2012) proposed a

screening procedure for bacterial biological control agents. In a first bioassay, the strains are tested for their activity against the weed. Selected strains that suppress the growth or germination of the weed are in the next step tested against several crop plants. Only bacterial strains that do not suppress the crop plants are tested in soil in the greenhouse and in the field. To speed up the initial screening against the weed, several alternative methods are proposed (Fig. 2). To avoid the labor-intensive propagation of the weeds, also protoplast culture or callus culture of weeds have been used. Souissi and Kremer (1998) used a callus culture from leafy spurge (*Euphorbia esula* L.) grown in multiwell plates to screen for phytotoxic bacteria. Vidal, Guermache and Widmer (2004) used callus cultures of yellow starthistle (*Centaurea solstitialis* L.) to rapidly screen for phytopathogens. However, such systems are not always available. As an alternative method for the screening against target weeds, the indicator technique for antimetabolite toxin production against *Escherichia coli* was proposed (Gasson 1980). The mechanism of *E. coli* growth inhibition is similar to the phytotoxin-induced chlorosis of plant tissue (Gasson 1980) In this assay, phytopathogenic bacteria or cell free filtrates produce

an inhibition zone on plates containing *E. coli* cells. Using cell-free filtrates gave the same result as whole cells, but by using the supernatant less contamination on plates was observed (Harris and Stahlman 1996). The assay only works for bacteria that produce secondary metabolites, but we did not see a correlation with deleterious effects on weeds. If seeds of weeds are not easily available, the lettuce (*Lactuca sativa* L.) seedling bioassay is widely used as an inexpensive and fast testing system for toxicity. The test shows consistently indication effects of growth inhibition by bacteria (Kremer 2013). Lettuce seeds assays are described as excellent phytotoxin detectors (Carvalho et al. 2007), but are not suitable to detect host-specific bioherbicides. However, in our experiments we have seen that only few bacterial strains, which cause growth inhibition on the target weeds, also cause a reduction in lettuce growth.

Detached leaf bioassays have been used to select fungal strains which were able to grow and colonize Johnsongrass (*Sorghum halepense*) leaves (Chaing, van Dyke and Leonard 1989). By applying different cell numbers to leaf pieces, the optimal cell density for later whole plant assays was selected (Saxena and Kumar 2010).

To narrow down the toxins produced by e.g. *Ps. syringae* PCR screens for coronatine (Bereswill et al. 1994) and tabtoxin (Lydon and Patterson 2001) production are available.

Interference with IAA production and signaling is an important mode of action of synthetic herbicides. Important herbicides such as 2,4 D and MCP are auxin growth regulators. Similarly, IAA production is an important mechanism of bioherbical microorganisms, which may be tested by a colorimetric method, e.g. using the Salkowski reagent (Sarwar and Kremer 1995).

Hydrogen cyanide (HCN), a volatile metabolite that negatively affects root metabolism and root growth, is produced by many *Ps. fluorescens* and *Ps. aeruginosa* strains (Blumer and Haas 2000). Bacteria produce different amounts of HCN and the production is very tightly regulated. Cyanide concentrations are usually below 1 mM and can be tolerated by many organisms (Blumer and Haas 2000). In cases where the plant is heavily colonized by *Pseudomonas* strains, the accumulated HCN concentration may have deleterious effects. Although colorimetric assays for the detection of HCN exist (Lorck 1948; Feigl and Anger 1966), its quantification is more laborious. The test proposed by Lakshmi et al. (2015) involves a paired plate assay and offers the possibility to screen bacteria for growth without knowing the volatile compound. Therefore, with this assay also unknown secondary metabolites will be detected. Carvalho et al. (2007) proposed a screening approach based on extracts from bacterial cultures obtained with methanol/ethyl acetate. The extracts were further processed by purification with silica gel columns, followed by different solution steps in methanol, ethyl acetate or water. Each fraction was tested on lettuce seeds.

For the application as bioherbicides, selected strains have to demonstrate that they are specific to the weed to prevent damage of economically important plants. The biological agent should be deleterious to the target species, but have no deleterious effects on non-target plants. Wapshere (1974) proposed a screening strategy based on a phylogenetic approach. Tests on native plants, crops and weed species of related families, tribes, subtribes and accessions closely related to the weed will determine the host range (Stubbs and Kennedy 2012). Until now six non-target species have been damaged outdoors by biocontrol agents, but the same agents damaged 107 non-target species in a pre-release test under controlled conditions (Barton 2012).

These data indicate that tests under controlled conditions are strict enough to predict the host range.

For commercialization, also other aspects of a successful bioherbicide are important, which include the viability in a formulation and the feasibility of mass production. The screening should be started as soon as positive strains are found, to avoid incorporating strains that are either susceptible to heat and drought or not suitable for mass production.

Like for other biocontrol agents, also for bioherbicides risk assessments have to be carried out prior to registration. The risk associated with bioherbicides can be categorized in the risk to humans and mammals, plant host range and effects on non-target organisms like competition or displacement of beneficial microbes. To address these issues, screenings for the toxin production and host range assays have been carried out. During the development of bioherbicide, strains with possible harm to humans and animals have to be excluded. By using naturally occurring plant-associated bacteria as bioherbicides, the risk of unbalanced growth or displacement of important microorganisms in the receiving environment is unlikely, but has to be monitored in course of the registration process. During the evaluation of CAMPERICO®, the control agent was not found after a certain time period (Imaizumi et al. 1997).

New approaches in molecular biology may facilitate the discovery of herbicidal compounds from metagenomic libraries targeting also microorganisms difficult to cultivate (Kao-Kniffin, Carver and DiTommaso 2013) (Fig. 2). It is predicted that metagenomics tools together with new sequencing technologies will provide the basis for the discovery of new antibiotics and enzymes in biomedicine and industrial fields (Li and Vederas 2009). The extraction of DNA or RNA directly from the environment bypasses the need for cultivation of single isolates. For the discovery of compounds with new mode of actions, structure and sequence functional screenings on the host have to be developed (Kao-Kniffin, Carver and DiTommaso 2013). Using different functional screenings on small plants like duckweed (*Lemna minor* L.), algae or leaf spot assays can improve the success rate (Kao-Kniffin, Carver and DiTommaso 2013). Selected clones or strains can then be tested at a later stage on a larger scale in the greenhouse. Using high-throughput sequencing techniques and advanced bioinformatics tools together with metabolomics analyses will allow the identification of genes and metabolites responsible for the production of herbicidal compounds.

## CONCLUSIONS AND FUTURE PROSPECTS

Weeds and invasive plants modulate soil microbiota in several ways. New plant species may bring along novel microorganisms and interact with natural microbiota to favor the growth and competitiveness of the invader. On the other hand, they also contribute to a higher microbial diversity. During domestication, crop plants have often lost their ability to interact with beneficial microorganisms and weed-associated microorganisms may increase the richness and expand the functional capacities of soil microbiota.

Understanding the functions of the microbiome may guide us to a microbial-based weed management. The challenge is to identify microbes or management practices, which reduce weed growth as well as promote the growth of crop plants or at least have no detrimental effects on non-weedy plants. It will be of high importance to further elucidate the role of soil and plant-associated microbial communities in weed establishment and growth as well as to better understand the mechanisms

involved in these interactions. Based on the comparison of the microbiomes from weed suppressive and non-suppressive soils, it may be feasible to elaborate recommendations for weed management systems. Similarly, it will be equally important to understand the modes of action of potential biocontrol candidates as well as the regulation of diverse mechanisms, such as the production of herbicidal secondary metabolites, to enable improved selection as well as application procedures. Any harm to humans, animals and the environment have to be avoided, and therefore aspects such as potential pathogenicity and toxicity, including phytotoxicity to non-target plants, have to be thoroughly assessed prior to any release to the environment. As with any other biocontrol agent, additional issues have to be addressed, including potential mutations or horizontal gene transfer leading to pathogenicity of non-target plants. However, the probability of such events is extremely rare.

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