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Synergy between Synthetic and Microbial Herbicides for Weed Control

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ABSTRACT

Synthetic herbicides have been investigated as tools to synergize mycoherbicides (fungal bioherbicides) for improved efficacy or management of hard-to-control weed problems. Herbicides may weaken weeds and impair their defence systems, thus making weeds more vulnerable to mycoherbicide infection. Despite many positive results, the practical value of synergy remains elusive. This review will discuss several fundamental aspects of synergy relating to development of this technology based on author's own experiences in biocontrol of green foxtail and scentless chamomile. These include application timing, dose effect, weed growth stage, and spray retention efficiency. Issues relating to the practicality, non-target risks, and cost of weed control are stumbling blocks to the adoption of synergistic technologies, and some tactics are proposed to address these challenges.

Keywords: adjuvant, biocontrol, compatibility, mycoherbicide tank mix

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INTRODUCTION

Microorganisms, especially host-specific fungal pathogens, have been studied extensively as potential weed biocontrol agents (mycoherbicides). Despite all the promise, successes at commercial levels have been limited (Boyetchko and Peng 2004; Gressel 2010). The most common challenge has been insufficient or inconsistent weed-control efficacy under field conditions (Gressel 2002; Hallett 2005). A weed population is generally heterogeneous, and a selected pathogen strain often is not universally virulent against all individuals in the weed population. To meet crop safety standards, most mycoherbicide candidates are highly host specific and this narrow-host spectrum can be perceived insufficient for weed control in crop fields where a range of weeds needs to be controlled with one spray application. Despite years of effort towards enhancing the field performance of mycoherbicides, the improvement has been incremental and the fundamental issue of insufficient virulence remains (Gressel 2002; Hallett 2005; Sands and Pilgeram 2009).

Inconsistent efficacy may also occur with synthetic herbicides, depending on weed targets and their growth stages, weather conditions, and/or rates used in a crop system (Kumaratilake and Preston 2005; Legere *et al*. 2006; James *et al*. 2007; Monnig and Bradley 2007). In some cases, a prominent weed problem is not controlled by a conventional herbicide or herbicide tank mixture, and additional options such as a mycoherbicide appears a reasonable proposition (Peng *et al*. 2005a, 2007). There is a large body of literature documenting the synergy between specific herbicides and mycoherbicides (Wymore *et al*. 1987; Wymore and Watson 1989; Caulder and Stowell 1988; Grant *et al*. 1990; Christy *et al*. 1993; Peng and Byer 2005; Graham *et al*. 2006b; Boyette *et al*. 2008a, 2008b), and several advan-

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Invited Review

tages have been suggested for using this synergy in weed control, including greater efficacy and/or lowered product rates. There is frequently a lack of understanding of mechanisms of action for herbicide-microbial interactions, otherwise the selection of synergistic components may be more efficient (Gressel 2010). Additionally, despite much research, little unilization of synergy is known in commercial situations. This review explores approaches used in discovery and application of synergy, strategies to optimize herbicide-microbial interations, and potential application of synergy for improved weed control.

ASSESSMENT OF SYNERGY

Theory and fundamentals

In most cases, the relationship between a mycoherbicide and its hosts likely entails insufficient virulence for the pathogen to achieve adequate weed control, otherwise the weed and pathogen would both have become extinct (Sands *et al.* 2001). Under greenhouse conditions, many weeds can be easily killed with mycoherbicides agents applied at high doses (Greaves and MacQueen 1992). In the natural environment or field conditions, an evolutionary balance may allow weed populations to withstand most pathogen attacks due to their genetic heterogeneity.

When attacked, plants defend themselves using a range of mechanisms, including structural barriers and induced secondary metabolites, when recognizing certain components of the pathogen or responding to a level of damage to their cells (Nimchuk *et al*. 2003). Many herbicides impair the ability of weeds to respond to pathogen attacks (Lydon and Duke 1989; Ahn *et al.* 2005a, 2005b). Herbicide-induced weakening can predispose the plant to the infection by facultative pathogens (Levesque and Rahe 1992). By blocking the synthesis of phenylalanine-derived phenols, glyphosate inhibits the production of phenolics, including lignin precursors and several classes of phytoalexins involved in plant defense responses (Levesque and Rahe 1992). On sicklepod [*Senna obtusifolia* (L.) Irwin & Barneby, syn. *Cassia obtusifolia*], glyphosate suppressed the biosynthesis of a phenylpropanoid phytoalexin elicited by the mycoherbicide agent *Alternaria cassiae* Jurair & Khan produced through the Shikimate pathway (Sharon *et al*. 1992), and this inhibition occurred even at reduced rates of glyphosate (Keen *et al.* 1982).

Suppression of callose production by herbicides may also diminish plant defense responses (Gressel 2002). Plant cells often deposit callose between the plasma membrane and cell wall in close proximity to the invading pathogen (Ryals *et al*. 1996), and these callosic deposits are commonly referred to as papillae. Although their precise function has not been determined, these structures act as a physical barrier that impedes pathogen penetration. By slowing down or immobilizing the invading pathogen, the host may be able to deploy additional mechanisms, including wall-degrading enzymes, phytoalexins, or initiating cascade responses involving specific resistance genes (Brown *et al*. 1998). For most of the synergy studies reported in the literature (Grant *et al*. 1990; Wyss and Muller-Scharer 2001; Yandoc *et al*. 2006; Weaver and Lyn 2007; Puja and Kumar 2008), the mechanism that mediates the effect was not clear. The main assumption was that herbicides or microbial-based phytotoxins had a general weakening effect on weeds that would make mycoherbicide agents more virulent (Vurro *et al*. 2001; Gressel 2010).

Herbicide-microbial synergy can vary with pathogen-weed systems, and is therefore difficult to generalize. For instance, the herbicide metribuzin was synergistic with the mycoherbicides *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc. f. sp. *malvae* (Grant *et al*. 1990) and *C. truncatum* (Schwein.) Andrus & W.D. Moore (Peng *et al*. 2005a) against round-leaved mallow (*Malva pusilla* Sm.) and scentless chamomile (*Matricaria perforate* Mérat) respectively, but showed no such effect with *C. truncatum* for control of Florida beggarweed [*Desmodium tortuosum* (SW.) DC.] (Caulder and Stowell 1988). Alternatively, Gressel (2010) suggested basing the detection of synergism on herbicide modes of action, and this strategy might offer an efficient approach for selecting synergistic components against certain some weed targets. A better understanding of herbicide modes of action and pathogen infection strategies may help to narrow the range of search.

Measuring synergy

To determine the nature of herbicide-microbial interactions, often the method based on the multiplicative survival model described by Colby (1967) is used to analyze the efficacy of mixtures where the components have different modes of action (Morse 1978). Synergism is anounced when the effect is mathematically greater than the sum of individual components applied separately (Peng and Byer 2005; Graham *et al.* 2006b). A statistical procedure such as Fisher's protected LSD may be applied for further validity of the results (Lanclos *et al*. 2002; Koger *et al.* 2005). Colby's method provides a useful criterion for selecting synergistic elements but this synergy may be of limited value in practice because it does not factor in product rates, costs, acceptable efficacy, and/or other weed-control considerations in a particular field situation (Owens and Gressel 2001). Gressel (1996) used the term "$ynergy" which would have economic and agronomic considerations for the approach, e.g. are there any dollar value for using the two components together? This approach is more complex and involves practical elements for utilizing the strategy. Ideally, an assessment on the nature of interaction should be conducted for real efficacy gains, and Colby's method can help narrow down the candidates efficiently. Sometimes even marginal synergism can turn weed control from suppressive (< 80%) to plant kill (Peng *et al*. 2005a).

BENEFITS

As Hoagland (1996) suggested, synergy may reduce the application rate of biological and synthetic herbicides required for effective weed control. Lowering mycoherbicide rates helps reduce the cost of biological control and decreasing the rate of herbicide reduces the load of pesticides in the environment. Additional benefits include a broadened spectrum of weed control and a widened window of application under field conditions.

Several examples show that the rate requirement for mycoherbicides can be reduced substantially via synergy. Sharon *et al*. (1992) were able to reduce the dose of *Alternaria cassiae* by 5× when using it with glyphosate at a sublethal dose on sicklepod. Glyphosate diminished defense responses of the weed by suppressing the biosynthesis of a phytoalexin elicited by fungal infection (Sharon *et al*. 1992b). Heiny (1994) also reported that adding 2,4-D or MCPA to conidial suspensions of *Phoma proboscis* Heiny attained as effective control of field bindweed as the fungus alone at a 10× higher dose. From the commercial perspective, this reduction in mycoherbicide rate represents significant cost savings because microbial production is generally more expensive than that of conventional herbicides (Boyetchko and Peng 2004; Bailey 2004). One of the issues with this strategy is that of herbicide registrants may be reluctant to reduce product rates substantially due to sales, liability considerations, and additional regulatory requirements for new labels. Extensive field trials are likely required to validate the new rates proposed in synergistic applications.

Many mycoherbicides, including Collego™, and BioMal®, have a narrow spectrum of weed targets, which may limit their potential in field crops where multiple weed problems often need to be tackled at the same time. There are a few cases where mycoherbicides are used as a broad-spectrum option based on non-host-selective fungal pathogens (Bourdot and Harvey 1996; Harper *et al.* 1999; Schnick *et al.* 2002), but non-host specificity can be perceived

as a risk factor for mycoherbicide due to potential impact on crops and non-target species. From the practical perspective, a broader weed-control spectrum is more appealing to commercial development due to greater market potential.

Several studies have demonstrated that synergy can broaden the range of weed control by mycoherbicides. Acifluorfen or bentazon in a tank mixture with Collego™ controlled both northern joint vetch (original target) and hemp sesbania (extra weed) in rice and soybean fields without any visible injury to the crops (Smith 1991). Similarly, *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar:Fr., which was highly virulent on sicklepod and hemp sesbania when formulated with the surfactant Silwet L-77 (Andersen and Hallett 2004), was also highly virulent on kudzu [*Pueraria lobata* (Willd.) Ohwi] but only moderately virulent on redvine [*Brunnichia ovata* (Walt.) Shinners] or trumpetcreeper [*Campsis radicans* (L.) Seem. Ex. Bureau] (Boyette *et al*. 2001). When the fungus was applied 2 days after a glyphosate treatment, however, both redvine and trumpetcreeper were effectively controlled in the greenhouse (Boyette *et al*. 2006) and in RoundUp®-resistant soybean fields (Boyette *et al.* 2008b). Neither glyphosate nor the fungus alone controlled the weeds at acceptable levels (< 80%). These results indicate that synergy may be used to control several important weeds in RoundUp®-resistant soybean in southern United States; the weeds that may be at risk of developing glyphosate herbicide resistance.

Mycoherbicides tend to be more effective when weeds are in seedling or early growth stages (Makowski 1993; Walker and Tilley 1997; Peng *et al*. 2004), and often this window of application is narrower than that for synthetic herbicides. For example, when *Plectosporium tabacinum* [(van Beyma) Palm, Gams et. Nirenberg] was used to control false cleavers (*Galium spurium* L.), the fungus killed seedlings rapidly but did not do so on older plants (Zhang *et al*. 2002). Similarly, *Phomopsis amaranthicola* Rosskopf, Charudattan, Shabana & Benny controlled smooth pigweed (*Amaranthus hybridus* L.) when plants were at younger than the 4-leaf stage. On older plants, weed control was much poorer and increasing the fungal dose by 100× did not improve the efficacy (Rosskopf *et al*. 2005). Variation in the growth stage is common within a natural weed population (Auld *et al*. 1990) and generally a greater dose of mycoherbicide is required to achieve sufficient efficacy against weeds in more advanced growth stages. This shows the non-traditional dose response of some mycoherbicide agents which will contribute to high costs of biocontrol (Boyetchko and Peng 2004).

OPTIMIZATION OF SYNERGISTIC INTERACTIONS

Treatment timing

A prior treatment with a herbicide, relative to tank-mix applications, may boost weed- control efficacy for a synergistic herbicide-microbial combination (Peng and Bayer 2005). Sometimes this sequential application may be preferred because incompatibility resulting from the inhibition to fungal spore germination or other infection processes by the herbicide or spray adjuvant (Hoagland 1996) diminishes synergy with tank-mix applications. For example, split applications allowed the use of *Colletotrichum coccodes* (Wallr.) Hughes (Hodgson *et al*. 1988) and *C. gloeosporioides* (Grant *et al.* 1990) with incompatible herbicides for control of velvetleaf and round-leaved mallow, respectively. The major drawback with the split application is added costs to the producer (Hatzios and Penner 1985).

Another factor to consider is the need to predispose weeds to fungal infection with herbicides, which impairs plant defense responses. This process may take time because the herbicides will have to be absorbed by the plant and some may have to be translocated to active sites for the effect. Therefore this predisposition may not reach an appreciable level until some time after. For example, only 16% and 2% of sethoxydim were absorbed by centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.] and goosegrass [*Eleusine indica* (L.) Gaertn.] two hours after application (McCarty *et al*. 1990). Sharon *et al*. (1992a) also found that phytoalexin production in sicklepod induced by *Alternaria cassiae* was not inhibited by glyphosate until 12 h after treatment. This implies that, in tank-mix applications, some early steps during fungal infection, including penetration and initial colonization, may not be synergized by herbicides. Conversely, when glyphosate was applied 1-3 days prior to the mycoherbicide agent *Microsphaeropsis amaranthi* Ell. & Barth (Smith and Hallett 2006), synergy was much more manifested relative to a co-application and much lower herbicide rates would be required for control of common waterhemp (*Amaranthus rudis* Sauer).

Dose effects

More information on this aspect will be provided in the first case studies to be presented a little later. One of the advantages of synergy is the potential to reduce the rate requirement for mycoherbicide (Hoagland 1996), and the herbicide rate may also be lowered substantially. Sharon *et al*. (1992a) demonstrated that adding the fungus *Alternaria cassiae* synergized glyphosate to kill sicklepod at a sublethal rate.

The growth stage of weeds

Plant growth stage can be a factor when designing a synergistic package for weed control because mycoherbicide and herbicide efficacy generally decline with the age of weeds. For example, when treating common lambsquarters (*Chenopodium album* L.) with *Ascochyta caulina* (Karst) Aa & Kest, the efficacy was substantially lower on older plants (Ghorbani *et al*. 2006). Similar observations were reported for biocontrol of false cleavers (Zhang *et al*. 2002) and smooth pigweed (Rosskopf *et al*. 2005). Adding herbicides, even at sublethal doses, may synergize mycoherbicides to achieve satisfactory efficacy at more advanced weed growth stages. These will be discussed in more detail in two case studies later. For efficacy and cost considerations, the choice of synergistic components and rates may vary depending on weed growth stage.

There are also examples where synergy still requires targeting young weeds. For example, Hodgson *et al.* (1988) reported that efficacy against velvetleaf with thidazuron plus *C. coccodes* was the highest at the seedling stage of the weed under field conditions. It is also possible that within a range of growth stages, little variation may be observed in efficacy for herbicide plus mycoherbicide mixtures (Peng and Byer 2005) because synergy often extends the treatment window. However, it is useful to know the treatment windows relative to weed growth stages in order to select synergistic components/rates judiciously.

Spray adjuvants

Efficacy of a synergistic mixture may be optimized through use of spray adjuvants, which can be different from those recommended for herbicides due to unique requirements of microbial components (Peng and Wolf 2008) and generally low efficiency of spray deposition or retention under field conditions (Matthews 2000). Spray adjuvants can alter the physiochemical properties of liquids, and consequently spray drop-size spectrum and velocity, in-flight and/or impaction behaviour, and deposit-target interactions (Miller *et al*. 2001). These traits can influence delivery efficiency of a synergistic mixture to the target. Ideally, these adjuvants should not only optimize deposition/retention efficiency, but also assist the survival and activities of microbial agents post application (Zidack and Quimby 1998; Bateman and Chapple 2001). For example, the surfactant Tween® 80 can release spores of *Colletotrichum* mycoherbicides from self-inhibition at high inoculum concentrations and stimulate conidial germination (Zhang *et al*. 2003). The surfactant

Silwet L-77 stimulated conidial germination and après-sorial formation of *C. truncatum* in a unrefined corn-oil formulation, lessening dew requirement for biocontrol of hemp sesbania under field conditions (Boyette *et al*. 2007). This adjuvant also benefited the mycoherbicide agent *C. gloeosporioides* in biocontrol of sicklepod and reduced the dew requirement for infection from 16 to 8 h (Boyette *et al*. 2006). Several commercial adjuvants and polyoxyethylene tridecyl ether (TDA) stimulated *Myrothecium verrucaria* in control of kudzu, redvine, and trumpetcreeper by altering the plant cuticle (Weaver *et al*. 2009). It is highly beneficial to use adjuvants which optimize the dose transfer to targets and also protect or stimulate mycoherbicide activities.

CASE STUDY 1 – SYNERGY OF *PYRICULAIA SETARIAE* WITH HERBICIDES FOR CONTROL OF GREEN FOXTAIL

The fungus *Pyriculaia setariae* Nisikado is a mycoherbicide candidate for control of green foxtail [*Setaria viridis* (L.) Beauv.] (Peng *et al.* 2004). However, its efficacy suffers from insufficient disease severity on emerging young leaves. Several factors were suspected for this; meristems of many grasses are protected by leaf sheathes (Greaves and MacQueen 1992) and younger tissues may be more tolerant to the pathogen (Moss and Trevathan 1987). As a result, green foxtail treated with *P. setariae* alone often recovered from initial injuries due to continuous growth of young leaves, unless extremely high fungal doses were applied in high water volumes (Peng *et al.* 2001). After extensive assessment based on the Colby's standard, several herbicides were found synergistic with the fungus, especially some of the graminicides at reduced rates that boosted virulence of the fungus substantially (Peng and Byer 2005). These herbicides generally target young grass leaves. For example, sethoxydim inhibits cell division and is particularly toxic to actively growing young tissues (Jain and Vanden Born 1989). For quinclorac, young grass leaves and apices can act as strong sinks (Lamoureux and Rusness 1995), which lead the accumulation of cyanide in these tissues (Grossman 1998). These herbicide effects seemed complementary to the mode of action of *P. setariae* on green foxtail.

The nature of herbicide-fungus interaction and efficacy of weed control can depend on the products and rates selected. Often a combination is more synergistic in nature when individual components are only moderately effective. Quinclorac at 0.1× label rate plus the fungus at 5×10^7 spores/mL killed most green foxtail under greenhouse conditions, and the same efficacy was also obtained with only < 10% of the fungal dose when the herbicide rate was increased to 0.5× (Peng and Byer 2005). A similar pattern of interaction was also observed with propanil and *P. setariae*. Propanil at reduced rates (0.1 to 0.5×) was less efficacious than sethoxydim, and often came across as "synergistic" (despite a moderate level of weed control), whereas sethoxydim was frequently found to be only "additive". In terms of efficacy, however, sethoxydim at 0.1× label rate killed green foxtail completely in the greenhouse when synergized by a low dose of *P. setariae* (**Fig. 1**). In field, the rates may need to be fine-tuned for optimal efficacy; sethoxydim at 0.25× label rate was needed for a noticeable impact on green foxtail and for sufficient weed control when tank mixed with *P. setariae* (**Fig. 2**).

The fungus *P. setariae* also caused slight infection on giant and yellow foxtail [*Setaria faberi* Herrm and *S. glauca* (L.) Beauv.], respectively (Peng and Byer 2009). In a further study, when the fungus was applied with quinclorac or propanil at 0.5×, or sethoxydim at 0.25× label rate, giant foxtail was killed (**Fig. 3A**) while yellow foxtail was injured only slightly (**Fig. 3B**) under greenhouse conditions. The herbicides at these rates suppressed the growth of both weeds substantially although no plant mortality occurred. Additional grass species were examined in a similar fashion, and only sethoxydim at 0.25× rate enhanced fungal infection slightly on Italian ryegrass and proso millet, but not on any other grass species evaluated (**Table 1**). This demonstrates that synergy can help expand the target of the mycoherbicide agent *P. setariae*.

Fig. 1 Effect of *Pyricularia setariae* plus sethoxydim on green foxtail in greenhouse conditions. From left to right: untreated control, herbicide alone, fungus alone, and fungus plus herbicide (0.1× label rate).

Fig. 2 Control of green foxtail by sethoxydim (0.25× label rate) (**A**) almost no damage) and sethoxydim plus *Pyricularia setariae* (**B**) severely damaged) under field conditions.

The rates of *P. setariae* and herbicide may be optimized for maximum efficacy. For quinclorac and propanil, higher rates combined with higher doses of *P. setariae* showed a

Table 1 Effect of the herbicide sethoxydim and fungus *Pyricularia setariae* on selected grassy species.

Test plant	Scientific name	Sethoxydim	*P. setariae*	Damage severity (%)[a]
Italian ryegrass	*Lolium multiflorum* Lam.	–[b]	–	0
		–	+	3.3
		+	–	15.0
		+	+	39.8**[c]
Proso millet	*Panicum miliaceum* L.	–	–	0
		–	+	1.5
		+	–	11.8
		+	+	26.1**
Goosegrass	*Eleusine indica* (L.) Gaertn.	–	–	0
		–	+	0
		+	–	32.2
		+	+	42.6
Barnyard grass	*Echinochloa crus-galli* (L.) Beauv.	–	–	0
		–	+	0
		+	–	32.0
		+	+	38.5
Large crabgrass	*Digitaria sanguinalis* (L.) Scop	–	–	0
		–	+	0
		+	–	34.6
		+	+	40.1
Wild oat	*Avena fatua* L.	–	–	0
		–	+	0
		+	–	6.1
		+	+	10.2

[a] Based on a 0 to 11 scale (Horsfall and Barratt 1945), which reflects the fact that human eyes distinguish small differences in percent best near zero or 100% and poorest near 50%.
[b] "+" and "–" indicate the component was present and absent in the treatment, respectively.
[c] **: Significant at 0.05 level (Protected LSD) for impact on weeds and synergy relative to efficacy of the herbicide and fungus.

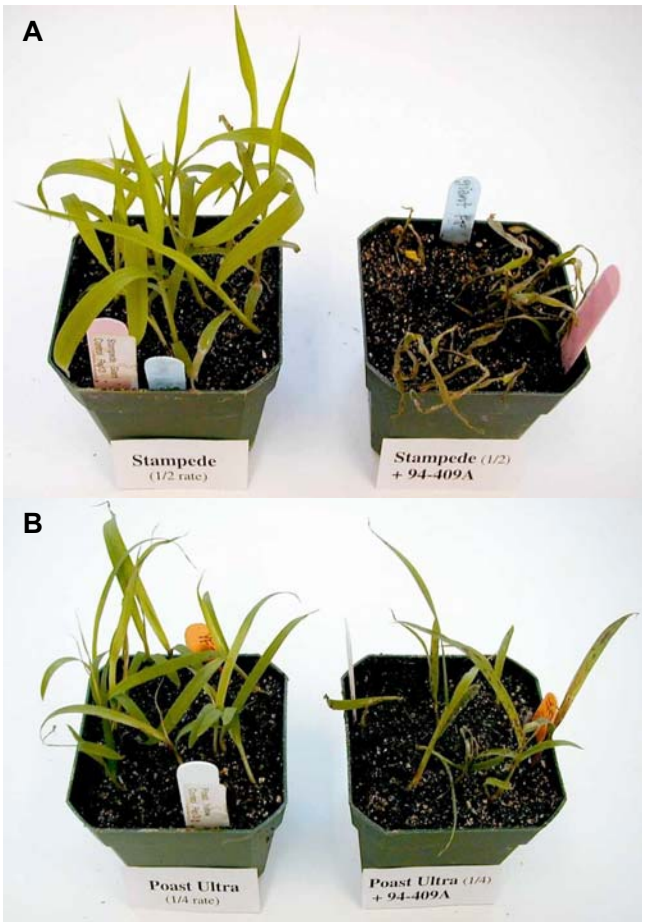

Fig. 3 The effect of *Pyricularia setariae* plus a herbicide at reduced rates on giant (**A**) and yellow (**B**) foxtail (right-hand pots), respectively. The pots on the left are herbicide-alone treatments.

trend of greater synergy, with efficacy peaking at 0.25× label rates when combined with the fungus at 2×10^7 spores/ml under controlled-environment conditions. When increasing the quinclorac rate to 0.5×, the fungal dose could be lowered to 5×10^6 spores/ml without compromising efficacy. A similar pattern was also seen with propanil plus *P. setariae* (Peng and Byer 2005). In contrast, sethoxydim at 0.1-0.5× label rates did not respond substantially to the addition of *P. setariae* in the greenhouse but the synergy was essential for the herbicide to control green foxtail under field conditions (**Fig. 2**). This case study highlights the potential of synergy in broadening weed targets of mycoherbicides and the need for fine tuning doses to maximize efficacy in field conditions.

CASE STUDY 2 – SYNERGY OF *COLLETOTRICHUM TRUNCATUM* WITH HERBICIDES FOR CONTROL OF SCENTLESS CHAMOMILE

The fungus *C. truncatum* is a mycoherbicide agent for control of scentless chamomile in western Canada (Peng *et al.* 2005a). When applied at high doses, the fungus killed the weed at seedling stages but, on older plants, it only suppressed the growth and attacked the old bottom leaves (Graham *et al*. 2006a). Like many other *Colletotrichum* species (O'Connell *et al.* 1993; Morin *et al*. 1996; Wei *et al*. 1997), this fungus is also a hemi-biotrophic pathogen and causes latent infection (Forseille *et al*. 2009). This latency may limit efficacy of weed control because the pathogen stays quiescent in young tissues until they start to senesce. As a result, plants at more advanced growth stages often survive the fungal attack, even under high inoculum doses (Graham *et al*. 2006a). A large number of herbicides were evaluated in combination with the fungus, and several products from Group-4 and Group-5, including 2,4-D, MCPA, clopyralid and metribuzin, synergized the fungus significantly (Graham *et al*. 2006b).

The literature offers only oblique clues to understanding the synergy occurred on scentless chamomile. It seems reasonable for the Group-5 herbicides to be synergistic because, as Photosystem-II inhibitors that block electron transfer through binding to D-1 proteins (Reade and Cobb 2002), these herbicides can destroy photosynthetic tissues by disrupting cell membrane and pigment formation, causing nutrient leakage and cellular dysfunction (Caulder and Stowell

1988). Consequently, tissues become "senescent" prematurely and the quiescent fungus in the plant tissue turns aggressive. On other hand, Group-4 herbicides are synthetic auxins that stimulate plant photosynthesis and mobilize carbohydrates and amino acids for increased protein synthesis. This effect tends to delay plant tissue senescence temporarily, making the condition unfavourable to aggressive tissue colonization by the pathogen. However, these plant growth regulators (herbicides) are applied in such a quantity that they over-stimulate cell division and differentiation, causing intracellular membranes to collapse and organelles to breakdown. Young tissues can be more sensitive to this effect than older tissues (Reade and Cobb 2002). Additionally, many Group-4 herbicides belong to the phenoxy family that affect plant growth and differentiation by causing cellulose-catalyzed cleavage of hemi-cellulose, cell wall loosening, and membrane leakage, thereby leading to the loss of water and nutrients (Cohen *et al*. 2002). This leakage may favor the pathogen by enhancing the availability of required nutrients and impairing mobilization of cellular defense responses. The herbicide 2,4-D inhibits phenylalanine ammonia-lyases that convert L-phenylalanine to t-cinnamate and ammonia as a key branch point in synthesizing several phenolic compounds critical to plant defense mechanisms (Davies 1972; Hoagland 1990). Possibly for these reasons, Group-4 herbicides was not observed to stimulate the aggressiveness of *C. truncatum* on scentless chamomile during first few days after treatment but did so two weeks later (Graham *et al* 2006b).

Synergy may widen the window for treatment application. None of the herbicides or the fungus alone was sufficiently effective on old scentless chamomile plants. For example, metribuzin at a rate recommended for control of most broadleaf weeds in pulse crops in western Canada (SAF 2006) did not kill any scentless chamomile plants at the 11-13 leaf stage. When the herbicide is combined with *C. truncatum*, the treatment resulted in 100% weed mortality (Peng *et al*. 2005a). In further studies, it was observed that efficacy of *C. truncatum* or herbicides declined with increasing growth stages of scentless chamomile (Graham *et al*. 2007); when applied to plants at the 8- to 11-leaf stages in the greenhouse, the fungus alone was generally ineffective and the herbicides clopyralid plus MCPA (Curtail® M) or metribuzin alone was merely suppressive (Graham *et al.* 2007). Clopyralid plus MCPA in combination with *C. truncatum* ($> 2\times10^7$ pores/ml) killed scentless chamomile at the 8-leaf stage but only metribuzin plus the fungus killed the weed at the 11-leaf stage. Furthermore, only 50% of the fungal dose was needed with metribuzin for the efficacy, and this represents substantial cost savings on the mycoherbicide (Graham *et al.* 2007). Here synergy provided an option to deal with older scentless chamomile which otherwise would not be controlled sufficiently. In addition, synergy may offer greater flexibility to field applications because over-wintered scentless chamomile seedlings can develop rapidly under warm spring temperatures which can often be accompanied by rainy and windy conditions in western Canada, making it difficult for timely spray applications (Peng *et al*. 2007; Hynes *et al*. 2010).

Although MCPA, clopyralid, and metribuzin were all synergistic with *C. truncatum* (Graham *et al.* 2006b), tank mixes of these individual herbicides with the fungus showed variable efficacy depending on the growth stage of scentless chamomile. On seedlings, MCPA plus the fungus may be sufficient. For plants at the 8-leaf stage, Curtail® M plus the fungus or metribuzin alone caused a high rate of plant mortality but for plants at the 11-leaf stage, metribuzin plus the fungus was required for satisfactory weed control (Graham *et al*. 2007). Besides, the latter option required lower doses of the fungus relative to other treatments. This case study further demonstrates that synergy can widen application windows for greater flexibility in weed control and its efficacy can be influenced by the growth stage of weeds.

UTILIZATION OF HERBICIDE-MICROBIAL SYNERGY

Synergy has been demonstrated in experimental plots and non-replicated large commercial fields. Collego™ in a tank mixture with acifluorfen controlled northern joint vetch and hemp sesbania in rice (Smith 1986) and soybean (Khodayari *et al*. 1987) plots. An aerial spray of this tank mixture also controlled these weeds in rice and soybean fields (Smith 1991). In Canada, acifluorfen, bentazon, and chlorimuron were found to enhance the activity of *C. coccodes* in control of velvetleaf in soybean fields (Wymore *et al*. 1987; Wymore and Watson 1989). Despite many positive results, synergy is under-utilized. The following issues relating to the practicality, risk, and cost will have to be addressed to facilitate the utilization.

Compatibility

In general, tank-mix applications are more practical for field delivery of synergistic active ingredients and this will require the components in the mixture to be compatible. Herbicide products can have a negative effect on mycoherbicide agents (Caulder and Stowell 1988), including delaying or even preventing spore germination or appressorial formation. This effect, however, is often specific to individual herbicide products. For example, propanil inhibited conidial germination of *P. setariae* on agar media, but sethoxydim was only slightly inhibitory (Peng and Byer 2005). Sometimes recommended surfactants/adjuvants have a larger negative impact on mycoherbicide agents than the active ingredient of herbicide. For example, sethoxydim at 0.1× label rate had little negative effect on *P. setariae*, but when the adjuvant Merge® was added to the mixture, conidial germination was significantly reduced and so was synergy and weed control (Peng and Byer 2005). When Merge® was replaced with the surfactant Tween® 80, the inhibitory effect diminished.

Most glyphosate products suppressed or abolished the germination of *Microsphaeropsis amaranthi* on common waterhemp. However, when testing the adjuvants used commonly in glyphosate products and technical-grade glyphosate salts separately, the inhibition occurred only with the adjuvants, but not with the active ingredient (Smith and Hallett 2006). In a separate study, several commercial glyphosate formulations, including Touchdown™ and RoundUp® HiTech®, were found compatible with *Myrothecium verrucaria* but RoundUp Weather MAX® killed *M. verrucaria* spores quickly at only 0.1× label rate (Smith and Hallett 2006).

The type of chemical (herbicides or adjuvants), its concentration, and duration of exposure may all affect mycoherbicide agents in a tank-mix situation (Grant *et al*. 1990). For example, the herbicides clodinafop, glufosinate, MCPA, and 2,4-D ester had only a minor effect on conidial germination of *P. setariae*, whereas bromoxynil, glyphosate, and the adjuvants Score®, Agral® 90 and Merge® were highly inhibitory (Peng and Byer 2005). This negative effect can be transient with some products (Grant *et al*. 1990), but more lasting for others. Lowering the dose may alleviate the negativity of some herbicides, but the antagonism from adjuvants is a bigger challenge because reducing their concentration will likely affect the spray quality and retention substantially (Peng and Wolf 2008). Prolonged exposure can exacerbate the negative impact (Peng and Byer 2005) and for this reason, some mixtures should be applied as soon as possible. Germination assays on agar media can often overestimate the impact because herbicides or adjuvants are possibly less antagonistic on plants due to active absorption and translocation (Zhang *et al*. 2003). Those herbicides unsuitable for tank mixing with mycoherbicides may be applied in a sequential order, as shown in the cases of controlling velvetleaf and round-leaved mallow (Hodgson *et al*. 1988; Grant *et al*. 1990), respectively. Of course, the concern to this approach is added costs to the producer

Table 2 Effect of *Colletotrichum truncatum* plus the herbicide metribuzin on lentil biomass under field conditions[a]

Treatment	Lentil biomass (g/m row)[b]
Untreated control	33 c[c]
Weed-free control	66 a
C. truncatum alone	36 c
Metribuzin alone	44 b
C. truncatum + metribuzin	47 b

[a] Data extracted from Peng *et al*. 2007.
[b] Taken 4 weeks after treatment and averaged over 8 plots (two trials).
[c] Means followed by the same letters do not differ (protected LSD, $P = 0.05$).

(Hatzios and Penner 1985).

Non-target effects

Predisposition of weeds by synergistic herbicides can decrease host resistance reactions, potentially broadening the spectrum of weed control. However, the same effect on non-target species may pose a risk to certain crops. For example, *P. setariae* was tested on 27 plant species belonging to the families *Asteraceae*, *Brassicaceae*, *Fabaceae*, *Linaceae*, and *Poaceae* to determine its host range. All of the species except *Zea mays* (field corn) were immune (Peng *et al*. 2004). In a further study, twelve of the crop species, including wheat, barley, oat, and canary grass, were treated with propanil at 0.5× or sethoxydim at 0.25× label rate 24 h prior to fungal inoculation. Most of the plants maintained the immunity, but necrotic flecking was observed on leaves of the barley plants pre-treated with propanil. These diseased leaves senesced prematurely, whereas the barley plants treated with propanil or fungus alone showed no such symptoms. This negative non-target effect will certainly disallow any use of the mycoherbicide candidate with propanil in barley crops. Quinclorac at 0.5× label rate, however, did not change the susceptibility of barley and none of the herbicides predisposed wheat, oat, or canary grass substantially.

The effect on non-target crops may vary from case to case, depending on the pathogen and herbicide involved. *Colletotrichum truncatum* caused slight infection on lentil (a non-target species) under controlled-environment conditions (Peng *et al*. 2005a; Gossen *et al*. 2009), but this impact appeared to be transient and the plants resisted the infection with a hypersensitive reaction (Forseille *et al*. 2009). To assess potential predisposition of lentil by metribuzin, a recommended herbicide for control of broadleaf weeds in pulse crops (SAF 2006), trials were conducted in greenhouse and field plots by applying the herbicide to lentil seedlings 24 h prior to fungal inoculation (Peng *et al*. 2007). In the field, lentil plots were infested with scentless chamomile at 25 plants/m^2. In greenhouse conditions, metribuzin did not increase the infection on lentil seedlings by the fungus relative to fungus controls. In field trials, no disease was observed on lentil plants while a modest level of disease occurred on scentless chamomile 1 week after treatment (Peng *et al*. 2007). Metribuzin plus the fungus did not affect lentil biomass when compared to untreated controls four weeks after treatment (**Table 2**).

The *C. truncatum* isolates used in these studies initially produced balloon-like infection vesicles in epidermal cells of scentless chamomile, from which thick primary infection hyphae originated within hours. These hyphae grow strictly within epidermal cells and can be distinguished readily from secondary infection hyphae which are much thinner and grow intercellularly in leaf tissues (Forseille *et al*. 2009). Fungus-colonized epidermal cells remained alive until a necrotrophic phase was triggered, and then secondary hyphae were initiated. This process is typical of the hemibiotrophic infection mechanism revealed with other *Colletotrichum* spp. (Goodwin 2001). The fungus was able to penetrate epidermal cells of lentil leaves but the colonization failed abruptly without production of infection vesicle or primary infection hyphae due probably to non-host responses including a hypersensitive reaction (Forseille *et al*. 2009). Metabolic interactions during the biotrophic phase are important to pathogen-host recognition for *Colletotrichum* spp. (O'Connell *et al*. 1993). The hyphae within the lentil epidermal cells may become dormant as in other cases of latent infection (Singh 1988; Viswanathan *et al*. 1998). The treatment of lentil with metribuzin did not seem to break this dormancy (Peng *et al*. 2007). As shown above, herbicide predisposition may change the susceptibility of 'non-host' species depending on the herbicicde, fungus, and species in question. As part of a risk assessment, crop safety must be evaluated carefully for synergy, especially for mycoherbicide agents that cause latent infection on crop species (Forseille *et al*. 2009). Mass applications of pathogen inoculum may be deemed a risky act, and therefore require science-based evidence to prove non-target safety (Barton 2004).

Optimized spray retention

When including a mycoherbicide in herbicides to control a specific weed problem, it also adds the cost to the treatment. This can make synergy a tough sale in practice, especially in field crops where input costs are being constantly squeezed. Several spray parameters may be optimized to maximize dose transfer, hence lowering the cost. Small droplets tend to enhance spray retention efficacy (Spillman 1984), but mycoherbicides can be a unique case where too fine droplets may carry few spores or even be "empty" (Jones 1998). For some mycoherbicides, targeting specific tissues or locations on the weed is of special importance. For example, BioMal controls round-leaved mallow by causing severe infection on lower stems (Mortensen 1998). Conventional spray systems are inefficient in targeting vertical lower stems due to interception of spray drops by the upper canopy (Chapple *et al*. 1996) or poor angling (Wolf and Caldwell 2004). Special application methods may be necessary to overcome these challenges. For example, an in-canopy spinning-disc sprayer can generate a narrow spectrum of fine droplets (Bateman 1999), potentially enhancing deposition on lower stems. Spray deposition is normally highest when targets are perpendicular to the droplet trajectory (Richardson and Newton 2000). Nozzle angling and travel speed may be adjusted to reduce the contact angle and enhance a more horizontal spray trajectory and deposition/retention on vertical surfaces (Nordbo *et al*. 1993; Wolf and Peng 2011).

Spray adjuvants, including surfactants, thickeners, stickers, and humectants, may also enhance the retention efficiency via effects on spray quality (Chapple *et al*. 1993; Stevens 1993). The effect of an adjuvant on retention efficiency can be concentration dependant (Wolf *et al*. 1997; Byer *et al*. 2006); high concentrations may increase retention, but sometimes inhibit mycoherbicide propagules as discussed earlier. It is important to ensure that the adjuvant concentration selected will not decrease the germination or appressorial formation of mycoherbicide agents. Otherwise, gains can easily be negated. Improvements on spray retention based on modification of spray quality and nozzle configuration may enhance the delivery efficiency, hence reducing material costs for synergy.

CONCLUDING REMARKS

One of the potential drawbacks with mycoherbicides is the inconsistent weed-control efficacy under field conditions. Herbicides have been investigated to synergize microbial agents for more effective and consistent weed control. Often the companion herbicide weakens the weed, impairing its defence responses to pathogen attacks. There are several potential benefits with synergy, including enhanced efficacy, lower product rates, a broadened spectrum of weed control, and wider windows of application. The reduction in mycoherbicide rates would mean significant cost savings

for biological control. Despite all the promise, little utilization of this strategy is known in practice. This is certainly related to the fact that few mycoherbicides are commercially available. To develop this technology, several aspects, including application timing, dose effects, weed growth stage, and spray efficiency should be considered to optimize efficacy. Issues relating to the practicality, non-target risks, and cost will have to be addressed on a case by case basis. This strategy will not be a panacea for all poor performances of mycoherbicides, but rather an option to tackle unique situations where a prominent weed problem is not controlled with regular herbicide programs, including herbicide-resistant species or those at risk of developing such resistance. Possibly such weeds can be managed effectively with amendment of mycoherbicides in mixture with herbicides as part of a control program.

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