Synergism between synthetic herbicides and *Nigrospora oryzae* (Berk. & Broome) Petch for the inundative biocontrol of pampas in New Zealand

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Summary South American pampas (*Cortaderia* spp.) are serious weeds in New Zealand, particularly in pine forests. Literature documenting the synergy between specific herbicides and mycoherbicides, identify benefits, including; greater efficiency and lowered product rates. Understanding herbicide-microbial interactions is an important step in the selection of synergistic partners. Organic herbicides are residue-free alternatives to synthetic herbicides, however, they are non-selective in activity. In Experiment 1, we studied the effect of three herbicides on the viability and growth of *Nigrospora oryzae* (Berk. & Broome) Petch;

1. 0.25 strength Gallant™ Ultra,
2. Greenscapes® and
3. Weedfree®.

The biomass accumulation was measured over 24 weeks, and the colony forming units (CFUs) and spore formation sampled on five occasions. No stimulation of fungal growth was shown by any of the herbicide treatments. Gallant herbicide at 0.25 strength neither interfered with fungal growth or sporulation as did both the organic herbicides. Weedfree was more fungitoxic than Greenscapes. For Experiment 2, we undertook a glasshouse spray-trial of the co-application of 0.25 strength Gallant and the organic herbicides with and without *N. oryzae.* Gallant at 0.25 strength killed the test plants (with or without *N. oryzae*). The organic herbicides initially killed the laminar of the test plants, but the plants re-sprouted from the pseudostem. The co-application of *N. oryzae* with each of the organic herbicides resulted in the death of the test plants. These results suggest that the co-formulation of *N. oryzae* with synthetic herbicides has the potential to kill pampas more effectively than the effect of the fungal pathogen alone.

Keywords Pampas, *Cortaderia* spp., *Nigrospora*, haloxyfop-P-methyl.

INTRODUCTION

The South American pampas grasses, *Cortaderia selloana* (Schult. & Schult.f.) Asch. & Graebn. and *C. jubata* (Lemoine) Stapf (Poaceae) are serious ecological weeds in New Zealand, particularly of low-growing and relatively open indigenous plant communities in habitats such as dunelands, cliffs and wetland margins (Gosling *et al.* 1999). Pampas grass is also one of the worst weeds of pine plantations in New Zealand (Richardson *et al.* 1996). Both species are very invasive and can form dense impenetrable stands, and as pampas grass spreads, other desirable vegetation is smothered. Pampas grass poses a fire risk in summer, is home to rats and mice, and affects visibility on roadsides. The range of areas under threat includes forests, conservation areas, scrublands, reserves and roadsides. A reduction in herbicide residues in forest products has necessitated the study for lower- and residue-free alternatives for weed control in pine plantations. Previous studies have demonstrated synergy between synthetic and microbial herbicides for weed control (Peng and Wolf 2011). 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 costs of biological control, and decreasing the rate of herbicides reduces the load of pesticide in the environment. Selected fatty acids such as pelargonic and caprylic acids are effective non-selective herbicides for a wide spectrum of annual weed species (Coleman and Penner 2008). Organic herbicides are available on the hardware-store shelf, and are advertised as soft alternatives to chemical herbicides because they do not have any residues.

As part of a natural enemy survey for pampas pathogens, Bellgard *et al*. (2011) recovered a rice pathogen from the laminar of pampas, *Nigrospora oryzae*. This study aimed to investigate the synergy between *Nigrospora oryzae* inoculum and three synthetic herbicides; 1) 0.25 strength Gallant™ Ultra and Greenscapes® (700 g L^{-1} Pelargonic acid) and Weedfree® (220 g L^{-1} pine oil and 560 g L^{-1} caprylic acid). In experiment 1, the *in vitro* activity of *N. oryzae* was studied to assess the effect of herbicides on; biomass accumulation, colony forming units, and spore production. The second experiment describes a glasshouse spray-trial to compare the performance of the three herbicides alone and together with *N. oryzae* in a fully replicated trial, using both *Cortaderia jubata* (ex Nelson) and *C. selloana* (ex Te Atatu) test plants.

METHODS

Experiment 1. *In vitro* interactions between *N. oryzae* and synthetic herbicides The design was fully randomised, with five replicates of each of the following combinations of 200 mL of each of three herbicides plus 300 mL *N. oryzae* (7.6×10^7 CFU mL^{-1}) and a negative control using 0.03% Tween on the 14/10/14. *Nigrospora oryzae* was grown in sterilised, clarified V8 juice liquid medium. Three measures were used to assess the effect of herbicides on the viability of *N. oryzae*:

1. Dry weight after 24 weeks,
2. Assessment of the number of colony forming units at five sampling times (4, 12, 16, 20 and 24 weeks), and
3. Spore formation at five sampling occasions (4, 12, 16, 20 and 24 weeks).

Mean dry matter accumulation was measured after samples were extracted under suction onto pre-weighed, sterilized, Whatman™ No. 1 filter papers. Filters and samples were weighed and then dried overnight at 60°C. The filter mass was then subtracted from the total dry mass to give the dry matter value. Mean numbers of colony forming units were determined by taking five, 50 µL replicate sub-samples from each pair of harvested flasks at each sampling occasion. The sub-samples were spread onto PDA plates amended with streptomycin sulphate (130 mg L^{-1}). A single dilution was established using 10 µL of sample to 90 µL of sterilized Reverse Osmosis (RO) water, and this was plated to a fresh plate of PDA with 100 ppm streptomycin sulphate (Leyronas and Raynal 2008). After four days, colonies were counted. Two replicates were harvested at each of the following occasions after inoculation; 4, 12, 16, 20 and 24 weeks. Spore numbers were estimated using a C-chip™ disposable haemocytometer. Five, 50 µL aliquots were removed from each pair of harvested flasks at each sampling occasion and placed in the haemocytometer. The spores in each of five replicate large squares were counted and the initial concentration calculated. Two replicates were harvested at each of the following occasions after inoculation; 4, 12, 16, 20 and 24 weeks.

Experiment 2. Glasshouse spray-trial *N. oryzae* was grown on wild rice which had been autoclaved with 10% V8 juice in 250 mL volumetric flasks. Inoculum was grown for two months at 18°C. The inoculum was removed from the flask, and combined with 500 mL of sterilized RO water. The suspension was blended at high speed in a Waring-type blender for five minutes. The suspension was made up to 1.5 L. The suspension was added to 1 L of 0.3% Tween (to achieve a final volume of 2.5 L). Five, 500 mL aliquots were added to each of the herbicides (in a 1:1 ratio), and applied to five replicates of each *C. jubata* (ex Nelson) and *C. selloana* (ex Te Atatu) plants using a hand held, Yates Plassay® Maxi 6 Pressurised spray unit. Five replicates of each species also received each of the herbicides, and one replicate set received only water (negative control). A positive control was also established, which only received the *N. oryzae* spore suspension in a 1:1 ratio with water.

RESULTS AND DISCUSSION

Experiment 1. *In vitro* interactions between *N. oryzae* and synthetic herbicides Biomass accumulation decreased over time as the inoculum resided in the herbicides. The organic herbicides based on fatty acid and fatty acid/pine oils were the most biocidal, with 0.25 strength Gallant apparently being the least biocidal (Figure 1). Both pelargonic and caprylic acid are known to have antibiotic properties (Gilbert and McBain 2003), and the anti-fungal activity of pine oil is well documented (Gilbert and McBain 2003).

After 24 weeks, there was a significant effect of herbicides for dry matter accumulation ($F_{1,3}$ 33.4, P <0.0001) of *N. oryzae* (Figure 1).

At the end of the 24 weeks, the number of CFUs in the control had increased by 15% (Table 1). Colony forming unit numbers were three to four times greater than spore numbers, as CFUs also contained a hyphal fragment fraction. The colony forming units showed a downward trend, with the organic herbicides displaying a strong biocidal effect (Table 1). After four weeks, CFU numbers had dropped by approximately 25% in the Weedfree® treatment and 15% in the Greenscapes® treatment (Table 1). Gallant™ Ultra had little or no effect after four weeks, with a drop in CFU numbers after 24 weeks of around 2% (Table 1). By the end of the 24 week period, the organic herbicides had reduced the CFU numbers by up to 33% (Table 1).

Spore numbers were also negatively impacted by the organic herbicides, with 0.25 strength Gallant having very little biocidal effect, and reducing spore numbers by a factor of six (Table 2). Spores were less sensitive than the CFUs to the biocidal effect, with the overall reduction in number after 24 weeks being between 13 and 15% (Table 2). After 24 weeks, the spore numbers had remained more or less static in the control (Table 2).

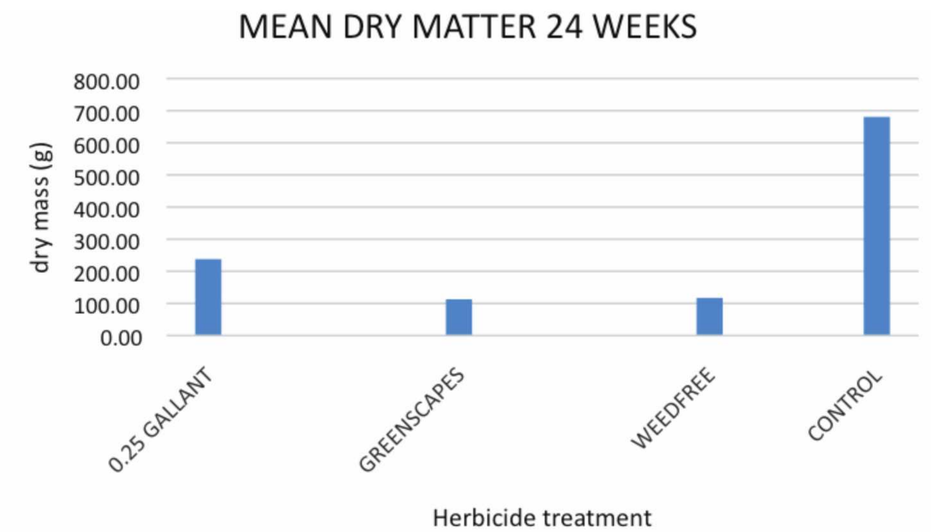

Figure 1. Fungal biomass after 24 weeks after addition of *N. oryzae* suspension to herbicide.

Table 1. Colony forming units CFUs per L after addition of *N. oryzae* suspension to herbicide (mean ± s.e.m. n = 5).

Herbicide	Initial concentration CFU per L	Concentration after 4 weeks CFU per L	Concentration after 24 weeks CFU per L
0.25 strength Gallant™ Ultra	7.6×10^7	$7.54 \times 10^7 \pm 2.2 \times 10^5$	$7.47 \times 10^7 \pm 8.21 \times 10^5$
Greenscapes®	7.6×10^7	$6.54 \times 10^7 \pm 4.22 \times 10^5$	$5.17 \times 10^7 \pm 5.18 \times 10^5$
Weedfree®	7.6×10^7	$5.72 \times 10^7 \pm 3.35 \times 10^5$	$4.98 \times 10^7 \pm 3.4 \times 10^5$
Control + Tween	7.6×10^7	$7.69 \times 10^7 \pm 8.96 \times 10^4$	$9.06 \times 10^7 \pm 8.69 \times 10^5$

Table 2. Spore numbers after addition of *N. oryzae* suspension to herbicide (mean ± s.e.m. n = 5).

Herbicide	Initial spore concentration CFU per L	Spore concentration after 24 weeks CFU per L
0.25 strength Gallant™ Ultra	$2.1 \times 10^7 \pm 1.6 \times 10^7$	$3.5 \times 10^7 \pm 1.6 \times 10^7$
Greenscapes®	$2.1 \times 10^7 \pm 1.6 \times 10^7$	$1.83 \times 10^7 \pm 2.6 \times 10^5$
Weedfree®	$2.1 \times 10^7 \pm 1.6 \times 10^7$	$1.78 \times 10^7 \pm 2.6 \times 10^5$
Control + Tween	$2.1 \times 10^7 \pm 1.6 \times 10^7$	$1.8 \times 10^7 \pm 2.2 \times 10^7$

Experiment 2. Glasshouse spray-trial The final spore suspension, as applied, was 2.0×10^7 CFU per L ($\pm 1.2 \times 10^5$ CFU per L) [s.e.m.] (Commenced 17th June 2015). The herbicides alone performed quite variably against *C. selloana* and *C. jubata*. After two weeks, the 0.25 strength Gallant™ caused a slight yellowing along the margins of the younger leaves. The initial impact of Weedfree and Greenscapes were very dramatic after two weeks on both *Cortaderia* species. However, the plants treated with the organic herbicides re-sprouted from the pseudo-stem. In comparison, by the end of the experiment, the 0.25 strength Gallant eventually killed the plants.

After two weeks, there was very little difference in the plants treated with *N. oryzae* alone compared to the uninoculated control for either *C. selloana* and/or *C. jubata* (Figures 2a,b). *N. oryzae* is a leaf spot pathogen and so, we were not expecting for the inundative application of *N. oryzae* alone to be able to kill pampas.

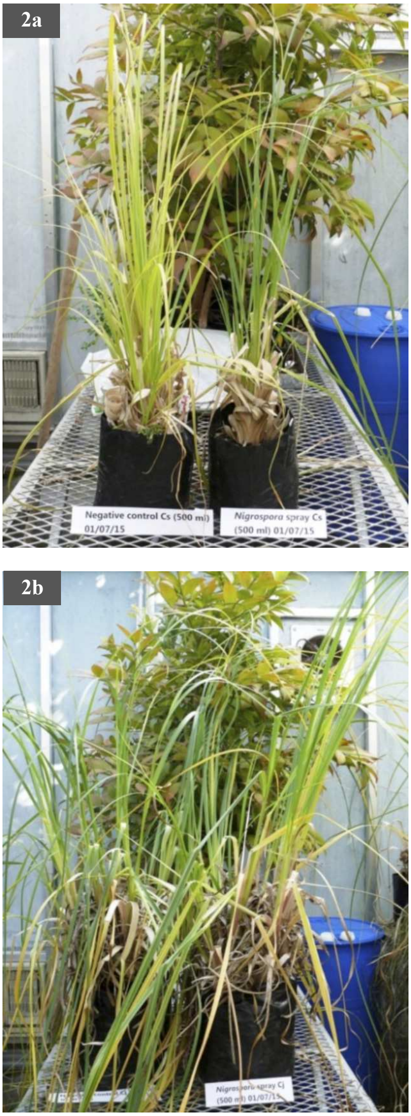

Figure 2. Response of a) *C. selloana* and b) *C. jubata* to *N. oryzae* (2.7×10^7 CFU per L) after two weeks (control on left).

In comparison, after two weeks, there was an observed synergy between the 0.25 strength Gallant and both the organic herbicides when co-applied with *N. oryzae* to *C. selloana*, compared to the control (Figures 3a,b,c). In a similar way, after two weeks, there was a very discernable difference in the *C. jubata* plants with the co-applied herbicides and *N. oryzae* inoculum compared to the control (Figures 4a,b,c).

The addition of *N. oryzae* to the herbicides increased the efficacy of all three herbicides. This synergism was associated with a reduced rate of Gallant. Also, the addition of the *N. oryzae* to the organic herbicides enabled both organic herbicides to kill pampas, which is beyond their biocidal capacity on their own. Previous studies by Peng and Byer (2005) reported an increase in pathogenicity of a leaf-spot pathogen *Pyricularia setariae* Y. Nisik. when added to the synthetic herbicide sethoxydim for the biocontrol of green foxtail.

Smith and Hallett (2006) reported that a range of chemical herbicides and adjuvants were strongly inhibitory to germination of *Microsphaeropsis amaranthi* Heiny & Mintz (Ell. and Barth.) conidia. They observed that *M. amaranthi* was compatible with a selection of post-emergent herbicides including; carfentrazone, chloransulfam and imazethapyr. However, most glyphosate products suppressed or abolished germination of *M. amaranthi* conidia.

Little biocidal interaction was observed between the 0.25 strength Gallant and *N. oryzae*, while the organic herbicides impacted upon the viability of *N. oryzae*. Successful synergy between herbicides and *N. oryzae* requires a spore with a resistant wall that protects it from the biocidal active ingredient, but does not impinge upon its infectivity. Enform Tech has applied its proprietary 'Surface Fermentation Technology' to produce a pure-spore formulation of *N. oryzae* with a hardened fungal spore which increases resilience in co-formulation with herbicides, improves product efficacy and extends shelf life.

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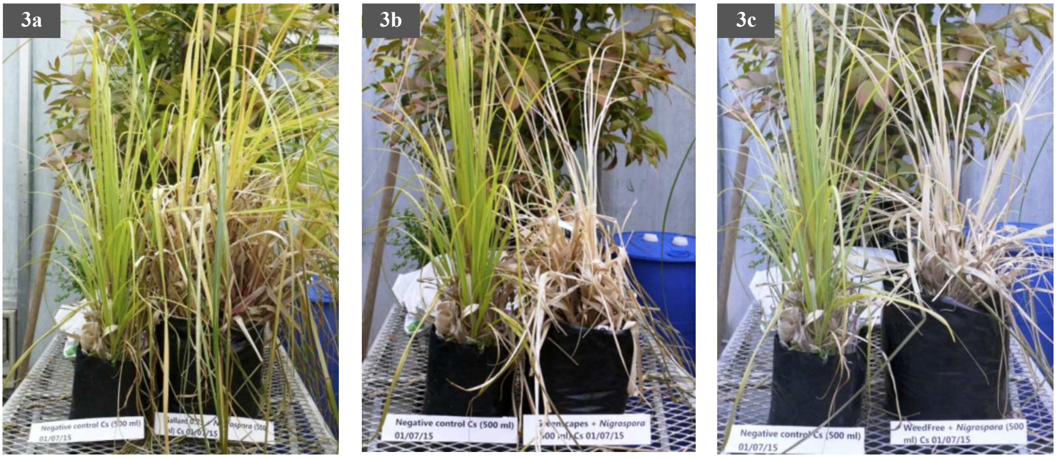

Figure 3. *C. selloana* responses to co-applied *N. oryzae* + herbicide (L–R): a) 0.25 strength Gallant, b) Greenscapes, c) Weedfree (control on left).

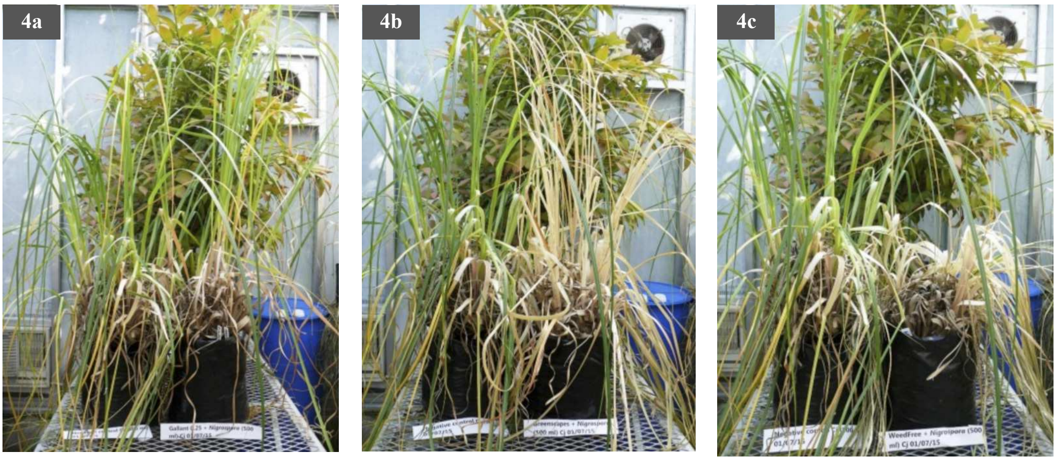

Figure 4. *C. jubata* responses to co-applied *N. oryzae* + herbicide (L–R): a) 0.25 strength Gallant, b) Greenscapes, c) Weedfree (control on left).

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