

Progress in formulation of a vegetable-oil-based invert emulsion for mycoherbicide control of bracken, *Pteridium aquilinum*

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Abstract. Spores of the bracken pathogen, *Ascochyta pteridis*, can be suspended (10^6 ml⁻¹) in an invert emulsion in which the oil phase consists of Soya oil (54% w/w), Marcol 52 (30% w/w), paraffin wax (10% w/w), Arlancel 780 (5% w/w) and Arlancel 989 (1% w/w) to circumvent the free-water requirements for host infection. The emulsion is highly stable and has excellent water-retaining capacity. The efficacy of this potential mycoherbicide can be greatly enhanced by the addition of very low concentrations (0.001% w/w) of the herbicide ioxynil. A single application of the emulsion together with ioxynil and spores at 100 l/ha achieved 73% control of bracken under controlled conditions (22°C and 70% relative humidity) compared to 10.5% in the absence of ioxynil, and 20% when ioxynil was present, but there were no spores. The relatively high viscosity of the emulsion necessitates air-assisted spray technology for field application. A further problem to be addressed arises from the observation that viscosity also appears to hinder gravitation of *A. pteridis*-spores through the emulsion droplets to the host surface, numerically reducing potential penetration sites. Work will continue to determine ways of reducing the viscosity of the formulation. However, there is evidence that the emulsion in its present state may be successful in other mycoherbicide systems.

Introduction

Bracken (*Pteridium aquilinum* L. Kuhn) is an important weed in upland areas of the United Kingdom where hill farming, grouse management and amenity-land are under significant threat from this aggressive, poisonous plant. No chemical herbicides, presently registered for use against bracken, are completely environmentally-acceptable and the least harmful compound (asulam) is an expensive material to use on low-value land. The costs at present for contract aerial-spraying of bracken are reported to be £120/ha (Robinson 1995), whilst weed control in arable agriculture is comparatively cheap. The need for a safe, relatively inexpensive alternative is clearly identified.

The bracken curl-tip-pathogen *Ascochyta pteridis* is a native fungus which has high potential as a mycoherbicide, and the isolate Ass 3v has proved to be a particularly virulent strain with great potential in mycoherbicide formulation. This strain possesses the typical morphological attributes of the fungus, and can be readily cultured on artificial media. It has a broad

temperature-tolerance with an optimum of 22°C and a minimum and maximum of 5 and 30°C respectively. Spores sprayed at 10^6 ml⁻¹ on to pot-grown bracken foliage can cause 50-70% damage to the plant, but only if free-water (dew) is made available to the spores for a period of about 20 hours.

The literature suggests that invert emulsions are the most successful formulations capable of providing sufficient water over the dew period, and the potential of these formulations for use with *A. pteridis* was demonstrated by Munyaradzi *et al.* (1991). Although this type of emulsion is usually of high viscosity, formulations have been developed with much reduced viscosities, thereby facilitating application with conventional spraying apparatus, including the air-assist spraying systems (Connick *et al.* 1991).

Previous reports on the evaluation of formulations, particularly those using non-polar oils as major constituents of the oil phase, indicate that fungi-toxic effects on the germ-tube growth of germinated spores are commonly expressed and are sufficient to prevent disease initiation on pot-grown bracken (Womack *et al.*

1995). Vegetable oils have proved to be suitable as the major constituents of new invert emulsions for use with *A. pteridis*, due to their relative low cost, low phytotoxicity and stimulatory properties to spore germination and germ-tube growth, with soybean oil exhibiting the greatest potential (Womack and Burge 1993). Furthermore, these oils form stable invert emulsions using very simple methods, when emulsified by the emulsifying agents Arlacel 780 and Arlacel 989 (ICI Speciality Chemicals, Leatherhead, England). Although these emulsifying agents are of relatively high cost, they are used in small proportions, are capable of forming stable emulsions with polar- or non-polar-oil phases, and are stimulatory to *A. pteridis* spore germination and germ-tube growth (Womack and Burge unpublished data).

The progress in formulation of a soybean-oil-based invert emulsion for mycoherbicidal control of bracken is presented here.

Materials and methods

Preparation of the emulsion

The oil phase of CPW5 consists of 48% w/w soya oil (Soya oil, Safeway, Glasgow, Scotland), 30% w/w paraffinic oil (Marcol 52, Wilsons, Glasgow, Scotland), 10% w/w paraffin wax (pastillated paraffin wax 125-130, Dussek Campbel Ltd, Bury, England), 10% w/w Arlacel 780 and 1% w/w Arlacel 989. The oil phase was prepared by heating all the ingredients to 60°C until all the wax had melted, followed by a short period of hand mixing to ensure dispersal of the ingredients. After cooling to room temperature, the formulation was homogenized for 10 min using a Silverson mixer-emulsifier (Fisons Ltd., Loughborough, England). The emulsion was prepared by adding distilled water (room temperature) to the oil phase at a 50:50 ratio (by weight) using a H.R. Flow inducer (Fisons Ltd.) delivering 5 ml per min, while mixing at 1000 rpm using a paddle stirrer (Fisons Ltd.). The mixing rate was increased to 3000 rpm for 20 min to complete the formation of the emulsion.

Verification, stability, and viscosity tests

An ammeter was used to verify the emulsion type by measuring the current passing between two electrodes immersed in the formulation, immediately after preparation. A zero reading would confirm a continuous oil phase. The stability of the emulsion was determined by sealing a 100 ml glass container of

prepared emulsion and incubating at 22°C. The nature of any observed instability was described, i.e. breaking, creaming, or inversion, and the degree of this instability measured at 24 h, 48 h, and two weeks after preparation. Viscosity measurements were taken at 25°C using a Ferranti-Shirley Cone and Plate Viscometer (Ferranti, Bracknell, England) fitted with a 7.0 cm diameter cone (0.4° angle). The viscometer was used in conjunction with an automatic flow curve recording unit designed to produce a standardized shear procedure with an attached X-Y plotter. The emulsion was examined, one hour after preparation, using a maximum shear-rate of 1684 sec⁻¹ and a sweep-time of 600 sec. An apparent viscosity value was determined from the apex of the flow curve (1684 sec⁻¹) generated by this viscometer. Practical experience suggests that formulations with an apparent viscosity value of up to approximately 200 mPa.s are suitable for application using the air-assist spraying systems. This flow curve was also examined to predict the behaviour of the formulation during the application process. Evidence of a high degree of shear thickening would indicate that application may be problematic. Three samples of the formulation were evaluated to determine the reproducibility of the results. Viscosity measurements were taken at 25°C using a Ferranti-Shirley Cone and Plate Viscometer in automatic mode with attached X-Y plotter, one hour after preparation.

Water-retaining properties of emulsion droplets

The emulsion was applied to pre-weighed glass plates (50x22 mm) using a Shandon Laboratory Spray Gun (Fisons Ltd.) at an application rate of approximately 100 l/ha. Ten replicates were carried out and the trial repeated three times. Incubation of the treated glass plates was carried out in Fisons Fi-totron 600H growth cabinets (Fisons Ltd.) with an internal environment of 22°C and 70% relative humidity. Water loss due to evaporation was assessed by gravimetric readings taken at hourly intervals over a six-hour period and a final reading taken after 20 h. After this time, the glass plates were incubated at 60°C to remove all the water and re-weighed after cooling to determine the amount of water lost during spraying. Gravimetric analysis of the oil phase was also carried out, over time, to confirm that weight loss was due to water loss.

Spore germination and germ-tube growth in emulsion droplets

An aqueous phase containing spores of *A. pteridis*

(10^6 spores ml^{-1}) was added to the oil phase at a 50:50 ratio (by weight) as described earlier. Glass plates were sprayed at approximately 100 l/ha and incubated at 22°C and 70% relative humidity. There were three replicates per treatment and the trial was carried out three times. Spores were examined microscopically at hourly intervals and germination assessed by examining 10 spores from five separate fields of view per replicate. Measurements of germ-tube length, from the point of emergence from the spore to the tip, were carried out using a micrometer eyepiece.

Growth-cabinet trials with pot-grown bracken

Pot-grown bracken was raised from 10-cm rhizome segments collected from a disease-free site in Dunbartonshire (NS 554764). At two to three weeks after frond emergence, when the second pair of pinnae were expanding, pots were transferred to the above-mentioned growth cabinets. Plants were allowed to acclimatize for 24 h prior to treatment. Duplicate trials consisted of four replicate pots of plants treated in one of the following ways: *A. pteridis* spore suspension (10^6 spores ml^{-1}) only; emulsion only; emulsion containing a spore suspension of *A. pteridis* (10^6 spores ml^{-1}); and untreated controls. Visual assessments were made at seven-day intervals up to 21 days after treatment. Damaged tissue was examined for fungal infection by standard re-isolation techniques.

Modification of the CPW5 formulation was then achieved by reducing the emulsifying agent content and increasing the vegetable oil content. This new formulation, designated CPWA, consisted of 54% w/w soybean oil, 30% w/w paraffinic oil, 10% paraffin wax, 5% w/w Arlacel 780, and 1% w/w Arlacel 989. CPWA was evaluated in a second set of growth-cabinet trials which incorporated sublethal concentrations of ioxynil (0.1, 0.01, 0.001% w/w) in the oil phase. The spores were provided with 0.1% malt broth and 0.5% sodium alginate in the aqueous phase, which is known to enhance disease establishment (McElwee 1987). The trial was carried out as described above.

Results and discussion

Stable water-in-oil emulsions were formed with the CPW5 formulation, which persisted in this stable state for at least two weeks. The apparent viscosity, derived from the shear-stress/shear-rate relationship at the apex of the rheogram (1684 s^{-1}), was determined to be 155.8 mPa.s at 25°C (Fig.1). This would suggest that this

formulation was not suitable for application with conventional hydraulic nozzles at the targeted 100 l/ha. However, Airtec nozzles (Cleanacres Machinery Ltd.), are more than adequate for the delivery of such formulations. The flow curve for this emulsion (Fig.1), which may be considered as a measure of the ability of the emulsion to resist structure-breakdown during the standardized shearing-cycle, is relatively simple. The flow curve shows that some shear-thinning is exhibited at low shearing forces, while shear-thickening is exhibited at higher shearing forces. The extent of these is minor and little effect is likely to be observed during the application processes.

Investigations on the water-retaining properties of the formulation confirmed that weight loss was due to water loss. During application of CPW5, a mean of 2.67% of the water content was lost. After 20 h, the amount of water lost by evaporation from sprayed emulsion droplets (Fig. 2) was very low, being 28% of the original 50%. This would ensure that the water available for fungal-spore germination is far in excess of the minimum requirement of 10% (Womack and Burge unpublished data). This was confirmed in trials that were used to examine the spore germination in sprayed emulsion droplets (Fig. 3). Germination of spores was first observed at seven hours and reached a maximum of about 78% after 12 h. The advantages of a rapid and early germination are obvious, and modifications to CPW5 have indicated that a reduction of three hours can be achieved for initial germination events (Womack *et al.* in press). Germ-tube growth in CPW5 was uninhibited, with a mean length of about 62 μm after 20 h (Fig. 4).

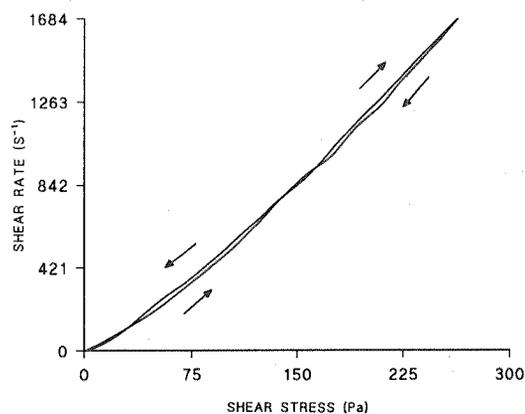


Fig. 1. Ferranti-Shirley Cone and Plate Viscometer flow curve obtained from a 50:50 CPW5 invert emulsion at 25°C; the arrows indicate the direction of the up and down curves.

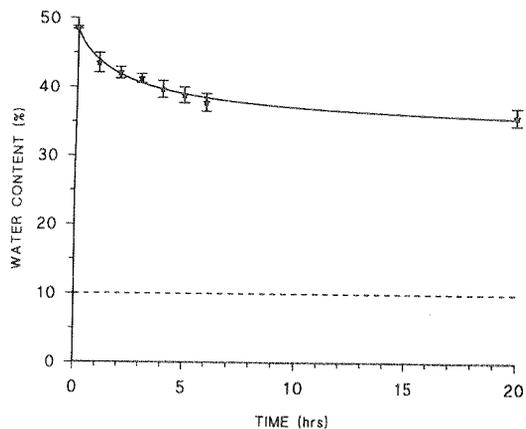


Fig. 2. Rate of water evaporation from a 50:50 CPW5 invert emulsion sprayed onto glass plates and incubated at 22°C and 70% relative humidity. The dashed line represents the minimum water content level for spore germination. The vertical bars represent the standard error.

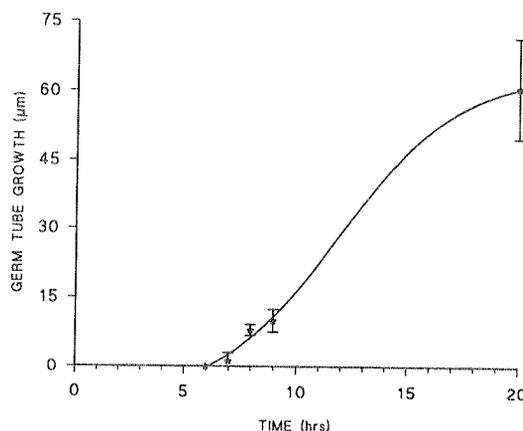


Fig. 4. Germ-tube growth of germinated *Ascochyta pteridis* spores in a 50:50 CPW5 invert emulsion sprayed onto glass plates and incubated at 22°C and 70% relative humidity. The vertical bars represent the standard error.

Growth-cabinet trials on pot-grown bracken indicated that the CPW5 formulation caused insignificant levels of phytotoxicity, i.e. <2%, thus reducing the potential of resistance to infection either by fungi-toxic host exudates or by competition with necrotrophic pathogens. However, the herbicidal efficacy of CPW5 was minimal. Necrotic spotting, which was observed at seven days, failed to develop further, though this damaged tissue was found to be infected with *A. pteridis*. This suggests that potential does exist for this emulsion as a mycoherbicidal formulation.

Provisional results from treatments incorporating ioxynil into a modified formulation of CPW5, designated CPWA, caused rapid mortality at 0.1%

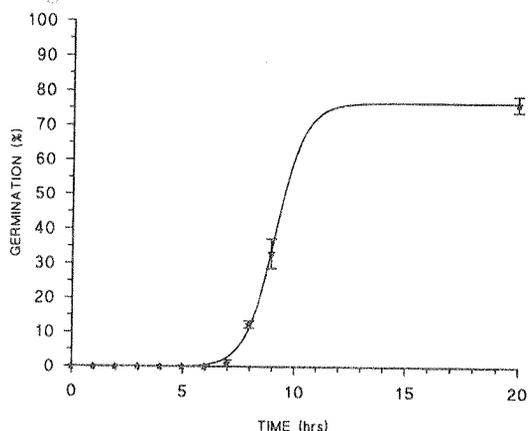


Fig. 3. Germination of *Ascochyta pteridis* spores in a 50:50 CPW5 invert emulsion sprayed onto glass plates and incubated at 22°C and 70% relative humidity. The vertical bars represent the standard error.

ioxynil irrespective of the inclusion of *A. pteridis* spores (Table 1). At lower concentrations, synergistic effects were observed between ioxynil and *A. pteridis*, with the greatest effect observed at 0.001% ioxynil. Twenty-eight days after application, a treatment of CPWA with 0.001% ioxynil and *A. pteridis* achieved 78% control compared to 20% when CPWA was applied with ioxynil alone. These results suggest that bracken is able to out-grow the effects of low concentrations of ioxynil. However, when formulated with spores of *A. pteridis*, the damage is progressive due to the invasive capacity of this fungus once disease is established.

Examination of treated pinnules revealed that a number of germ-tubes were not directed positively toward the host surface, suggesting that there may be some interference with the ability of the fungus to recognize host sites for infection. The greatest constraint on this criterion is thought to be the viscosity of the emulsion. Spores of the fungus have a greater density than water, and hence would sink to the host surface during natural infection by rain-splash, thus enabling host-site recognition. Reduction in the viscosity of the invert emulsion formulation should significantly improve the incidence of spore/host contact, thereby reducing the need for additional adjuvants. Modifications to CPW5, to reduce the viscosity of the emulsion, have already led to an earlier stimulation of spore germination. It is hoped that these modifications will provide further evidence for the viability of this mycoherbicidal approach and will fulfill the potential that it so clearly possesses.

Table 1. Effect of ioxynil on the efficacy of the CPWA formulation of *Ascochyta pteridis* on pot-grown bracken. Percentage necrosis of pot-grown bracken treated with 50:50 invert emulsion CPWA only (CPWA); 50:50 invert emulsion CPWA containing a spore suspension (SP) of *A. pteridis* at 10^6 spores ml^{-1} ; 50:50 invert emulsion CPWA containing ioxynil (I) at 0.001%, 0.01% or 0.1% w/w; 50:50 invert emulsion CPWA containing a spore suspension of *A. pteridis* at 10^6 spores ml^{-1} and ioxynil at 0.001%, 0.01% or 0.1% w/w; untreated controls (CONTROL). DAA - days after application. Means with common postscripts within columns are not significantly different at the $p = 0.05$ level (Duncan's multiple range test).

Treatment	Necrosis (%)			
	7 DAA	14 DAA	21 DAA	28 DAA
CONTROL	0.10 d	1.00 d	1.25 d	1.25 d
CPWA	0.13 d	1.25 d	1.63 d	1.50 d
CPWA + SP	2.50 d	5.28 d	10.50 cd	13.00 cd
CPWA + I ^{0.001%}	23.75 c	22.50 c	20.00 c	20.00 c
CPWA + I ^{0.001%} + SP	43.75 b	66.25 b	72.50 b	77.50 b
CPWA + I ^{0.01%}	88.75 a	88.75 a	85.00 a	83.75 ab
CPWA + I ^{0.01%} + SP	100.00 a	100.00 a	100.00 a	100.00 a
CPWA + I ^{0.1%}	100.00 a	100.00 a	100.00 a	100.00 a
CPWA + I ^{0.1%} + SP	100.00 a	100.00 a	100.00 a	100.00 a

The reliance on additional adjuvants such as ioxynil to improve the level of efficacy achieved by the CPWA formulation of *A. pteridis* to acceptable levels is not considered to be the optimum solution. The inclusion of ioxynil at 0.01% w/w resulted in 20% necrosis of bracken in the absence of *A. pteridis*. It is likely that this level of damage on bracken will also be expressed on non-target species, which is an obvious disadvantage for the use of such a specific mycoherbicide. The environmental advantages of using these formulations over conventional pesticides therefore become somewhat diminished. It can be concluded that efforts should concentrate on modifications of the emulsion CPWA to enable the maximum efficacy of *A. pteridis* in the absence of phytotoxicity expressed by the carrier formulation. Work will continue on other materials which express synergistic relationships with *A. pteridis* to provide a formulation which satisfies this criterion.

This work has resulted in the formulation of a promising emulsion which, not only could be used with *A. pteridis* to control bracken, but also with other biological control agents with similar requirements to *A. pteridis*. The potential of this formulation for use with other mycoherbicides is presently under investigation (Auld personal communication).

One further point to consider is that the success of the CPWA formulation of *A. pteridis* for the control of bracken, and other mycoherbicide formulations may depend on their ability to integrate into overall management schemes, rather than as direct competitors to conventional pesticides. It is therefore suggested that

the development of formulations of new mycoherbicides should be carried out in a way that is sympathetic to integrated pest management strategies.

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