SESSION 2A

NEW SOLUTIONS FOR CROP PRODUCTION

Chairman: Professor Phil Russell
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Session Organiser: Dr Leonard G Copping
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Platform Papers: 2A-1 to 2A-8

Poster Papers: P2A-9 to P2A-13

Aminopyralid, a new active substance for long-term control of annual and perennial broad-leaved weeds in grassland

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ABSTRACT

Aminopyralid is a new systemic herbicide in the pyridine carboxylic acid class under development by Dow AgroSciences. Globally, aminopyralid can be used for weed control in range and pasture situations, plantations and non-crop areas. In addition, uses in oilseed rape and cereals are being explored. Overall, aminopyralid has a favourable toxicity profile, with no evidence of teratogenicity, mutagenicity, carcinogenicity, endocrine or adverse reproductive effects. Because of the low toxicity of aminopyralid the risks to workers handling aminopyralid formulations are low. Aminopyralid produces no significant soil or water metabolites except CO_2 and exhibits very low acute or chronic toxicity to mammals, birds, fish and aquatic invertebrates and passes the EU ecotoxicological risk assessment for algae and aquatic plants. GF-839 is a combination of the new active substance, aminopyralid, and fluroxypyr. It is the first new product to be developed primarily for the grassland market in many years. GF-839 offers reliable long term control of annual and perennial broad-leaved weeds in grassland including *Rumex obtusifolius* (broad-leaved dock), *R. crispus* (curled leaf dock), *Cirsium arvense* (creeping thistle), *C. vulgare* (spear thistle), *Urtica dioica* (common nettle), *Ranunculus repens* (creeping buttercup), *Taraxacum officinale* (dandelion) and *Stellaria media* (chickweed), whilst also offering a high degree of selectivity to grass.

INTRODUCTION

Aminopyralid is a new pyridine carboxylic acid herbicide under development by Dow AgroSciences. Aminopyralid is a synthetic auxin type herbicide, it is systemic and rapidly absorbed by leaves and roots. In susceptible plant species aminopyralid induces an epinastic response leading to cessation of growth and rapid necrosis.

The new herbicide, GF-839, is a combination of a new active substance aminopyralid and the fully approved active substance fluroxypyr in the quantities 30 g a.e./litre aminopyralid + 100 g a.e./litre fluroxypyr. It is an emulsion, water in oil formulation (EO), and will be sold as foliar applied herbicide acting on leaves and roots for the long-term control of annual and perennial broad-leaved weeds in grassland

AMINOPYRALID

Chemical and physical properties
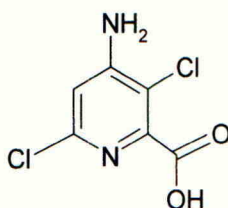
Structural formula of aminopyralid

Chemical Class:	Pyridine carboxylic acid
Chemical name (IUPAC):	4-amino-3,6-dichloropyridine-2-carboxylic acid
Dow AgroSciences Codes:	XR-750, XDE-750, DE-750
Common name:	Aminopyralid
Empirical Formula:	$C_6 H_4 Cl_2 N_2 O_2$
Molecular weight:	207 g/mole
Melting point:	163.5 ^{o}C
Vapour pressure at 20°C:	9.52×10^{-9} Pa
Aqueous solubility (pH 7.0):	2.48 g/litre (18°C)
Dissociation constant (pK$_a$):	2.56
Partition coefficient: Log P	0.201 (19°C) Unbuffered water

Toxicology

Acute

Oral:	LD_{50} Rat >5000 mg/kg
Dermal:	LD_{50} Rat >5000 mg/kg
Inhalation:	LC_{50} Male Rat >5.50 mg/litre
Skin irritation:	Negative study results (Rabbit)
Skin sensitisation:	Negative study results (Guinea pig)
Eye irritation:	Irritating – active substance

Chronic/Sub-chronic

Carcinogenicity:	Negative study results
Mutagenicity: AMES + CHO/HGPRT	Negative study results
Toxic effects for reproduction:	Negative study results

These data show that aminopyralid is very low in acute oral toxicity. It does not present an inhalation hazard. It is essentially non-irritating to skin and was negative in skin sensitisation tests. Aminopyralid poses a low risk for the user under normal agronomic conditions

Ecotoxicology

Birds
Acute oral:	14 d LD_{50} quail > 2250 mg a.e./kg b.w.
Short term dietary:	LD_{50} quail > 5620 mg a.e./kg b.w.
	LD_{50} duck > 5620 mg a.e./kg b.w.

Aquatic organisms
Acute toxicity: fish	96 h LC_{50} rainbow trout >100 mg a.e./litre
	96 h LC_{50} sheepshead minnow >120 mg a.e./litre
Acute toxicity: *Daphnia*	48 h EC_{50} (immobilization): >100 mg a.e./litre
Acute toxicity: algae	72 h EC_{50} freshwater green algae 30 mg a.e./litre
	120 h EC_{50} freshwater blue-green algae 27 mg a.e./litre

Non Target Organisms
Acute contact: honey bee	48 h LD_{50} > 100 mg a.e./bee
Acute oral: honey bee	48 h LD_{50} > 120 mg a.e./bee
Acute toxicity: earthworm	14 d LC_{50} >1000 mg a.e./kg soil

Aminopyralid is of low or no toxicity to terrestrial and aquatic organisms except for slight toxicity to algae. Studies on non-target arthropods, epigaeic beneficial insects, non-target soil micro-organisms and earthworms showed no effects or very low toxicity. No effects on sewage bacteria have been identified.

Environmental Fate

Soil Half Life
Laboratory half life (DT_{50}) 20°C:	18 to 143 days (mean 67 days)
Field half life (DT_{50}):	8 to 35 days (mean 25 days)
Field half life (DT_{90}):	26 to 116 days (mean 84 days)
Adsorption coefficient (K_{OC}):	0.0 to 38.9 ml/g (mean 10.8 ml/g)

Soil

The primary route of degradation in soil is aerobic microbial degradation. Laboratory studies have shown that the only major metabolite observed was CO_2 indicating that the phenyl ring of aminopyralid is mineralised. No other degradation products were detected.

Field DT_{90} values of 26 to 116 days (mean 84 days) were determined, average field soil half life was calculated as 31 days, and thus aminopyralid is not expected to accumulate in soil.

Aminopyralid is weakly adsorbed to soil with an adsorption coefficient, normalised for organic carbon content in the range of 0.0 to 38.9 ml/g (mean 10.8 ml/g) from eight soils. These sorption results indicate that aminopyralid would be considered potentially mobile. However, FOCUS groundwater modelling showed that under typical use conditions, the PECGW was <0.1 µg/litre, indicating the risk of leaching is low.

Air

Aminopyralid has a very low vapour pressure of 9.5×10^{-9} Pa at 20°C, suggesting that only very low amounts of aminopyralid would be present in air. This has been confirmed in a wind tunnel study.

Water

The primary route of degradation is photolysis. The estimated DT_{50} under environmental conditions was 0.6 days at latitude 40°N in the summer. Photodegradation occurred via de-chlorination and ring cleavage.

Mode of action

Aminopyralid is a synthetic auxin type (growth regulator) herbicide. It is systemic and rapidly absorbed by leaves and roots. In susceptible plant species, aminopyralid induces an epinastic response (i.e. stimulation of cell elongation and premature senescence, particularly in meristematic tissue) leading to cessation of growth and rapid necrosis.

GF-839

The new herbicide, GF-839, is a combination of the new active substance aminopyralid and the fully approved herbicide fluroxypyr in the quantities 30 g a.e./litre aminopyralid + 100 g a.e./litre fluroxypyr. It is an emulsion, water in oil formulation (EO), and will be sold as a foliar acting herbicide for the long-term control of annual and perennial broad-leaved weeds in grassland.

Aminopyralid is the most active halopyridine yet discovered and as a synthetic hormone it poses a low risk of resistance. *Rumex obtusifolius* (broad-leaved dock), *R. crispus* (curled leaf dock), *Cirsium arvense* (creeping thistle), *C. vulgare* (spear thistle), *Urtica dioica* (common nettle), *Ranunculus repens* (creeping buttercup), *Taraxacum officinale* (dandelion) and *Stellaria media* (common chickweed) are all pernicious, persistent weeds of grassland in Europe. If left unchecked, they can lead to significant reductions in sward quality and quantity and can spread to neighbouring areas.

In the UK alone, 1.1M ha of grassland are infested with *Cirsium* spp, and of these 400,000 ha have infestation levels of more than 1 plant/m^2, equating to a potential loss of 1 million tonnes of dry matter per year. As little as 10% ground cover by *Rumex* spp, can cause a potential 10% silage loss. There are currently various products on the market for control of these weeds, but GF-839 differs in that it is the first new compound to be developed primarily for the grassland market for many years, and offers reliable long-term control of all of these weeds, in combination with good grassland management practice, whilst also offering a high degree of selectivity to grass.

METHODS

During 2002 and 2003, 125 efficacy trials were carried out in established grassland (grass more than 1 year old) to evaluate the spectrum of activity and dose rate of GF-839. Twenty yield trials were carried out in established grassland and new leys. All field trials were carried out in accordance with EPPO guidelines, with a minimum plot size of 12 m^2 with a minimum of 3 replicates per treatment.

RESULTS

From the 125 efficacy trials, in-season control of all target weeds with 2 litre/ha GF-839 was over 95%. Figure 1 shows that long-term control (12-18 months after application) of perennial weeds was also excellent compared to market standards. GF-839 is most efficacious when applied to actively growing weeds in grassland situations of either perennial weeds or new sown leys this can be throughout the calendar year, Figure 2 demonstrates that equivalent long term efficacy is achieved following early season (March – June) or late season (August – October) applications for the representative species of *Rumex obtusifolius* and *Stellaria media*.

Yield and quality data from the 20 yield trials which included the label rate of 2 litre/ha and the double rate of 4 litre/ha of GF-839, demonstrated that GF-839 may be used on new or established grass from the three true leaf stage.

Phytotoxicity data from 145 trials showed no long-term injury in any trial and data from 6 screens on 14 of the most commonly sown and invasive grass species in the UK showed that GF-839 at the label rate and double label rate is safe to apply to new and old grass pastures. Data from 9 cutting interval trials show that there is no negative effect on efficacy when grass is cut as little as 7 days after application.
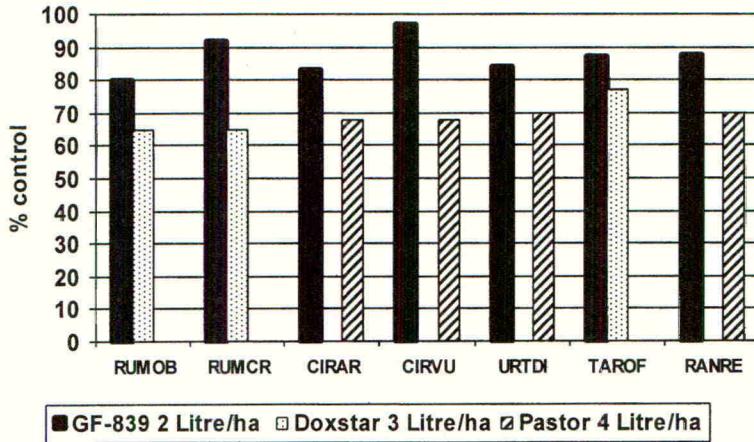
CONCLUSION

GF-839 at the proposed label rate of 2 litre product/ha gives both excellent in-season and long-term weed control, whilst being very selective to grass. GF-839 has a wide window of application which makes it fully adaptable to grassland husbandry practices. These compatibilities together with good husbandry and management techniques, demonstrate that GF-839 is a novel, useful and effective tool that can be used in an integrated approach to improving the quality of grassland.
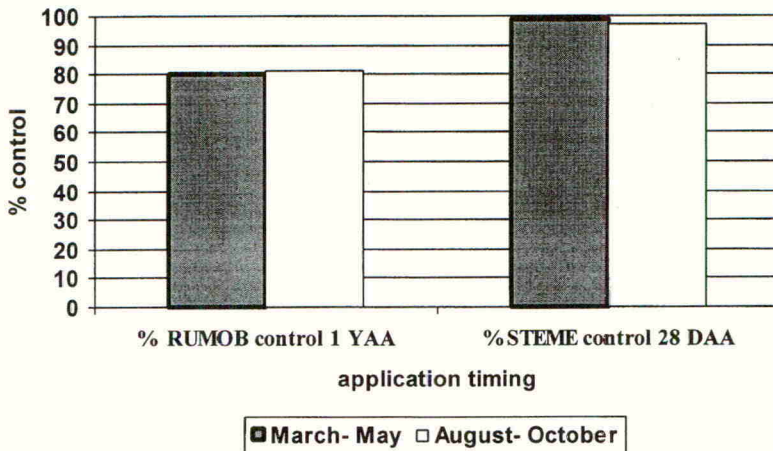
SUMMARY

Aminopyralid from Dow AgroSciences is the most active halopyridine yet discovered. It offers effective broadleaved weed control in a range of crop situations, including range and pastures, plantations, cereals, oilseed rape and non-crop areas. Aminopyralid produces no significant soil or water metabolites except CO_2 and exhibits very low acute and chronic toxicity (practically non-toxic) to mammals, birds, fish, and aquatic invertebrates. The product GF-839 is a combination of aminopyralid and fluroxypyr and has been developed specifically for use in pasture for the effective long-term control of annual and perennial weeds, such as docks, thistles and nettles, whilst being very selective to grass.

Figure 1: Long term percent control of perennial weeds in grassland

Categories (x-axis): RUMOB, RUMCR, CIRAR, CIRVU, URTDI, TAROF, RANRE

Legend: ■ GF-839 2 Litre/ha □ Doxstar 3 Litre/ha ▨ Pastor 4 Litre/ha

Doxstar contains 100 g a.e./litre fluroxypyr + 100 g a.e./litre triclopyr. Pastor contains 50 g a.e./litre clopyralid + 75 g a.e./litre fluroxypyr + 100 g a.e./litre triclopyr.

Figure 2: % Weed Control Following Early or Late Season Application of 2 litre/ha GF-839

x-axis: % RUMOB control 1 YAA   % STEME control 28 DAA

application timing

Legend: ▨ March- May □ August- October

Bifenazate, a new acaricide for use on ornamentals in Europe and Africa

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ABSTRACT

The acaricide bifenazate 240 SC has recently been introduced in European and African countries for use in the production of ornamentals. The target species for bifenazate is *Tetranychus urticae* (two-spotted spider mite), of which eggs and all motile stages are susceptible. Laboratory and field data on *T. urticae*, and side effects on other mite species are discussed. Resistance management is part of the introduction program, and results of cross-resistance studies are presented. Bifenazate has not shown any phytotoxic effect on a wide variety of cut flower and potted plant species, which were tested at concentrations up to twice the label rate. Bifenazate fits well in Integrated Pest Management programs, and effects on the main predators and parasitoids used in IPM are discussed.

INTRODUCTION

Ornamentals include a variety of covered and outdoor cultures of cut flowers, pot plants, container plants, perennials, bedding plants, and bulbs. The main acaricidal market is on cut flowers and potted plants. For an acaricide, it is important that it does not cause phytotoxic symptoms, does not leave undesirable visible residues on the crop, and that it fits into Integrated Pest Management (IPM) programs. To meet the latter demand, side effects on biological control agents should be limited. In spite of the fact that many spider mite species may occur on ornamentals, only a few species are of economic importance. *Tetranychus urticae* (two-spotted spider mite) is the most polyphagous, and by far the most economically important species. *T. cinnabarinus* (carmine spider mite), is sometimes considered to be a separate species, but its taxonomic position is not clear. For spider mites, the main factors for high pest incidence are the often insufficient spraying techniques, the decreasing number of non-phytotoxic acaricides, and the increasing resistance to many acaricides. The increasing area of ornamental crops under IPM, further strongly limits the potential number of acaricides, as several acaricides show high activity against many beneficials. For these reasons, new acaricides, which combine high efficacy on spider mites with crop safety, absence of visual residue, and low effects on beneficials, are required in order to implement resistance management strategies by alternating acaricides with different modes of action.

Bifenazate is an invention of Crompton Corporation. The compound was introduced by Dekeyser *et al.* at the BCPC Conference in 1996, under the code number D2341. The first registration on ornamentals was granted in the USA in 1999, where the compound is classified by the Environmental Protection Agency (EPA), as a 'Reduced Risk' pesticide, a

classification that expedites review of safer or reduced risk pesticides to help them reach the market more quickly. Other registrations for the WP formulation on ornamentals have been obtained in Asia and South America. In Europe and Africa, the bifenazate formulation for use on ornamentals is an SC, containing 240 g a.i./litre sold under the trade name 'Floramite'. In June 2005, bifenazate received a unanimous positive vote by the EU legislative meeting, for Annex I inclusion under Council Directive 91/414/EEC.

BIOLOGICAL PROPERTIES AND MODE OF ACTION OF BIFENAZATE

On *Tetranychus* species, bifenazate is active on all stages (eggs, larvae, nymphs and adults) of mites, with the highest activity on larvae and nymphs. Bifenazate is a contact acaricide and has no systemic or translaminar effects. Consequently, thorough coverage of the foliage is essential for good mite control. Laboratory trials with *T. urticae* at 15, 25 and 35°C, showed that the activity at 15°C was slightly lower than at 25 and 35°C.

Preliminary results from studies on the mode of action of bifenazate in insects, indicate that, at high test concentrations, bifenazate acts on the postsynaptic GABA receptor in the insect nervous system. GABA is an inhibitory transmitter, and a disturbance of the balanced action of excitatory and inhibitory transmitters results in over-excitation or paralysis of the insect muscles. The mode of action of bifenazate has not yet been confirmed in mites, and it may be that GABA receptors in insects and mites are different. Mites, which have been sprayed with bifenazate, will become hyperactive after approximately 3 h, and will not feed anymore. Subsequently, the activity of the mites gradually decreases, and after 3 - 4 d the maximal effect on the population is reached. Bifenazate has no side effects on insects at the recommended label rates.

CROP SAFETY

Up to 185 varieties of the ornamental species were tested for crop safety, at up to double the label rate (80 ml bifenazate 240 SC/100 litres), and no signs of phytotoxicity were observed (Crompton Europe Ltd, Technical Brochure of bifenazate 240 SC. PM 127, undated). In northern Europe, trials were performed in the winter season. In southern Europe, trials were performed in the spring/summer season. In most trials, 2 applications were made with an interval of 7 d between applications. As roses represent the most important market segment of ornamental cut flowers, 119 varieties of roses were tested. Other cut flower species tested were carnations (6 varieties), chrysanthemums (2 varieties), *Gerbera* (18 varieties), and *Alstroemeria* and *Zantedeschia* (1 variety, each). Additionally, 19 varieties of pot plants (belonging to 13 different species) and 19 varieties of bedding plants (belonging to 12 different species) have been tested.

In addition to studies on possible phytotoxic effects, the effect on the crop stand has also been evaluated in cut flowers and pot plants grown in glasshouses or under plastic cover. In these studies, 2 applications with bifenazate were made at an interval of 7 d. No effect on crop stand was observed in any of the trials.

In the above mentioned studies, where applications were made to full coverage of the crops, any visible residue was assessed. After drying of the spray liquid, no visible residues were observed on flowers or foliage.

Quantitative recordings of yield were made by Singleton (Crompton Europe Ltd., internal report, 2005), with 2 cultivars ('Mirimar' and 'Little rock') of pot plant chrysanthemums. Plants were treated with a foliar spray to full coverage, at up to double the label rate (80 ml bifenazate 240 SC/100 litres). At weekly intervals, up to 4 weeks after spraying, the number of flowers was assessed. Bifenazate had no effect on flower production.

Though bifenazate rapidly degrades in soil and water, possible effects of the parent compound or its degradation products were studied on succeeding crops. Findak (Ricerca Inc., USA, internal report, 2000) studied the nature and amount of bifenazate residue in rotational crops grown in soil treated with radiolabelled bifenazate a rate of 560 g a.i./ha. The parent compound (bifenazate) or reference metabolites were not detected in any of the plants. Porch and Krueger (Wildlife International Ltd., USA, internal report, 1999a), additionally studied the effects of bifenazate on seedling emergence of 10 plant species at 1.122 kg a.i./ha. No effects on any of the test species were observed. A further study by Porch and Krueger (Wildlife International Ltd., USA, unpublished report, 1999b) was made to assess the vegetative vigour of the same 10 plant species. Application was made on seedlings, and observations on plant height, plant weight, and plant condition, were made up to 14 DAT. The study showed no treatment-related effects on growth or condition of the seedlings. These data indicate that effects of bifenazate on succeeding crops are highly unlikely.

CONTROL OF MITES ON ORNAMENTALS IN EUROPE AND AFRICA

In Europe, the label rate for control of *T. urticae* (and *T. cinnabarinus*) is 40 ml of the bifenazate 240 SC formulation/100 litres spray liquid. This rate is based on a large number of trials in many European countries. Crops tested belonged to the major cut flower and ornamental pot plant species. In Israel, the label rate is 50 ml 240 SC/100 litres spray liquid. For good acaricidal activity, the first application has to be made when populations reach levels of 2 - 3 mites per leaf or leaflet. If the level of control is below 95% after 7 d, a second application should be made. The need for a second application depends on several factors, such as the mite population pressure, the speed of reproduction (which is related to the temperature), the density of the crop, and the penetration of the spray liquid within the crop. In trials where a second application was needed, control significantly decreased when the interval was extended from 7 to 14 d. Rose cut flower cultures on artificial substrate always need a second application. In this culture, thin stems are bent sideways into the crop, to ensure high photosynthetic capacity of the plants. This cultural practice results in a high density of plant material in the lower crop segment, which is very difficult to penetrate with the spray liquid. In European registration trials, the acaricidal activity of bifenazate was equal to or better than the registered rate of abamectin 1.8 EC (25 ml/100 litres). The residual activity of bifenazate, at 40 ml/100 litres, depends on the crop. On cut flower roses, complete control of *T. urticae* can be achieved for at least 3 weeks. On pot plants, control can last considerably longer.

In Europe and Africa, the registrations of bifenazate in ornamentals are limited to *T. urticae.* However, effects on other mite species in ornamental crops have also been tested:

Oligonychus ununguis (conifer spinning mite or spruce spider mite). In 2002 and 2003, outdoor trials were undertaken in Denmark by Paaske (Denmarks Jordbrugs Forskning, internal reports) with *Picea glauca* as a host plant. At 60 ml bifenazate 240 SC/100 litres

spray liquid, population reductions were 82 and 84%, at 7 and 14 DAT, respectively. At 80 ml bifenazate 240 SC/100 litres, population reductions were 79 and 91%, at 7 and 14 DAT, respectively.

Panonychus citri (citrus red mite). This species is a major pest of citrus, but also occasionally attacks ornamental plants. Bifenazate 480 SC has been widely tested on citrus. The rate for registration will be about 30 g a.i./100 litres spray liquid. *P. citri* has not been tested in glasshouses.

Panonychus ulmi (fruit tree red spider mite). This mite also occurs in tree nurseries. Bifenazate 480 SC has been widely tested in commercial fruit orchards in Europe. The recommended rate for a future registration in commercial orchards will probably be 48 g a.i./100 litres, based on a spray volume of 1000 l/ha.

Polyphagotarsonemus latus (broad mite). This mite has a broad spectrum of host plants in glasshouses. In 2004, trials were made in Poland by Labanowski (Instytut Sadownictwa i Kwiaciarstwa, internal report). On *Hedera*, the activity of bifenazate 240 SC (40 ml/100 litres) was 96.5% population reduction at 7 DAT, and 86 % at 14 DAT. In the trial on *Gerbera*, the same rate gave 92.6% at 7 DAT, and 90% population reduction at 14 DAT.

Steneotarsonemus ananas (pineapple tarsonemid mite). In The Netherlands, Boogaard, Weijers & 't Hoen (PPO, internal report, 2002) studied effects of bifenazate in a glasshouse trial on *S. ananas* on the Bromelia *Guzmania minor*. An application with 10 g a.i./100 litres was repeated 3 times, at a weekly interval. One week after the final application, the activity of bifenazate was equal to abamectin at 0.05%, with 75 and 74% control, respectively.

Steneotarsonemus pallidus (cyclamen or strawberry mite). This mite is a destructive pest of many ornamental flowers and shrubs, including *Cyclamen*, *Gerbera*, and *Begonia*. In 2003 and 2004, tests with *S. pallidus* on strawberry were undertaken in Denmark by Paaske (Denmarks Jordbrugs Forskning, internal reports). In field trials, bifenazate 240 SC (up to 0.6 litre product in 600 litres/ha) gave moderate control (maximum of about 60%) of *S. pallidus*. When leaves were sprayed and kept in Petri-dishes in the laboratory, almost complete control was obtained. The moderate control in the field is probably due to behaviour of the mites, occupying sites on the plants that are difficult to reach with the spray liquid (unopened leaflets, between tightly packed young leaves in the buds, etc.).

Tyrophagus putrescentiae (mould mite). This mite occurs on several ornamental species in glasshouses. In a laboratory trial, mites were introduced into Petri-dishes with moist tissue and bran. The mites feed on a fungus that grows on the bran. The mites were sprayed with bifenazate 240 SC at 10 g a.i./100 litres. At the final observation at 5 DAT, almost complete mortality was reached. (Boogaard, Weijers & 't Hoen, PPO, internal report, 2002).

CROSS-RESISTANCE AND RESISTANCE DEVELOPMENT

In horticulture, new acaricides are needed because several registered acaricides have lost, or are losing, their effectiveness due to resistance. Bifenazate has been evaluated extensively for cross-resistance with other acaricides in laboratory and field trials. In unpublished trials in Japan, bifenazate proved to be effective on *T. urticae* resistant to amitraz, hexythiazox,

clofentezine, fenpyroximate, pyridaben and tebufenpyrad. In US trials, bifenazate showed excellent performance against abamectin-resistant strains of *T. urticae* (McDonald, Crompton, internal report, 1999). Van Leeuwen *et al.* (2004) reported that no cross-resistance was found between chlorfenapyr and bifenazate. The chlorfenapyr-resistant *T. urticae* strain used in this study was selected in the laboratory. It had a resistance ratio of 580 at the LC_{50} level. In a field-collected Belgium strain of *T. urticae*, with strong resistance to bifenthrin, dicofol and fenbutatin oxide, Van Leeuwen *et al.* (2005) found very low cross-resistance to bifenazate (Resistance Ratio 2.2 at the LC_{50} level).

In Europe/Africa, the general label states that the number of 'treatment-programs' per year should not exceed two. A treatment–program can consist of either a single spray, or two sprays with a 7-d interval between sprays. It is advised to monitor regularly, and to apply bifenazate as soon as mite infestations are observed. Further advice is to alternate with at least two products from different chemical classes between treatment-programs of bifenazate. An additional recommendation is to incorporate IPM techniques in the insect and mite control program, as a means of reducing the number of applications with acaricides.

POSSIBLE IMPACT ON BENEFICIAL INSECTS AND MITES

Pollinators

Apis mellifera (honey bee). Kelly & Allan (Inveresk Research, Scotland, unpublished report, 2001), investigated acute contact and oral toxicity of bifenazate on the honey bee. The 48 h acute contact and oral LD_{50} values are greater than 98 and 110 µg bifenazate/bee, respectively. The 48 h contact and 48 h oral no-observed-effect levels are 98 and 55 µg bifenazate/bee, respectively. The hazard quotients for oral and contact toxicity, assuming a maximum application rate of 144 g active substance/ha, are < 1.46 and < 1.31, respectively. As these hazard quotients are well below the critical value of 50, the normal horticultural use of bifenazate will not have any adverse impact on honey bees.

Bombus terrestris (bumble bee). Sterk *et al.* (2004) tested effects on the bumble bee by direct contact, and by oral feeding of bifenazate in mixtures with either pollen or a sugar solution. In the direct contact test, 50 µl of a concentration of 0.04% bifenazate 240 SC was applied to bumble bee workers. In the oral tests, feeding was *ad libitum.* Effects were measured by assessing the offspring. In all tests, bifenazate was harmless (IOBC class 1; <25% effects).

Predators and parasitoids

Amblyseius californicus (predatory mite). In laboratory tests by van de Veire & Tirry (2003), eggs were placed on a sweet pepper leaf with a dry spray deposit (1.5 mg spray liquid per m², spray liquid containing 150 mg bifenazate/litre). At 6 DAT, adults of the predatory mite had emerged, and mortality was checked. Bifenazate was classified as 'harmless'.

Aphidius colemani (parasitic wasp). Gouldman & Steinberg (2002) tested the susceptibility of adults of the aphid parasitoid *A. colemani* in laboratory tests. A sweet pepper leaf disc was placed on agar, and sprayed in a Potter Tower with 5 ml of a liquid with 120 mg bifenazate/litre. The test insects were introduced after drying. At 5 DAT, effects on mortality,

egg-hatch, hatching of the pupae, and effects on fertility were assessed. Bifenazate was categorized as 'harmless'.

Aphidius rhopalosiphi (parasitic wasp). A laboratory study on this Braconid was made by Buttle & Walker (Ecotox, internal report, 2001). Adult wasps were exposed to glass plates, treated in a Potter Tower sprayer at a spray volume of 200 litres/ha. Mortality and effects on fecundity were evaluated. The 48 h median lethal application rate (LR$_{50}$) of bifenazate to adult *A. rhopalosiphi*, is estimated to be 752 g/ha, which is far above the maximum application rate of bifenazate in practice. Surviving females of *A. rhopalosiphi* which were exposed to either 10 or 100 g bifenazate/ha, were transferred to units with pots of barley, infested with cereal aphids. After 24 h, the females were removed and 12 days afterwards, the numbers of aphid mummies were counted. No effects on fecundity of surviving females of *A. rhopalosiphi* exposed to 10 and 100 g bifenazate/ha were observed.

Chrysoperla carnea (lacewing). Buttle (Ecotox, internal report, 2000) performed a laboratory study on the lacewing, *C. carnea*. First instar larvae were placed on dry residue of bifenazate on glass plates, treated in a Potter Tower sprayer at a volume equivalent to 200 litres/ha. When applied at the highest test rate of 300 g bifenazate/ha, only 3% mortality was observed. Also, no effect on egg production or egg hatch was observed at this rate. Bifenazate is classified as 'harmless' to *C. carnea*, at rates up to and including 300 g/ha.

Diglyphus isaea (parasitic wasp). Gouldman & Steinberg (2002) tested the susceptibility of adults of this leafminer parasitoid in laboratory tests. A sweet pepper leaf disc was placed on agar, and sprayed in a Potter Tower with 5 ml of a liquid with 120 mg bifenazate/litre. The test insects were introduced after drying. At 5 DAT, effects on mortality, egg-hatch, and hatching of the pupae were assessed. Bifenazate was categorized as 'harmless'.

Encarsia formosa (parasitic wasp). Effects of bifenazate on *E. formosa*, a species important in whitefly control in glasshouses, were evaluated in a Tier 2 laboratory study on a bean leaf substrate (Hargreaves & Walker, Ecotox, internal report, 2002). After 48 h of exposure at rates of 75, 150 and 300 g a.i./ha, at a volume of 200 litres/ha, mortality in bifenazate treated units was less than or equal to the control mortality of 5.2%. Parasitism rate of *E. formosa* exposed to the 300 g bifenazate/ha exceeded that in the control treatment. In laboratory tests by van de Veire & Tirry (2003), adult females were exposed to a dry spray deposit on a glass plate (1.5 mg spray liquid per m^2, spray liquid containing 150 mg bifenazate/litre). In this test, bifenazate was classified as 'harmless' at 3 DAT.

Feltiella acarisuga (gall midge). Larvae of *F. acarisuga* are important introduced predators of spider mites in ornamental crops in glasshouses. An efficacy trial with bifenazate (2 applications with 9.6 g/100 litres spray liquid at an 8-d interval) on cut flower roses in The Netherlands (Hendriks, Certis Europe, unpublished report, 2002) had to be terminated prematurely by the increasing abundance of *F. acarisuga*. The average number of larvae and pupae before treatment was 0.9 per 50 leaves in the control plots, and 1.7 per 50 leaves in the plots to be treated with bifenazate. At 6 d after the second treatment, the average number of larvae and pupae was 19 in the control plots, and 27 per 50 leaves in the plots treated with bifenazate

Macrolophus calliginosus (predatory bug). In laboratory tests by van de Veire & Tirry (2003), 2nd and 3rd instar nymphs were exposed to a dry spray deposit on glass (1.5 mg spray

liquid/m², containing 150 mg bifenazate/litre). Bifenazate was classified as 'harmless' at the final observation at 3 DAT.

Orius laevigatus (predatory bug). Buttle (Ecotox, internal report, 2001) performed a laboratory study on 2nd instar nymphs of the Anthocorid bug *O. laevigatus.* Treatments of glass coffin cells were made in a Potter Tower at a spray volume of 200 litres/ha. When applied at 300 g a.i./ha, no mortality by bifenazate was found when nymphs were introduced once the tested surfaces had dried. Also, no effect on egg production or egg hatch was observed. Bifenazate is classified as 'harmless' to *O. laevigatus,* according to the IOBC scheme, at rates up to and including 300 g/ha. In laboratory tests by Gouldman & Steinberg (2002), sweet pepper leaf discs were placed on agar, and sprayed in a Potter Tower with 5 ml containing 120 mg bifenazate/litre. The test insects were introduced after drying. At 5 DAT, effects on mortality, egg-hatch, and hatching of the pupae were assessed. Bifenazate was categorized as 'harmless'. In laboratory tests by van de Veire & Tirry (2003), 1st and 2nd instar nymphs were exposed to a dry spray deposit on a glass plate (1.5 mg spray liquid/m², containing 150 mg bifenazate/litre). In this test, bifenazate was also classified as 'harmless' at the final evaluation at 12 DAT.

Phytoseiulus persimilis (predatory mite). Gouldman & Steinberg (2002) tested the susceptibility of adult *P. persimilis* in the laboratory. A sweet pepper leaf disc was placed on agar, and sprayed in a Potter Tower with 5 ml of a liquid with 120 mg bifenazate/litre. The mites were introduced after drying. At 5 DAT, effects on mortality, egg-hatch and hatching of the pupae were assessed. Bifenazate was categorized as 'harmless'.

Poecilus cupreus (Carabid beetle). Potential effects of bifenazate on the Carabid *P. cupreus* were investigated in a laboratory study by Walker & Sprosen (Ecotox, internal report, 2002). Beetles were contained on a sand substrate treated with bifenazate at 75, 150 and 300 g/ha. Mortality was evaluated over a period of two weeks. Beetles were fed *Musca* sp. pupae, to evaluate effects on prey consumption. In the bifenazate treatment, no effects on mortality or prey consumption were observed, and bifenazate was classified as 'harmless'.

Typhlodromus pyri (predatory mite). A laboratory study on *T. pyri* was made by Walker & Hargreaves (Ecotox, internal report, 2001). Adults were exposed to a dry spray deposit on glass plates, treated in a Potter Tower at an equivalent of 200 litres/ha. At 7 DAT, 25 g bifenazate/ha (equivalent to 12.5 g a.i./100 litres) gave 72% mortality. The LR_{50} of bifenazate to *T. pyri* was 27.8 g/ha. To investigate effects on a natural substrate further, a test with *T. pyri* on bean leaves and similar laboratory methodology was made by Buttle (Ecotox, internal report 2001). At rates up to 600 g a.i./ha (equivalent to 300 g a.i./100 litres) negligible mortality was found. Fecundity was also assessed in this test. At rates equivalent to 75 g a.i./ha (37.5 g a.i./100 litres) and above, reductions in egg production were observed. However, the concentration recommended for use on ornamentals (9.6 g a.i./100 litres) is well below the range tested in the laboratory and would not be expected to result in significant effects on fecundity. Effects on populations of *Typhlodromus* sp. were further evaluated in a field study in an apple orchard in southern France by Lagrasse (Promo-Vert, internal report, 2003). Bifenazate was applied in July 2002 at a rate of 48 g/100 litres, and a spray volume of 1000 litres/ha. Effects of the bifenazate treatment were evaluated at 3, 6, 20, 47 and 62 DAT, and compared to a water control treatment. At 3 and 6 DAT, numbers of Phytoseiidae in the bifenazate treated plots and the water controls were not significantly different. At 20 DAT, numbers in the bifenazate plots were 33.7% lower than in the control

plots, but populations had recovered fully by 47 DAT, and remained at or above control levels up to 62 DAT. Effects of bifenazate on *Typhlodromus* sp. in this field study, can be described as limited and of short duration.

We may conclude that trials have shown that bifenazate is very effective against phytophagous mites, and that it does not cause any crop phytotoxicity. Furthermore, as shown in this paper, it also has a very low toxicity to beneficial arthropods at recommended application rates.

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Flubendiamide - a new insecticide for controlling lepidopterous pests

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ABSTRACT

Flubendiamide, 3-iodo-N '-(2-mesyl-1,1-dimethylethyl)-N-{4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]-o-tolyl}phthalamide is a novel class insecticide having a unique chemical structure compared to existing insecticides and acts through a totally new mode of action. Flubendiamide has high activity against a broad spectrum of lepidopterous insect pests. It shows excellent activity against adults and larvae, fast acting properties, and extended residual activity, mainly by ingestion. After foliar application on vegetables, fruits, rice, and cotton in field studies, flubendiamide provided superior efficacy over standard compounds. Since it shows no cross-resistance with conventional insecticides and is safe for beneficial arthropods in addition to superior field performance, flubendiamide will be an essential tool in IPM and insect resistance management programs.

INTRODUCTION

Resistance has often been a problem or a potential problem for insecticides and this is one of the most important reasons why insecticides with new modes of action have been always desired. Although it is quite a difficult task to find such insecticides, Nihon Nohyaku Co. Ltd discovered flubendiamide among benzenedicarboxamide derivatives (Tohnishi *et al.* 2005) at its Research Center in Japan. Flubendiamide is being co-developed by Nihon Nohyaku Company and Bayer CropScience globally (Nishimatsu *et al.* 2005). This paper describes the biological properties and performance of flubendiamide under both laboratory and field conditions, against major lepidopterous insect pests.

CHEMICAL AND PHYSICAL PROPERTIES

Common name:	Flubendiamide (ISO proposed)
Code name:	NNI-0001
Chemical name:	3-iodo-N '-(2-mesyl-1,1-dimethylethyl)-N-{4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]-o-tolyl}phthalamide
Molecular formula:	$C_{23}H_{22}F_7IN_2O_4S$
Molecular weight:	682.39
Appearance:	White crystalline powder

Structure:
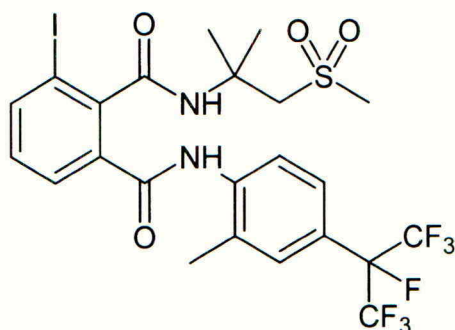
Melting point:	217.5-220.7°C
Vapor pressure:	$<10^{-4}$ Pa (25°C)
Solubility in water:	29.9 µg/litre (20°C)
Partition coefficient:	$\log P_{o/w} = 4.2$ (25°C)

TOXICOLOGY

Acute oral: Rat LD_{50}	Male & Female	>2000 mg/kg
Acute dermal: Rat LD_{50}	Male & Female	>2000 mg/kg
Eye irritation:		slight
Skin irritation:		none
Mutagenicity:		Ames Negative
Aquatic organism:	Carp LC_{50}	>548 µg/litre (96 h)
Honeybee:	Oral / Contact LD_{50}	>200 µg/bee (48 h)
Bird (Bobwhite quail):	Oral LD_{50}	>2000 mg/kg body weight

BIOLOGIAL PROPERTIES

Spectrum of activity

EC_{50} values for flubendiamide against major insect and mite species are shown in Table 1. Flubendiamide provided high activities against all lepidopteran insect pests, and its EC_{50} values were under 1.0 mg a.i./litre. Flubendiamide showed broad-spectrum activity against lepidopterous pests, but was inactive against insect species other than Lepidoptera.

Table 1.   Insecticidal spectrum of flubendiamide on major insect pests in agriculture

Scientific name	Common name	Tested stage	DAT	EC_{50} (mg a.i./litre)
Lepidoptera				
Plutella xylostella	Diamond-back moth	L3	4	0.004
Spodoptera litura	Tobacco cutworm	L3	4	0.19
Helicoverpa armigera	Old World bollworm	L3	4	0.24
Agrotis segetum	Turnip moth	L2-3	7	0.18
Autographa nigrisgna	Beet semi-looper	L3	4	0.02
Pieris rapae crucivora	Common cabbage worm	L2-3	4	0.03
Adoxophyes honmai	Smaller tea tortrix	L3	5	0.38
Homona magnanima	Oriental tea tortrix	L4	5	0.58
Hellula undalis	Cabbage webworm	L3	5	0.01
Chilo suppressalis	Rice stem borer	L3	7	0.01
Diaphania indica	Cotton caterpillar	L3	3	0.02
Coleoptera				
Sitophilus zeamais	Maize weevil	A	4	>1000
Hemiptera				
Nilaparvata lugens	Brown rice planthopper	L3	4	>1000
Myzus persicae	Green peach aphid	All stages	7	>1000
Pseudococcus comstocki	Comstock mealybug	L1	7	>100
Acarina				
Tetranychus urticae	Two-spotted spider mite	All stages	4	>100

L2, L3, A: second instar, third instar and adult   DAT: Day(s) after treatment

Activity on different developmental stages of lepidopterous pest

The activity of flubendiamide on three developmental stages of *Plutella xylostella* was evaluated (Table 2). Flubendiamide is most effective on larvae followed by adults, but it has no ovicidal activities. The activity of flubendiamide on three larval stages of *Spodoptera litura* was also evaluated. Flubendiamide is most effective on first instar larvae followed by third and fifth instar larvae (Table 3). Flubendiamide shows higher activity, even against fifth instar larvae, than four existing insecticides, but application to young larval stages is recommended to give more effective control in practical use.

Table 2.   Insecticidal activity of flubendiamide on three developmental stages
            of *P. xylostella*

Treatment	EC_{50} (mg a.i./litre)		
	Egg (1-2 days old, 2 DAT)	Larva (3rd instar, 4 DAT)	Adult (1 day old, 2 DAT)
Flubendiamide 20% WDG	>500	0.004	0.21
Cyhalothrin 5% EC	0.81	0.24	0.20

DAT: Day(s) after treatment

Table 3.   Insecticidal activity of flubendiamide on three different larval
            stages of *S. litura*

Treatment	EC_{50} (mg a.i./litre, 3 DAT)		
	1st instar	3rd instar	5th instar
Flubendiamide 20% WDG	0.033	0.19	0.51
Cyhalothrin 5% EC	0.08	0.36	0.72
Methomyl 45% WP	13.8	17.3	15.4
Profenophos 45% EC	1.38	17.3	54.8
Spinosad 25% WDG	0.67	45.5	54.8

DAT: Day(s) after treatment

Mode of action

A novel mode of action was first suggested by the characteristic symptoms induced by flubendiamide. Typical symptoms in lepidopterous larvae are gradual body contractions, which are unlike the symptoms induced by conventional insecticides. Similar symptoms are also seen in insects treated with ryanodine, a modulator of a calcium release channel. It is well known that calcium release channels play a pivotal role in muscle contraction. This evidence strongly suggests a calcium channel such as the ryanodine sensitive calcium release channel could be involved in the insecticidal activity of flubendiamide. The results of these mode of action studies will be reported in due course.

Cross-resistance

The activity of flubendiamide against third instar larvae of *Plutella xylostella* resistant to synthetic pyrethroids, benzoylphenylureas, organophosphates and carbamates is shown in Table 4. Flubendiamide provided the same EC_{50} values against both the resistant and the susceptible strains.   The absence of cross-resistance between flubendiamide and conventional insecticides is probably because of the new mode of action of flubendiamide.

Table 4.   Biological activity of flubendiamide against the 3rd instar larvae of *P. xylostella* resistant to pyrethroid, benzoylphenylurea, organophosphate and carbamate insecticides

Treatment	EC_{50} (mg a.i./litre, 4 DAT)		R/S ratio
	Resistant strain	Susceptible strain	
Flubendiamide 20% WDG	0.002	0.004	0.5
Cyhalothrin 5% EC	869	0.24	3621
Flufenoxuron 10% EC	233	0.05	4660
Methomyl 45% WP	287	14.5	19.8
Profenophos 45% EC	24.2	3.78	6.4

DAT: Day(s) after treatment

Field evaluations

Field evaluations of flubendiamide have been conducted in many areas on various crops including vegetables, top fruit, and cotton. Data from the trials conducted through JPPA (Japan Plant Protection Association) during 2000-2003 in Japan and data in India and Thailand in 2004 are shown in Tables 5-7 as examples of field performance. Flubendiamide showed excellent performance in controlling the major lepidopterous pests on each crop at the recommended doses and its efficacy was comparable to or better than those of standard insecticides.

Flubendiamide (20% WDG) shows no phytotoxicity to any crop tested at the recommended doses.

Table 5.   Control efficacy of flubendiamide against major lepidopterous pests on cabbages in Japan (JPPA field trials)

Treatment	Dose (mg a.i./litre)	*P. xylostella*		*P. rapae crucivora*	*S. litura*	*H. undalis*	
		Kagoshima	Gunma	Aichi	Saitama	Hyogo	Aichi
		2001	2001	2001	2001	2001	2002
		7 DAT	7 DAT	7 DAT	7 DAT	7 DAT	7 DAT
Flubendiamide 20 WDG	100	100	100	98.5	98.3	100	96.8
Emamectin-benzoate 1 EC	5	86.6	-	96.8	93.1	93.2	-
Chorfenapyr 10 SC	50	75.0	74.1	-	-	-	-
Indoxacarb 10 SC	50	-	-	-	-	73.7	-
Chlorfluazuron 5 EC	25	-	-	-	-	-	68.7

DAT: Day(s) after treatment

Table 6.   Control efficacy of flubendiamide against major lepidopterous on apple in Japan (JPPA field trials)

Treatment	Dose (mg a.i./litre)	*Carposina niponensis*		*Phyllonorycter ringoniella*		*Lyonetia. prunifoliella malinella*	
		Ishikawa	Akita	Nagano	Akita	Akita	Ishikawa
		2002	2002	2000	2001	2001	2003
		20 DA3T	21 DA2T	20 DAT	17 DAT	12 DAT	10 DAT
Flubendiamide 20 WDG	50	93.6	93.1	93.2	99	82	96
Chlorpyrifos 250 WP	250	85.1	-	-	-	-	-
Permethrin 20 WP	100	-	89.1	-	-	-	-
Nicotine-sulfate 40 EC	400	-	-	94.1	99	-	-
Dinotefuran 20 WSP	100	-	-	-	-	88	-
Clothianidin 16 WSP	40	-	-	56.8	-	-	-
Imidacloprid 10 WP	100	-	-	-	-	79	95

DAnT: Day(s) after n times treatments   DAT: Day(s) after treatment

Table 7.   Efficacy of flubendiamide against major lepidopterous pests on cotton and rice in India and Chinese kale in Thailand, 2004

Treatment	Dose (g a.i. /ha)	Cotton		Rice		Chinese kale
		% of fruit damage		% of white ears	Damaged leaves/10 hills	% of crop damage
		Bollworms		Stem Borer	Leaf folder	*P. xylostella*
		India (1)	India (2)	India	India	Thailand
Flubendiamide 20 WDG	50	3.30	19.10	-	-	11.3
Flubendiamide 20 WDG	25	-	-	4.98	7.4	16.3
Lambda-cyhalothrin 5 EC	25	4.37	-	-	-	-
Lambda-cyhalothrin 5 SC	12.5	-	-	5.43	8.3	-
Spinosad 45 SC	90	--	-	-	-	31.3
Spinosad 12 SC	75	3.39	19.18	-	-	-
Indoxacarb 15 SC	75	-	23.05	-	-	38.8
Indoxacarb 15 EC	30	-	-	5.35	6.4	-
Monocrotophos 36 WSC	500	-	-	5.20	13.6	-
Untreated	-	6.43	29.15	10.72	21.6	58.8

Toxicity to beneficial arthropods

Acute toxicity of flubendiamide to several species of beneficial arthropods is shown in Table 8. Flubendiamide was inactive against beneficial arthropods tested at rates from 100 to 400 mg a.i./litre. This result indicates that flubendiamide would be compatible with integrated pest management (IPM) programs.

Table 8.   Toxicity of flubendiamide on beneficial arthropods

Common name	Scientific name	Test stage	Test method	EC_{30} (mg a.i./litre)
Lady beetle	*Harmonia axyridis*	Adult	ID	>200
	Coccinella septempunctata bruckii	Adult	ID	>200
Parasitoid wasp	*Encarsia formosa*	Adult	DF	>400
	Aphidius colemani	Adult	DF	>400
	Cotesia glomerata	Adult	DF	>100
Green lacewing	*Chrysoperla carnea*	Larva	S	>100
Predatory bug	*Orius strigicollis*	Adult	S	>100
Predatory midge	*Aphidoletes aphidimyza*	Larva	S	>100
Predatory mite	*Amblyseius cucumeris*	Adult	S	>200
	Phytoseiulus persimilis	Adult	S	>200
Spider	*Pardosa pseudoannulata*	Adult	ID	>100
	Misumenops tricuspidatus	Adult	ID	>200

ID: Insect dipping method, DF: Dry film method, S: Spraying on food & insect

CONCLUSIONS

Flubendiamide was discovered as a novel class insecticide having a unique chemical structure. All data obtained indicate that flubendiamide is classified into a new class of insecticide in view of the chemistry, the biology, and the biochemical mode of action. Flubendiamide provides excellent activity against a broad spectrum of lepidopterous insects and shows no-cross resistance to conventional insecticides. In addition, it is much safer against natural enemies. With these properties it is suggested that flubendiamide will be very suitable for insecticide resistance management (IRM) and integrated pest management (IPM) programs.

ACKNOWLEDGMENTS

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Potential for novel, metallo-ion products in inducing pathogen resistance in crops, and their use as post-harvest crop preservatives and disinfectants

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ABSTRACT

Environmental concerns and increasingly stringent regulations are placing ever greater demands on conventional growers using fungicides. Biorational fungicidal products are also required for use in resistance management strategies, e.g. late blight on potato. Furthermore, organic standards in Europe require decreased inputs of copper products over the coming years, which could result in reduced efficacy. Preliminary trials with a novel, highly-charged, cationic, copper electrolyte used at sub-35 ppm Cu rates have demonstrated the product has a range of distinct usages both for foliar and systemic applications, and show considerable promise with regard to the control of common crop diseases. Preliminary data from trials with closely allied copper-based metallo-ion products for use as post-harvest crop preservatives and disinfectants are also disclosed, and demonstrate their potential commercial value.

INTRODUCTION

Organic and conventional growers are under increasing pressure to reduce cost of inputs to maintain price competitiveness, while ensuring products meet organic standards for certification. Conventional growers must ensure that their crops do not exceed maximum residue levels (MRLs).

Copper in the form of various salts (hydrochloride, hydroxide, oxide and sulfate) is widely used as a fungicide by both conventional and organic farmers in the UK and other European countries. New EU regulations will require reduced usage, with a maximum of 6 kg copper per ha per year being allowed from 1 January 2006.

There have been increasing concerns over the effect of copper compounds used as fungicides on soil (Merrington *et al.*, 2002; Van-Zwieten *et al.*, 2004) and aquatic organisms (Rice *et al.*, 2002; de Oliveira *et al.*, 2004). These toxic effects are caused by long-term usage of copper in specific locations in combination with excessive run-off to the soil and groundwater.

Additionally, there is limited evidence of non-target impacts of copper products to beneficial predatory or parasitic insects on crops (Michaud & Grant, 2003).

Longer-term risk assessments are presently being undertaken under the EU Pesticide Directive 91/414/EEC, and such assessments are likely to be critical of high dosage inorganic pesticide inputs owing to risk of environmental accumulation.

Furthermore, there have been increased consumer concerns over the use of chlorine as a disinfection agent for use in the post-harvest washing of salads in the 'ready-to-eat' category. Metallo-electrolyte products could potentially be used as a substitute.

Accordingly, there is a demand for copper-based products which are both effective and safe to the environment, while not exceeding future maximum limits set by regulation. The use of metallo-electrolytes may hold considerable promise for a range of pre- and post-harvest uses.

In the present studies, the use of a range of patented copper-electrolyte products is investigated in relation to both pre-harvest and post-harvest use. The concentration of elemental copper used in such products is very low, generally between 10 and 30 ppm, little more than the concentration of copper that occurs naturally within crop plants (OMRI, 2001).

MATERIALS AND METHODS

Pre-harvest 'ear-wash' on wheat

Low concentration copper-electrolytes (30 ppm Cu) were applied as an ear-wash during the growth of wheat crops at Throws Farm, Dunmow, Essex. Efficacy in disease management and effects on overall yield will be collated at the end of the season.

Pre-harvest use on potato

Copper-electrolyte products were incorporated into a randomised block design trial on evaluating their efficacy in blight (*Phytophthora infestans*) control against standard fungicides (mancozeb plus propamocarb hydrochloride, mancozeb plus zoxamide, fluazinam) at the Scottish Agricultural College.

Three different copper electrolytes were tested, each having different binders. One product (CU PC 33) has systemic properties.

Post-harvest wash trials

Spinach leaves (young) were exposed to a 2 minute potable water pre-wash and spin followed by a further 2 minute wash in a 20 ppm solution of copper-electrolyte (product code CU AL 42). Microbiological assessments (Total Viable Count and Enterobacteriaceae) were made (10 replicates) at intervals on Days 0 and 4.

RESULTS

Results from the pre-harvest trials were not complete at the time of finalisation of this manuscript. However they will be delivered at the conference.

Post-harvest wash trial

Two minute exposure to product CU AL 42 resulted in a 3 log reduction in common micro-organisms present on salad leaf surfaces following the copper wash (Table 1, Day 0). This level of efficacy exceeds the usual 1.5 log reductions normally achieved through the use of chlorine (sodium hypochlorite). The recolonisation of micro-organisms (Table 1, Day 4) suggest that any residual effects of the copper in this trial were negligible. Accordingly, copper-electrolytes may provide a viable alternative to chlorinated products for produce washing and disinfection.

Table 1.   Summary of microbiological count data following Cu washing of spinach leaves (TVC = Total Viable Count / bioburden [total number aerobic bacteria, including Enterobacteriaceae, and total yeast]; ENT = Entobacteriaceae count [medically important gram-negative aerobic and anaerobic bacteria, 12 genera in total]). Bracketed figures refer to % reductions from raw count

Treatment	Day 0 TVC	ENT	Day 4 TVC	ENT
Raw	2.1×10^7	2.5×10^4	-	-
Pre-wash	3.7×10^6 (17%)	1.6×10^3 (5%)	-	-
Cu-wash	2.8×10^4 (0.13%)	22 (0.09%)	4.3×10^6 (20%)	9.6×10^3 (38%)

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Bacillus subtilis strain QST 713, a new biological tool for integrated and organic disease control programs

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ABSTRACT

Bacillus subtilis QST 713, a naturally occurring bacterial strain, was discovered in a California orchard by AgraQuest, Inc., USA. *B. subtilis* QST 713 has been shown to possess significant efficacy against a broad spectrum of bacterial and fungal pathogens and is not toxic to beneficial and non-target organisms. As determined by US-Environmental Protection Agency and international regulatory authorities, *B. subtilis* QST 713 is exempt from the requirement of a tolerance because there are no synthetic chemical residues, and it is safe to workers and the environment. As a result, treated fruit and vegetables can be exported throughout the world without restrictions. *B. subtilis* QST 713 works through novel, multiple modes of action that involve the competitive biological action of *B. subtilis* in addition to fungicidal, lipopeptide compounds produced by the bacterium. *B. subtilis* QST 713 has been shown to be an effective tool for disease control in organic crop production and in integrated disease control programs contributing to resistance management and, overall, reducing dependency on synthetic fungicides.

DESCRIPTION AND REGISTRATION STATUS

Bacillus subtilis is a rod-shaped, gram positive, aerobic, motile bacterium which is ubiquitous in nature. The bacterium can also produce an endospore. *B. subtilis* is commonly found in various ecological niches including soil, water and air. The US-Environmental Protection Agency and international regulatory authorities have classified *B. subtilis* QST 713 as a microbial fungicide. The commercial formulated product and QST 713 technical product contain living *B. subtilis* strain QST 713 as the active ingredient. *B. subtilis* QST 713 was approved in the United States as a foliar fungicide in 2000 and is currently registered in 16 countries under the trade name, Serenade (Table 1).

Table 1.   *Bacillus subtilis* QST 713 global registration status.
Bold type indicates regulatory approval for commercial use

1999	2000	2001	2002	2003	2004	2005	2006>
Chile	**US**	**Mexico** **New Zealand** **Puerto Rico**	**Costa Rica**	**Japan** **Israel** **Philippines**	**Guatemala** **Honduras** **Switzerland** **Turkey** **Argentina**	**France** **Italy** Korea Ecuador Columbia	Africa Germany Canada Spain Greece Belgium Australia UK

CROP USES AND DISEASE SPECTRUM

Bacillus subtilis QST 713 efficacy has been demonstrated on over 30 crops in 20 countries against a broad spectrum of fungal and bacterial pathogens. Currently, the major commercial uses are in the crops listed in Table 2. *B. subtilis* QST 713 can be applied alone, in tank mixtures or in rotation programs with other fungicides. Therefore, it is ideally suited for "high" value, fruit and vegetable production particularly for export markets with requirements for organic or reduced chemical-input certification.

Table 2.   *Bacillus subtils* QST 713: global commercial use

Crop	Disease	Pathogen
Tomato/Pepper	Bacterial Leaf Spot	*Xanthomonas* spp.
	Powdery Mildew	*Leveillula taurica*
	Early Blight	*Alternaria solani*
Grapes	Gray Mold	*Botrytis cinerea*
	Powdery Mildew	*Uncinula necator*
	Sour Rot	Multiple pathogens
Cucurbits	Powdery mildew	*Erysihe/Sphaerotheca* spp.
	Gummy Stem Blight	*Didymella bryoniae*
Banana	Black Sigatoka	*Mycosphaerella fijiensis*
Mango	Anthracnose	*Colletotrichum gloeosporioides*
Lettuce	Leaf Drop	*Sclerotinia* spp.
Apples/Pears	Fire Blight	*Erwinia amylovora*
Beans	White Mold	*Sclerotinia sclerotiorum*

MODES OF ACTION

Bacillus subtilis QST 713 works through novel modes of action that are manifested by the bacterium colonizing the leaf surface and competing with the pathogen for nutrients and space and physically preventing attachment and penetration of the pathogen. In addition, *B. subtilis* QST 713 produces three groups of metabolites known as lipopeptides, (iturins, agrastatins/plipastatins, and surfactins) that act in a synergistic manner to destroy pathogen germ tubes and pathogen membranes. The iturins and plipastatins have been reported to have fungicidal activity. *B. subtilis* QST 713 is the first strain reported to produce iturins, plipastatins and sufactins and two new compounds, the agrastatins. Studies at AgraQuest on the effects of the individual groups of lipopeptides on pathogen spore germination compared to mixtures of the groups, provided a better understanding of the role of these metabolites. Morphological differences were observed in spores treated with different lipopeptide groups. The iturin group resulted in inhibition of spore germination and was dependent upon the concentration of iturins present. The iturins were most effective on *Botrytis cinerea* spores with an EC_{50} as low as 15 ppm (50% inhibition of spore germination). The EC_{50} of *Monilinia fructicola* spores occurred at 30 ppm and for *Alternaria brassicicola* the level required was 25 ppm. Exposure of the spores to the iturin/plipastatin group resulted in an abnormal appearance in which the spore had a large bubble-like growth replacing the normal appresorium. This effect was most notable with *A. brassicicola* spores in which the EC_{50} was 5 ppm. Investigation of the effects of combining the groups of lipopeptides gave further explanation for the efficacy observed with *B. subtilis* QST 713. Addition of concentrations as low as 1

ppm agrastatin/plipastatin to 10 ppm iturin provided a significant reduction in spore germination; reduced to approximately 5% spore germination for *M. fructicola*. The surfactin group was found to have no effect on spores at the highest rate tested (250 ppm). Addition of 25 ppm surfactin to 20 ppm iturin reduced spore germination from 85% to less than 5%. Surfactin at 25 ppm added to agrastatin/plipastatin reduced germination from 100% to 10%. Given the novel, multiple, modes of action, *B. subtilis* QST 713 is utilized as a resistance management tool in rotation programs with chemical fungicides, such as the strobilurin and triazole groups, which are highly susceptible to resistance development due to a specific metabolic site, modes of action.

FORMULATION

Bacillus subtilis QST 713 is formulated as a wettable granule and aqueous suspension product containing from 1 to 7 X 10^9 cfus/gram depending upon the formulation. It can be applied in conventional application equipment, requires no special storage conditions and has a shelf life of more than 2 years. *B. subtilis* QST 713 formulations have been shown to be compatible in mixtures with commonly used synthetic fungicides (e.g., sulfur, copper hydroxide, mancozeb, chlorothalonil, azoxystrobin, myclobutanil) and are approved for use in organic agriculture under the guidelines established by the Organic Materials Review Institute (OMRI-USA), Institute for Marketecology (IMO-Switzerland) and BCS Öko-Garantie (BCS-Germany).

USE IN INTEGRATED DISEASE MANAGEMENT PROGRAMS

Because of the excellent environmental profile, broad disease control spectrum and safety to non-target, beneficial organisms (Table 3), *Bacillus subtilis* QST 713 is ideally suited for use in integrated pest management (IPM) programs that utilize many approaches such as cultural practices, classical biological control and other fungicides.

Table 3. Summary of *Bacillus subtilis* strain QST 713 ecological toxicity studies

Non Target Test Organism	Toxicity	Toxicity Rating[1]
Avian oral (quail)	LD50 > 5000 mg/kg	5
Freshwater fish (trout)	LC50 = 162 ppm	3
Honey bee larvae	LC50 >10,000 ppm	5
Daphnia	EC50 = 108 ppm	3
Hymenoptera parasitic wasp	LC50 > 30,000 ppm	5
Lady beetle	LC50 > 60,000 ppm	5
Lacewing larvae	LC50 > 60,000 ppm	5

[1] Rating according to Hodge and Sterner scale of 1 to 6 where 1 is "Extremely Toxic" and 6 is "Relatively Harmless"

The benefits of *Bacillus subtilis* QST 713 in programs utilizing chemical fungicides are shown in the following studies. The objective of these studies were (1) to demonstrate the efficacy of *B. subtilis* QST 713 used alone against economically important diseases and (2) to document the effectiveness of *B. subtilis* QST 713 in strategically replacing conventional fungicide applications in rotation and tank mix programs. In a 2003 cucumber study conducted in Japan, *B. subtilis* QST 713 provided effective powdery mildew (*Sphaerotheca fuliginea*) control when

applied alone on a weekly schedule, although it was slightly less effective than the chemical standard, quinomethionate (Table 4). However, rotational programs with *B. subtilis* QST 713 and quinomethionate provided excellent powdery mildew control, comparable to weekly applications of quinomethionate alone. The *B. subtilis* QST 713/quinomethionate programs provided a 50% reduction in chemical fungicide use without compromising disease control.

Table 4. *Bacillus subtilis* QST 713 integrated pest management program for cucurbit powderymildew (*Sphaerotheca fuliginea*) control, Japan 2003 Chemical fungicide = quinomethionate

| | Treatment Schedule | | | | Powdery Mildew % Control |
	0 Day	7 Day	13 Day	20 Day	
Untreated	Water	Water	Water	Water	0
Chemical	Fungicide	Fungicide	Fungicide	Fungicide	98.8
B. subtilis	*B. subtilis*	*B. subtilis*	*B. subtilis*	*B. subtilis*	86.9
Chemical / *B. subtilis*	Fungicide	*B. subtilis*	Fungicide	*B. subtilis*	98.6
B. subtilis / Chemical	*B. subtilis*	Fungicide	*B. subtilis*	Fungicide	98.4

B. subtilis QST 713 is also effective in tank mix programs with reduced use rates of chemical fungicides as shown in a banana/black sigatoka (*Mycosphaerella fijiensis*) study conducted in Costa Rica during 2003 (Table 5). Black sigatoka causes significant reductions in functional leaf area resulting in yield losses and premature ripening of harvested fruit. Successful sigatoka control programs depend heavily on synthetic fungicides. As many as 30 to 50 fungicide applications per year are required for acceptable sigatoka control creating concerns for adverse affects on local ecosystems and potential health risks associated with human exposure. Systemic fungicides, such as strobilurin, sterol biosynthesis and sterol demethylation inhibitors, provide effective sigatoka control, but are highly susceptible to disease resistance development if not properly managed. In this study, evaluations parameters such as Youngest Leaf Infected and Functional Leaves at Shooting and Harvest showed that *B. subtilis* QST 713 provided effective control of sigatoka, particularly in tank mix combinations, with reduced rates of the standard protectant fungicide, mancozeb.

Table 5. *Bacillus subtilis* QST 713 integrated pest management program for black sigatoka (*Mycosphaerella fijiensis*) control in bananas, standard fungicide = mancozeb 60 OS formulation. Mindanao Philippines (March to August 2003)

Treatment (rate / hectare)	Visible Streaks	Youngest Leaf Spotted	Functional Leaves at Shooting Stage	Functional Leaves at Harvest
B. subtilis – 2 liters	3.9 a	9.5 c	13.3 a	7.8 a
B. subtilis + mancozeb (2 + 0.9 liters)	3.9 a	10.1 bc	13.5 a	7.4 a
Standard Program (mancozeb at 1.8 liters)	4.1 a	11.4 a	13.4 a	8.5 a
Untreated	3.4 b	8.6 d	12.9 a	4.9 b

Bacillus subtilis QST 713 has been shown to be an effective tool in rotational and tank mix programs with other fungicides contributing to resistance management and overall, reducing dependency on synthetic fungicides.

Cyflufenamid: its development and potential for powdery mildew control

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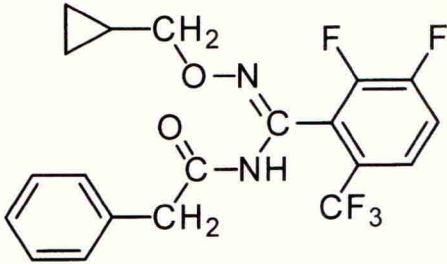*Email: horgan@certiseurope.co.uk*

ABSTRACT

Cyflufenamid is a specific powdery mildew fungicide which has been developed in the UK for the cereal market. Its different mode of action offers growers alternative chemistry against powdery mildew. It will now be developed for other crops.

INTRODUCTION

The UK cereal market is the third largest market in Western Europe with nearly 3,500,000 ha. Of this area, winter wheat is the largest UK cereal crop with over 2,200,000 ha grown. Consequently, cereal disease control is crucial for profitable production. Powdery mildew infestations in cereals are caused by the obligate parasitic fungus *Blumeria graminis* (formerly *Erisyphe*) which has specialised forms largely restricted to its own particular host, i.e. wheat, barley and oats. A new fungicide, cyflufenamid, from Nippon Soda, has been developed and registered for its use on cereals against powdery mildew in the UK.

CHEMICAL AND PHYSICAL PROPERTIES

Common name: cyflufenamid

Chemical name: (Z)-N-[α-(cyclopropylmethoxyimino)-2,3-difluoro-6-
                        (trifluoromethyl)benzyl]-2-phenylacetamide

Molecular formula: $C_{20}H_{17}F_5N_2O_2$

Molecular weigh: 412.36

Molecular structure:

Melting point: 61.5 – 62.5°C
Water solubility: 5.20×10^{-4} g/litre (20°C)
Partition coefficient n-octanol/water: log P_{ow} = 4.70
Vapour pressure: 3.54×10^{-5} Pa (20°C)

TOXICOLOGY (EW 50 g/litre)

Mammalian toxicity:
Oral (rat):	LD_{50}:	>5 g/kg
Dermal (rat):	LD_{50}:	>2 g/kg
Inhalation (rat):	LC_{50}:	>4.41 mg/litre (4h) (maximum practicable concentration)

Irritation:
Eyes (rabbit):   Slight irritant
Dermal (rabbit):   Moderate
Sensitization:
Dermal (guinea pig): Negative
Mutagenicity:
Ames test: Negative (as active ingredient)

Ecological toxicity:
Acute toxicity for fish (rainbow trout):	LC_{50}:	9.84 mg/litre (96h)
Acute toxicity for *Daphnia*:	EC_{50}:	9.48 mg/litre (48h)
Acute toxicity for Algae:	EbC_{50}:	0.701 mg/litre (72h)

FORMULATION

In development, the principal cyflufenamid formulation has been an emulsion in water formulation (EW) containing 5% active. This formulation has now been commercialised and launched in 2005 on cereal crops in the UK. This is the first country in Europe to receive registration. The product has been introduced for the control of *Blumeria graminis* in winter and spring wheat, winter and spring barley, rye and triticale. A new formulation is now being developed for use on other crops in the UK.

BIOLOGICAL KEY FEATURES

Cyflufenamid is in the amidoxime group and no cross resistance is likely to occur with available fungicides commercialized or in development. Cyflufenamid is active against all strains of cereal powdery mildew including those strains resistant to demethylation inhibitor, strobilurin-type and benzimidazole-type fungicides.

Cyflufenamid has shown both protectant and curative activity on powdery mildew and in addition has both a vapour and translaminar action which contributes to the overall efficacy of the product. At very low rates of use cyflufenamid performs well.

MODE OF ACTION

At present, the mode of action is not fully understood. Modes of actions that affect sterol, phospholipids, chitin, and protein biosynthesis have all been ruled out, as have effects on mitochondrial respiration and cell membrane function. The actual mode of action is still to be determined and studies are currently on-going.

MATERIALS AND METHODS

Initial efficacy trials were carried out in the laboratory to establish the range of activities with cyflufenamid. Then cereal field trials were carried out in the UK and Europe to test the efficacy of cyflufenamid under a range of different conditions and different cereal crops. Between 1999 and 2002, 119 efficacy trials in the UK, France and Germany were executed for data submission. All these results were collated and used within the registration package for the UK. Other data were obtained with regard to toxicological impacts, environmental fate, rainfastness, crop safety and crop residues.

In addition to these registration trials, other investigations were carried to establish curative activity under high disease pressure at likely user rates. This involved growing winter wheat under glass. Further work was also carried in the field to compare cyflufenamid with alternative chemistry, especially recently introduced chemistry such as metrofenone. On-going cereal development work is also being carried out for an eventual submission in other European countries. Submissions have been made in France and Germany and registrations are awaited. Following rate response trials, 0.5 litres product/ha was chosen as the maximum statutory rate. Treatments were applied to all trials using a foliar plot sprayer, calibrated to apply water volumes of between 200 and 300 litres/ha. Since cyflufenamid is specific to powdery mildew, it has been tested in mixture with broad-spectrum cereal fungicides to provide protection against a range of cereal diseases. In all cereals trials, applications were never applied beyond the full emergence of the ear (GS60) of the crop. In the UK, further development is now being undertaken on other crop sectors such as top fruit, soft fruit, grapes, stone fruit, cucurbits, tomatoes and ornamentals.

RESULTS AND DISCUSSION

Table 1 shows the results from a series of laboratory studies which established that cyflufenamid has curative, preventative, residual, vapour and translaminar activity. These studies were carried out to determine the field label rate of use and was determined to be equivalent to 0.5 litre/ha. In the majority of tests, 100% control was achieved before the equivalent label rate was reached, indicating strong activity at very low rates.

Table 1. Laboratory investigation to establish activities with cyflufenamid on winter wheat.

Dose ppm	Equivalent field rate (g a.i./ha) [1]	% Control with cyflufenamid under laboratory conditions					
		Curative Activity		Prevention	Residual	Vapour	T [2]
		Latent infection	Expressed infection	Inoculated immediately	Inoculated 7 days later		
800	160	-	-	-	-	-	-
200	40	-	100	-	100	40	-
50	10	100	100	100	100	35	94
12.5	2.5	100	100	100	100	22.5	17
3.1	0.6	100	75	99	94	10	-
0.8	0.16	75	-	91	-	-	-
0.2	0.04	50	-	75	-	-	-

[1] 25 g a.i./ha is the maximum label rate   [2] Translaminar

Table 2 shows the excellent curative activity following a single application of cyflufenamid at rates of 25 and 50 g a.i./ha against powdery mildew on winter wheat. Total infection degrees reached 1.0 for the 25 g a.i./ha rate 27 DAT and 1.7 for the 50 g a.i./ha rate at the same assessment time. Good activity was also shown at the lowest rate tested of 12.5 g a.i./ha cyflufenamid (total infection degree 8.0 at the 27 DAT assessment). The curative efficacy of cyflufenamid was superior at all rates tested to that of the current commercial standard, tebuconazole.

Table 2.　　　Curative activity of cyflufenamid on winter wheat (1997)

Treatment	Rate (g a.i./ha)	Infection degree					
		Leaf 4 – 30.8; Leaf 5 – 39.0					
	DAT	13			27		
	Leaf	4	3	Total	2	1 (flag)	Total
cyflufenamid	50.0	16.3	1.3	17.6	0.3	1.3	1.7
(50 g/litre EW)	25.0	17.8	0.5	18.3	0.2	0.8	1.0
	12.5	23.5	4.2	27.7	3.2	4.8	8.0
tebuconazole 23.5% EC	235.0	38.3	6.7	45.0	15.4	15.8	31.2
Untreated	-	62.7	27.5	90.2	60.4	29.4	89.8

In 2004, a glasshouse pot-grown winter wheat trial was carried out to establish the curative activity with lower than label rates. Figure 1 shows the results from a trial looking at a range of rates lower than the full label rate of cyflufenamid. Powdery mildew was established, between 5-10%, at the time of application. Note that up to 21 days after treatment, the low rates of cyflufenamid persisted well despite the high mildew pressure. These and field trial results were subsequently used to provide user rates.
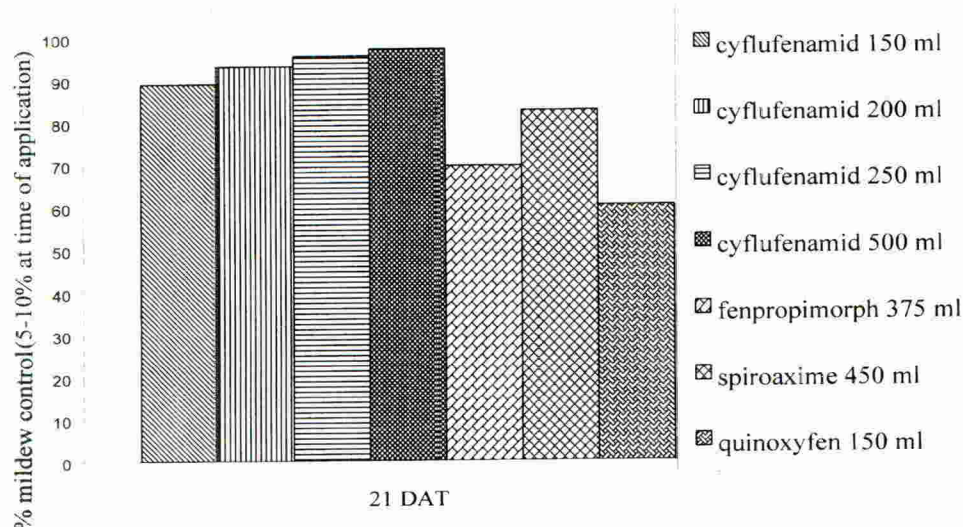

Figure 1.　　　UK Glasshouse Trials on winter wheat

In 2004, metrafenone was launched so a field trial was set-up to compare cyflufenamid with metrafenone. Figure 2 shows the percentage of mildew on leaves 1, 2 and 3, 38 DAT. The untreated levels of mildew at the time of assessment were 5.3% mildew on L1, 10% on L2 and 13.7% on L3.

All the treatments were significantly different to the untreated. When comparing cyflufenamid to metrafenone, all treatments showed cyflufenamid with less mildew. For example, on L1 when comparing cyflufenamid at 150 ml/ha to metrafenone at 200 ml/ha, cyflufenamid showed half the level of mildew compared to the metrafenone treatment.

Leaf 1, Leaf 2 and Leaf 3 with treatments: LSD = 2.26, 4.52 AND 4.49 respectively.
Mildew at 1% on L3 and 2% on L4 at time of treatments.
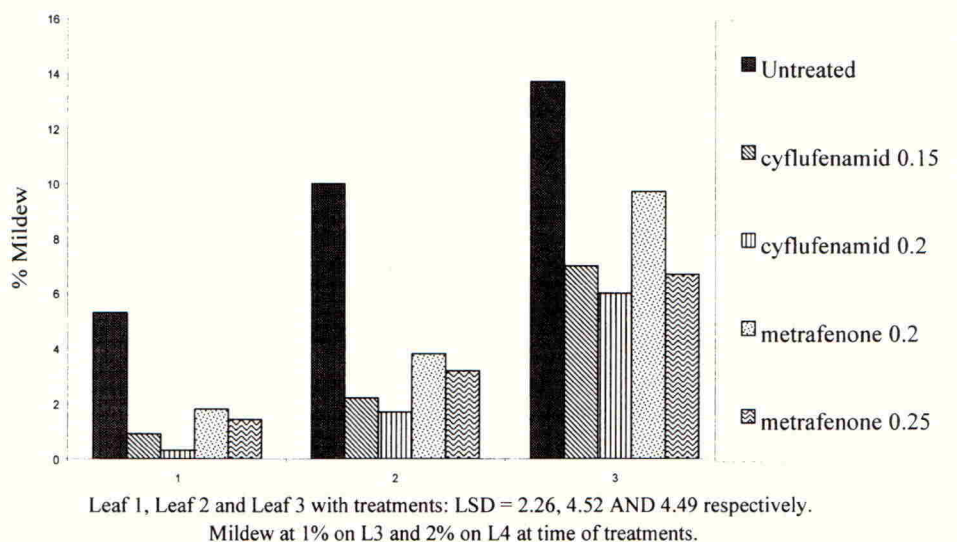
Figure 2.   Powdery mildew control comparing cyflufenamid with metrafenone
                      % Mildew on L1, L2 and L3. 38 DAT, 24 June 04 (Location: Morley.
                      Variety Claire)

Table 3 shows results from pot trials conducted by Nippon Soda Co., Ltd where the preventative efficacy of cyflufenamid (NF 149) on powdery mildew on various crops was evaluated. These results helped to direct future development where there may be potential for cyflufenamid on other crops.

Table 3.   Preventative efficacy of cyflufenamid (NF 149) on powdery mildew on
                      various crops (pot trials)

Crop	Pathogen	$EC_{\geq 75}$ (ppm)
Wheat	*Blumeria graminis* f.sp. *tritici*	0.2
Cucumber	*Sphaerotheca fuliginea*	0.2
Strawberry	*Sphaerotheca humuli*	0.2
Apple	*Podosphaera leucotricha*	0.8
Grape	*Uncinula necator*	0.8

CONCLUSIONS

Cyflufenamid EW formulation is now branded as Cyflamid on the-UK market for cereal growers. Both field trials and recent grower experience with the product has shown that cyflufenamid has a part to play in the defence against cereal powdery mildew. This benefit will be now developed for mildew control in alternative crops.

ACKNOWLEDGEMENTS

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Fluopicolide, a novel fungicide with a unique mode of action, setting a new standard for Oomycete control

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ABSTRACT

Fluopicolide is a novel fungicide of the new chemical class, acylpicolides, discovered and developed by Bayer CropScience. Fluopicolide exhibits a high level of efficacy against a wide range of Oomycete diseases such as potato late blight caused by *Phytophthora infestans* and grapevine downy mildew caused by *Plasmopara viticola*. Fluopicolide shows translaminar and systemic properties in plant tissues and offers strong, long-lasting protectant disease control at low dose rates. Fluopicolide has a novel biochemical mode of action and no cross resistance with other chemical families. Protectant foliar applications of fluopicolide consistently demonstrated excellent long-lasting disease control during five years of field trials, in a range of crops including grapevine and potato.

INTRODUCTION

Fluopicolide is a novel active ingredient of the new chemical class, the acylpicolides, discovered and patented by Bayer CropScience. Fluopicolide is highly active on Oomycete organisms responsible for a number of economically important diseases in crops such as grapevine, potato, fruit, vegetables and ornamentals.

Fluopicolide is being developed worldwide, in combination with other fungicides, for use in a wide variety of crops. The first commercial launches of fluopicolide will be in co-formulation with propamocarb hydrochloride for use in potatoes and vegetables, under the trade name Infinito, and with fosetyl-aluminium for use in grapevine.

This paper summarizes the physical, chemical, toxicological and environmental properties of fluopicolide. The biological performance of fluopicolide-based co-formulations in laboratory and field trials will be presented.

CHEMICAL AND PHYSICAL PROPERTIES

ISO name: Fluopicolide
Chemical class: Acylpicolide
Code number: AE C638206
Chemical name (IUPAC):   2,6-dichloro-N-{[3-chloro-5-(trifluoromethyl)-2-
                                  pyridinyl]methyl}benzamide
CAS registration N°:   [*239110-15-7*]

Structural formula:

Molecular formula: $C_{14}H_8Cl_3F_3N_2O$
Molecular weight: 383.59 g/mol
Melting point: 150°C
Vapour pressure: 3.03×10^{-7} Pa at 20°C
Partition coefficient: 2200 (log P = 2.9) Octanol/water

Water solubility: 2.8 mg/litre at 20°C (pH 7)
DMSO solubility: 183 g/litre at 20°C

Photolytic stability: Stable
Hydrolytic stability: Stable over pH range 4 to 9

TOXICOLOGY

Mammalian toxicity

Acute oral toxicity (rat): $LD_{50} > 5000$ mg/kg
Acute dermal toxicity (rat): $LD_{50} > 5000$ mg/kg
Eye irritation, rabbit: not irritating
Skin irritation, rabbit: not irritating
Acute inhalation, 4 h, LC_{50} (rat): > 5160 mg a.i./m³ air
Sensitisation (guinea pig):   not sensitising
Carcinogenicity (mice, rat):   no carcinogenic potential
Mutagenicity: no genotoxic effects
Developmental: no embryotoxic potential
Teratogenicity, rat, rabbit: no teratogenic potential

Toxicity to wildlife

Avian
Bird (bobwhite quail):	acute oral, LD_{50}	> 2250 mg/kg
Bird (mallard duck):	acute oral, LD_{50}	> 2250 mg/kg

Aquatic
Fish (rainbow trout):	acute (96 h), LC_{50}	0.36 mg/litre
Fish (bluegill sunfish):	acute (96 h), LC_{50}	0.75 mg/litre
Invertebrate (*Daphnia magna*):	acute (48 h), EC_{50}	> 1.8 mg/litre
Algae (*Selenastrum capricornutum*):	growth inhibition (72 h), ErC_{50}	> 4.3 mg/litre
Plant (*Lemna gibba*):	growth inhibition (7 d), EC_{50}	> 3.2 mg/litre

Beneficials
Earthworm:	acute (14 d), LC_{50}	> 1000 mg a.i./kg soil
Honeybee:	contact, LD_{50}	> 100 µg/bee
Typhlodromus sp.:	acute, LR_{50}	0.313 kg/ha
Aphidius sp.:	acute, LR_{50}	0.419 kg/ha

Due to a very favourable toxicological and environmental profile, fluopicolide is a candidate for use in integrated pest management systems (IPM) in a wide range of crops.

BIOLOGICAL PROPERTIES

Physiological characteristics

In vitro studies conducted on *P. infestans* and *P. viticola* demonstrated that fluopicolide is active at several stages of their lifecycles. Fluopicolide affects release of zoospores and cyst germination. Against *P. infestans*, it is effective on both "indirect germination" (release of zoospores) and direct germination of sporangia which occurs under high temperature conditions. Fluopicolide provides a strong inhibition of mobility of *P. infestans* zoospores at very low concentrations (LC_{90} = 2.5 ppm). Microscopic observations demonstrate that these zoospores stop moving in less than one minute after contact with fluopicolide. Zoospores then swell and burst.

Laboratory studies demonstrated that fluopicolide is also active in plant tissues by inhibiting sporulation and mycelial growth. Lysis of mycelium of *P. infestans* and *Pythium ultimum* is observed after treatment.

Biochemical mode of action

The biochemical mode of action of fluopicolide is novel. Biochemical studies conducted following standard methods (Beffa, 2004) showed that it is clearly different from fluazinam (oxidative phosphorylation), metalaxyl (rRNA synthesis) and strobilurins or other respiration complex III inhibitors, such as fenamidone. In addition, fluopicolide has no effect *in vitro* on tubulin polymerization (zoxamide).

Recent biochemical studies have shown that fluopicolide has an effect on spectrin-like proteins, believed to play a role in maintaining the membrane stability in Ascomycete fungi or Oomycetes, especially during hyphal tip extension. Microscopy studies demonstrate that fluopicolide induces a quick redistribution of these proteins from the membrane to the cytoplasm in both hyphae and zoospores. None of the fungicides tested (fenamidone, dimethomorph, metalaxyl, zoxamide and iprovalicarb) showed a similar effect on spectrin-like proteins. Work on the biochemical mode of action of fluopicolide is ongoing.

Uptake and redistribution in plants

Glasshouse tests and radio-labelled studies conducted on various plant species demonstrated that fluopicolide is xylem systemic and has good translaminar properties. Application to the upper surface of a leaf provides protection of the lower surface and *vice versa*. When applied to the base or petiole of a leaf, fluopicolide rapidly moves towards the leaf tip. In grapevine, treatment of immature buds protects developing leaves from infection. The molecule is not phloem mobile. Fluopicolide is not volatile and redistribution *via* the vapour phase is not anticipated.

Spectrum of activity and fungicidal properties

Fluopicolide is highly active in controlling a wide range of Oomycete diseases in crops of major economic importance. Glasshouse tests using artificial inoculations as well as field experiments demonstrated the protectant and translaminar properties of fluopicolide (Table 1).

Table 1.   Fungicidal properties of fluopicolide against different pathogens.

Pathogen	Crop	Test conditions	Glasshouse* IC_{90} (ppm)	Effective dose rates** (g a.i./ha)
Phytophthora infestans	Potato	Protectant activity	1-5	75-100
Phytophthora infestans	Potato	Translaminar activity	-	75-100
Phytophthora infestans	Tomato	Protectant activity	1-5	75-110
Plasmopara viticola	Grapevine	Protectant activity	1-5	100-125
Plasmopara viticola	Grapevine	Translaminar activity	1-5	100-125
Bremia lactucae	Lettuce	Protectant activity	10-20	85-100
Pseudoperonospora cubensis	Cucumber	Protectant activity	< 20	75-90
Peronospora parasitica	Cabbage	Protectant activity	-	90-110
Phytophthora porri	Leek	Protectant activity	-	90-130
Peronospora sparsa	Roses	Protectant activity	-	75-110
Peronospora tabacina	Tobacco	Protectant activity	-	75-100

(*) IC_{90} assessed in the glasshouse, 7 days after applications
(**) dose rates needed to equal the reference fungicides, field trials, 7-day spray intervals

Anti-resistance strategy

Although fluopicolide has no cross-resistance with other fungicides, the risk of resistance development has been considered from the beginning. Pro-active, anti-resistance management is an essential part of the marketing strategy of fluopicolide: fluopicolide will

be recommended for use with a limited number of applications per season and exclusively in combination with fungicides from different chemical classes.

Performance in the field

Field trials summarised in this paper were carried out in the most economically important crops affected by Oomycete diseases: grapevine and potato.

In 1999, experiments were conducted in order to assess the fungicidal performance of fluopicolide alone. Fluopicolide-based combinations were tested in 2000-2003 in field trials following relevant EPPO guidelines (European and Mediterranean Plant Protection Organisation) or local guidelines (EPPO, 1996a & b).

RAUDPC calculation (Tukey, 1977) was used to compare activity against potato late blight. Level of tuber blight is expressed as the mean percentage of infected tubers.
Potato

Fluopicolide alone was evaluated as a protectant treatment against potato late blight in 1999 (Table 2). Fluopicolide provided good control of potato late blight from 82.5 g a.i./ha and rates above 110 g a.i./ha outperformed the standard fluazinam.

Table 2.   Protectant activity of fluopicolide against *P. infestans* in potato.
Assessment on leaves, summary of 6 trials conducted in Europe, 1999

Treatment	dose rates (g ai/ha)	mean RAUDPC
Untreated control	-	59.5
fluopicolide SC	55	11.5
fluopicolide SC	82.5	9.3
fluopicolide SC	110	7.2
fluopicolide SC	165	5.6
fluazinam SC	200	10.4

Due to high synergy between fluopicolide and propamocarb hydrochloride for control of potato late blight, a co-formulation of both molecules (SC, ratio 1:10) was widely tested in field trials. Propamocarb hydrochloride is a well- established potato and vegetable fungicide with systemic and curative properties. Efficacy trials carried out in Northern Europe in 2002-2003 in protectant spray programmes at 7- to10-day spray intervals are summarised in Table 3.

Table 3.   Protectant activity of fluopicolide + propamocarb hydrochloride against *P. infestans* in potato. Summary of 26 registration trials in Europe in 2002-2003, 7- to 10-day spray intervals

Treatment	Dose rates (g a.i./ha)	Leaf protection (RAUDPC)	Stem protection (RAUDPC)	Tuber blight (% infected tubers)
Untreated		61.8 (26)	53.9 (4)	7.45 (15)
fluopicolide + propamocarb hydrochloride	75 + 750	6.8 (26)	5.5 (4)	0.81 (15)
fluopicolide + propamocarb hydrochloride	100 + 1000	5.0 (24)	4.6 (4)	0.75 (15)
Fluazinam	200	12.8 (18)	8.9 (3)	3.52 (15)
propamocarb hydrochloride + chlorothalonil	750 + 750	12.1 (14)		
Mancozeb	1570	28.8 (7)	-	-

( ) number of data points

The combination of fluopicolide at rates of 75 and 100 g a.i./ha with propamocarb hydrochloride at 750 and 1000 g a.i./ha respectively, provided excellent protection of leaves, stems and tubers. These treatments gave consistently high levels of blight control, better than the standard fungicides applied at 7-day and particularly 10-day intervals.

Grapevine

Field experiments conducted with fluopicolide alone for the control of *P. viticola* in grapevine are summarised in Table 4. Fluopicolide showed a dose response and provided good protection of both leaves and bunches from 10 g a.i./hl under long spray interval conditions.

Table 4.   Protectant efficacy of fluopicolide against grapevine downy mildew (*P. viticola*). Summary of 22 trials, Europe, 1999, 10- to 14-day spray interval

Treatment	dose rates (g ai/hl)	% efficacy on leaves (Abbott)	% efficacy on bunches (Abbott)
fluopicolide *	5.0	86.9 (16)	91.3 (14)
fluopicolide *	7.5	90.5 (16)	92.6 (14)
fluopicolide *	10.0	92.9 (16)	96.3 (14)
fluopicolide *	15.0	95.5 (11)	98.6 (11)
azoxystrobin	25	93.2 (18)	99.4 (14)
fosetyl-Al + mancozeb	103 + 65	86.9 (10)	88.5 (10)
untreated control (% disease intensity)	-	79.1 (22)	65.5 (17)

* fluopicolide SC formulation + adjuvant   ( ) number of trials

During investigations to identify the best mixing partner for fluopicolide in long spray intervals, fosetyl-aluminium showed the best potential for control of *P. viticola* in grapevine (Figure 1). Fosetyl-aluminium is a highly systemic fungicide used successfully for more than 25 years in this crop.
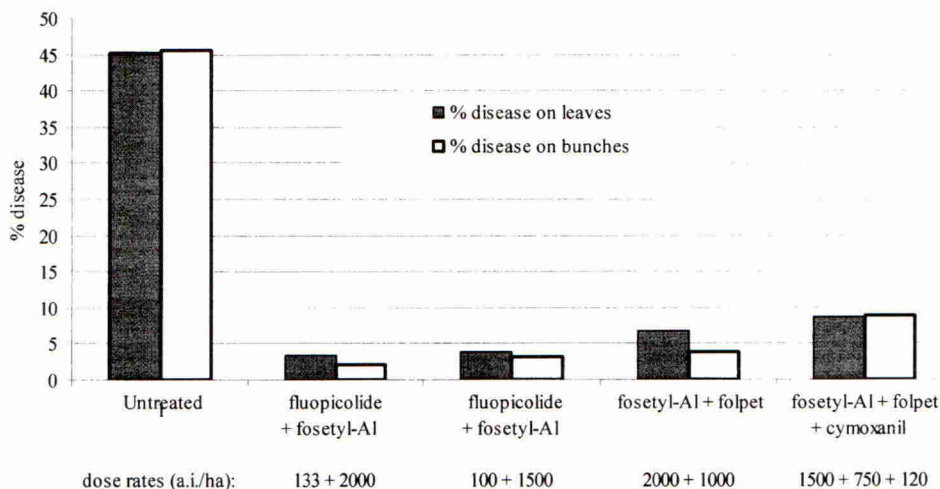

Figure 1.   Protectant efficacy of fluopicolide + fosetyl-aluminium for control of grapevine downy mildew (*P. viticola*). Summary of 36 trials, Europe, 2001-2003

The co-formulation of fluopicolide with fosetyl-aluminium (WG formulation, ratio 1:15) provided outstanding protection of both leaves and bunches with fluopicolide rates of 100-133 g a.i./ha, with a shallow dose response. This combination of two long-lasting fungicides was more effective than the commercial standards. The consistently high level of activity against *P. viticola* is remarkable, even under high disease pressure.

Crop selectivity

In all the field trials conducted since the beginning of its development, fluopicolide-based formulations have demonstrated excellent crop safety at dose rates required for disease control under practical conditions.

CONCLUSIONS

Fluopicolide is a versatile fungicide, highly effective against Oomycete diseases, belonging to the novel chemical class of acylpicolides. Fluopicolide has a unique mode of action showing no cross-resistance with other fungicides and thus provides an important new tool for effective resistance management.

Field trials carried out on grapevine and potato consistently demonstrate excellent protection against downy mildew and late blight diseases. Fluopicolide exhibits strong protectant

activity with translaminar and systemic properties. It provides remarkably quick effects on zoospores and long-lasting disease protection in the field.

Fluopicolide has been developed in combination with fosetyl-aluminium for the control of *P. viticola* in grapevine and with propamocarb hydrochloride against *P. infestans* in potatoes.

ACKNOWLEDGEMENTS

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Mandipropamid a new fungicide against Oomycete pathogens

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ABSTRACT

Mandipropamid is a new fungicide developed by Syngenta. It is the first derivative of the chemical class of mandelamide fungicides to be commercialized. It is highly effective against most foliar Oomycete pathogens. The main targets include economically important plant diseases such as: *Plasmopara viticola* in grapes, *Phytophthora infestans* in potatoes and tomatoes and *Pseudoperonospora cubensis* in cucurbits. Mandipropamid is highly effective in preventing spore germination. It also inhibits mycelial growth and sporulation. Mandipropamid is best used as a preventive spray against the target diseases, but also provides curative activity during the incubation period. Following foliar application, a large proportion of mandipropamid is adsorbed into the wax layer of plant surfaces. Uptake of mandipropamid into plant tissue assures good translaminar activity. Mandipropamid is fully rainfast as soon as the spray deposit has dried. Mandipropamid provides consistently excellent disease control even under adverse weather conditions. It is highly effective at low application rates of 100-150 g a.i./ha or 10-15 g a.i./100 litres spray solution. The compound is characterized by excellent crop safety. It also has a very favourable profile with regard to human safety and safety to wildlife and the environment.

INTRODUCTION

Mandipropamid is a fungicide against foliar Oomycete pathogens developed by Syngenta. It is the first derivative of the chemical class of mandelamide fungicides to be commercialized. Since its discovery in 1999, it has undergone extensive international evaluation in laboratory tests and in extensive global field testing programs. This paper describes its chemical and physical properties, its safety profile, its biological properties and performance in the field.
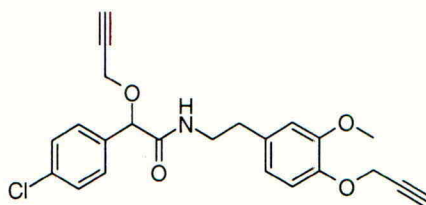
CHEMICAL AND PHYSICAL PROPERTIES

CAS number	*[374726-62-2]*
Common name:	Mandipropamid (proposed ISO common name)
Chemical class:	Mandelamides
Code number:	NOA 446510
Chemical Name (IUPAC):	2-(4-chloro-phenyl)-*N*-[2-(3-methoxy-4-prop-2-ynyloxy-phenyl)-ethyl]-2-prop-2-ynyloxy-acetamide

Molecular Formula: $C_{23}H_{22}ClNO_4$

Structural Formula:

Molecular weight:	411.9
Physical state:	light beige powder
Melting Point:	96.4 to 97.3 °C
Boiling point:	thermal decomposition starts at about 200 °C
Vapour pressure:	$<9.4 \times 10^{-7}$ Pa at 25°C
Partition coefficient n-octanol / water:	$\log P_{ow}$: 3.2 at 25°C
Solubility in water:	4.2 mg/litre at 25°C

PRODUCT SAFETY

Mammalian toxicity:

Acute oral LD_{50}	Rat	> 5000 mg/kg
Acute dermal LD_{50}	Rat	> 2000 mg/kg
Acute inhalation LC_{50}	Rat	> 5000 mg/m^3
Eye irritation	Rabbit	slight irritant
Skin irritation	Rabbit	mild irritant
Skin sensitisation (LLNA)	Guinea Pig	non-sensitising
Mutagenicity	5 tests	no mutagenic potential
Teratogenicity	Rat, Rabbit	no teratogenic potential
Chronic toxicity/Carcinogenicity	Rat, Mouse	no carcinogenic potential
Reproduction	Rat	no adverse effects
Neurotoxicity	Rat	no neurotoxic potential
Metabolism	Rat	rapid absorption and elimination

Toxicity to Wildlife:

Birds	Bobwhite quail	LD_{50}	> 2250 mg/kg
Fish	Rainbow trout	LC_{50}	> 2.9 mg/litre
Bees (contact and oral)		LD_{50}	> 200 µg/bee
Earthworm		LC_{50}	> 1000 mg/kg
Aphidius		LR_{50}	830 g/ha
Typhlodromus		LR_{50}	> 900 g/ha

Environmental Fate:

Hydrolysis in water		hydrolytically stable at pH 4-9
Photolysis in water	DT_{50}	1.7 days at pH 7 and 25°C
Mobility in soil	K_{oc}	mean 847ml/g (range 405-1294)
Degradation in soil (field studies)	DT_{50}	mean 17 days (range 2-29)

Mandipropamid has a very favourable profile with regard to human safety and safety to wildlife and the environment. It is safe to applicators, consumers and the environment and is suitable for use in integrated pest management programs.

BIOLOGICAL PROPERTIES

Mandipropamid is highly active against spore germination. It also inhibits mycelial growth and sporulation. Mandipropamid is best used as preventive spray against the target diseases but also provides curative activity during the incubation period (Knauf-Beiter & Hermann, 2005).

Mandipropamid has a high affinity to wax layers of plant surfaces. After the spray liquid reaches plant surfaces, the major part of the active ingredient is adsorbed into the wax layer and is fully resistant to wash-off by rain as soon as the spray deposit has dried. A small amount of active ingredient penetrates into the plant tissue. Due to its high intrinsic activity, the amount taken up into the plant tissue is sufficient to stop mycelial growth inside the plant and to protect the opposite leaf surface by translaminar movement (Hermann *et al.*, 2005).

These properties of mandipropamid ensure consistently excellent, long lasting disease control.

FIELD TRIALS

Methodology

Small plot field trials reported here were carried out in areas where strong epidemics of the target diseases could be expected. Some trials were artificially inoculated with the target diseases. Trials were established according to EPPO (European and Mediterranean Plant Protection Organisation) guidelines. Mean percent disease control values reported here were calculated from evaluations made towards the end of the trials, when differentiation between different treatments was most obvious.

Grapes

Mandipropamid applied alone or in mixtures with mancozeb or folpet provides excellent control of downy mildew (*Plasmopara viticola*) in grapes on leaves and bunches. The reliability of disease control is very high, particularly on bunches. This is due to the high affinity of the compound to the wax layer of grape berries. Results from a series of development trials carried out in France, Italy and Spain are summarized in Table 1.

Table 1. Mandipropamid for the control of downy mildew on leaves and bunches of grapes, France, Italy and Spain, 2002-2004

Treatment [1]	Rate [2]	% Efficacy			
		Leaves (12 trials)		Bunches (12 trials)	
		Mean	Min.-Max.	Mean	Min.-Max.
Mandipropamid	15	96.3	91.1-99.5	99.0	93.4-100
Mandipropamid + mancozeb	10 + 120	93.7	88.4-99.4	98.8	94.8-100
Mandipropamid + mancozeb	12.5 + 150	95.1	88.3-98.7	99.5	97.5-100
Dimethomorph + mancozeb	22.5 + 150	93.8	87.6-99.1	98.3	92.7-100
Mancozeb	150	75.1	40.0-95.9	86.0	34.4-99.6
Untreated (% area infected)		(52.1)	(22.0-80.0)	(54.1)	(21.4-97.9)

[1] 6-9 (mean 7.5) applications at 10-12 d intervals (mean 10.7)
[2] application rate g a.i./100 litres

Potatoes

Mandipropamid is highly effective against late blight (*Phytophthora infestans*). Applied alone or in combination with mancozeb or chlorothalonil a high level of protection of foliar infections is achieved even under conditions of very high disease pressure. Results from a series of development trials carried out in France, Germany, Great Britain and Switzerland are summarized in Table 2.

Table 2. Mandipropamid for the control of leaf infections of late blight in potatoes, France, Germany, Great Britain and Switzerland, 2002-2004

Treatment [1]	Rate (g a.i./ha)	% Efficacy on leaves	
		Mean (17 trials)	Min.-Max.
Mandipropamid	100	89.4	68.3-99.6
Mandipropamid	150	94.2	81.2-100
Mandipropamid + mancozeb	100 + 1200	94.1	85.9-100
Mandipropamid + mancozeb	125 + 1500	96.1	88.0-100
Dimethomorph + mancozeb	180 + 1200	83.4	54.8-98.5
Mancozeb	1500	67.7	25.9-92.6
Cyazofamid + adjuvant	80 + adj.[2]	89.6	77.0-100
Untreated (% leaf area infected)		(82.6)	(26.9-100)

[1] 5-11 (mean 7.5) applications at 7-10 d intervals (mean 9.1)
[2] adjuvant 150 ml product/ha

Treatments based on mandipropamid also provide good control of tuber blight caused by *P. infestans*, at least equal to the market standard fluazinam. The results of trials carried out in France, Belgium and The Netherlands are summarized in Table 3.

The absolute level of tuber infection is higher in these trials than would be acceptable in commercial practice. In these trials, foliar infections were deliberately not fully controlled towards the end of the season to be able to differentiate between treatments. Also desiccation of the foliage was later than would be general practice under commercial conditions.

Table 3. Mandipropamid for the control of tuber blight in potatoes,
France, Belgium and The Netherlands, 2004

Treatment [1]	Rate (g a.i./ha)	% Infected tubers [2]	
		Mean (7 trials)	Min.-Max.
Mandipropamid	100	2.7	0.5-8.8
Mandipropamid	150	1.5	0.1-4.4
Mandipropamid + mancozeb	100 + 1200	0.9	0.2-2.8
Mandipropamid + mancozeb	125 + 1500	1.1	0.2-3.0
Dimethomorph + mancozeb	180 + 1200	4.8	0.5-10.5
Mancozeb	1500	9.7	1.9-25.8
Fluazinam	200	2.6	0.2-8.0

[1] 10-13 (mean 11.7) applications at 7-10 d intervals (mean 7.6)
[2] total weight of infected tubers at harvest plus after 1-4 months in storage in relation to the total weight of tubers harvested

Vegetable crops

Results from trials in Indonesia against late blight in tomatoes and downy mildew (*Pseudoperonospora cubensis*) in cucumbers are reported in Tables 4 and 5 respectively.

Table 4. Mandipropamid for the control of late blight in tomatoes, Indonesia, 2002-2004

Treatment [1]	Rate (g a.i./100 litres)	% Efficacy on leaves	
		Mean (9 trials)	Min.-Max.
Mandipropamid	10	87.4	79.3-93.0
Mandipropamid	15	93.2	89.1-97.8
Mandipropamid + chlorothalonil	10 + 100	91.4	83.7-97.4
Dimethomorph + mancozeb	18 + 120	55.0	18.3-86.8
Cyazofamid + adjuvant	8 + adj.[2]	90.0	78.8-96.4
Untreated (% leaf area infected)		(89.4)	(77.3-96.4)

[1] 6-7 (mean 6.7) applications at 7-10 d intervals (mean 7.7)
[2] adjuvant 15 ml product/100 litres

Table 5. Mandipropamid for the control of downy mildew in cucumbers, Indonesia, 2002-2004

Treatment [1]	Rate (g a.i./100 litres)	% Efficacy on leaves	
		Mean (9 trials)	Min.-Max.
Mandipropamid	10	78.7	60.8-95.1
Mandipropamid	15	88.1	73.3-99.4
Mandipropamid + chlorothalonil	10 + 100	81.9	72.8-90.5
Dimethomorph + mancozeb	18 + 120	78.4	62.6-87.2
Cyazofamid + adjuvant	8 + adj.[2]	83.1	53.0-95.2
Untreated (% leaf area infected)		(52.9)	(35.0-69.6)

[1] 4-7 (mean 5.3) applications at 7-10 d intervals (mean 8.0)
[2] adjuvant 15 ml product/100 litres

Mandipropamid-based treatments also provide excellent disease control in tomatoes and cucumbers. Under tropical conditions, frequent rain and intense disease pressure, especially during the wet season, make it difficult to control these diseases effectively at the stretched spray intervals of 7-10 days. In commercial practice in Indonesia, products are usually applied at 4 day intervals.

In addition to results reported here, treatments based on mandipropamid also provide excellent control of a range of other foliar Oomycete pathogens in vegetable crops: *Peronospora parasitica* in brassicas, *Bremia lactucae* in lettuce, *Peronospora effusa* in spinach and *Peronospora destructor* in onions. Mandipropamid also effectively controls foliar infections of *Phytophthora capsici* in peppers and cucurbits. It is recommended to use mandipropamid against this disease in a spray program in conjunction with an effective treatment against soil-borne infections.

CONCLUSIONS

Extensive global field testing since spring 2002 has shown that treatments based on mandipropamid at 100-150 g a.i./ha or 10-15 g a.i./100 litre spray solution provide highly effective and reliable control of most foliar Oomycete pathogens even under adverse weather conditions. Mandipropamid-based treatments provide excellent protection of fruits and tubers. They are most effective when applied as preventive applications and have shown excellent crop safety.

Mandipropamid will be available as a solo formulation and as ready-mix products containing mancozeb, folpet or chlorothalonil depending on market requirements.

To prevent the development of resistance appropriate management strategies will be implemented.

ACKNOWLEDGEMENTS

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The behaviour of mandipropamid on and in plants

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ABSTRACT

Mandipropamid is a new fungicide developed by Syngenta. It is targeted especially against foliar Oomycetes such as: *Phytophthora infestans* on potatoes and tomatoes, *Plasmopara viticola* in grapes and *Pseudoperonospora cubensis* in cucurbits. Following foliar applications on grapes or potatoes, a significant proportion of the applied active ingredient binds immediately to the wax layer of the plant surface where it is stable for an extended period of time. The stability of the surface deposit results in excellent and long-lasting activity under glasshouse and field conditions. The adsorption to the wax layer also protects the active ingredient from being washed off by rain as soon as the surface spray deposit has dried. The excellent rainfastness was confirmed in bioassays with *P. infestans* on potatoes and *P. viticola* on grapes. A small amount of the applied active ingredient is taken up into the host tissue, contributing to curative disease control as well as providing translaminar activity.

INTRODUCTION

Mandipropamid is a new fungicide highly effective against many foliar Oomycete pathogens.

The distribution of mandipropamid following foliar application to grapevine and potato foliage was determined visually and quantitatively and used to interpret results from disease control experiments.

Uptake and subsequent redistribution of mandipropamid was visualised using 14C radiolabelled material and phosphorimage analysis of leaves. Quantitative distribution of mandipropamid between the leaf surface, epicuticular wax layer and foliar tissue following a foliar spray was determined by sequential washing of leaves with water followed by organic solvent prior to the ultimate extraction of foliar tissue to recover absorbed fungicide. Analysis was done by LC-MS.

MATERIALS AND METHODS

Radiolabelled experiments

14C radiolabelled mandipropamid was applied to the adaxial surface of leaves of outdoor grown vine plants as 50 x 0.5 µl droplets of a 250 g per litre SC formulation diluted to give a concentration of 498 mg a.i./litre; droplets were dispersed evenly over the leaf surface. The plants were further maintained under outdoor conditions until the leaves were sampled. Three replicate treated leaves were taken at set timings 1 to 17 DAT and prepared for visualisation of 14C distribution by phosphorimage analysis (Hamaoka 1990) using a Fujifilm BAS-500 imager. A typical example from the variant with sampling 14 DAT is presented.

Unlabelled analytical experiments

Potato plants (cv. Russet Burbank) were grown outdoors in pots to the 6-8 leaf stage. Leaf 4 was sprayed to maximum retention at a rate of 15 g a.i./100 litres using a hand-held sprayer. A sample of each spray solution was retained for analysis to confirm starting concentration. After 1.5 h, ten plants from each treatment were sampled. This procedure was repeated at 3 and 7 DAT.

Each treated leaf was cut from the plant and placed in a polythene tube containing 20 ml of water plus 0.05% Tween 20 to aid wetting. The tube was shaken vigorously for 20 s to remove any unabsorbed compound remaining on the leaf surface and the leaf was then removed and dried before being placed in a tube containing 20 ml chloroform to dissolve epicuticular waxes and recover any fungicide adsorbed. The tube was again shaken for 20 s and then the leaf removed and frozen at -20°C until ready for extraction. Absorbed mandipropamid was extracted from the plant tissue by homogenising in 15 ml of acetonitrile for 20 s using an Ultra-Turrax homogeniser.

Grapevine plants (cv. O'Hanez) were grown outdoors in pots. When 7-8 leaves were present, leaf 5 was sprayed to maximum retention at 15 g a.i./100 litres using a hand-held sprayer. A sample of each spray solution was retained for analysis to confirm starting concentration. After 1.5 h, ten plants from each treatment were sampled. This procedure was repeated 5 and 10 DAT. Sampling and extraction procedures were as described for potato leaves.

For analysis of the water wash, a 350 µl aliquot was removed into a glass hplc vial, 650 µl acetonitrile added, and samples analysed by hplc. Samples from the chloroform wash and the extractions were taken to dryness under an air flow, re-suspended in 65:35 acetonitrile:water and analysed by hplc. Analysis was carried out using a Hewlett Packard HP1100 hplc with a 25 cm Ace5 C18, 3.2 mm i.d. column, a mobile phase of acetonitrile:water 65:35 at a flow rate of 0.5 ml/min and UV detection at a wavelength of 210 nm.

Bioassay for uptake and distribution into grape foliage

This experiment was carried out on glasshouse grown grape seedlings (cv. *Gutedel*) with 5 leaves per plant. Five leaves from different plants were used as replicates. Ten 5 µl droplets, each containing 200, 60, 20 or 6 mg a.i./litre of mandipropamid, were applied to a defined 2 cm segment located in the centre of each leaf. In order to determine the local activity and acropetal or basipetal translocation, the droplets were applied to the abaxial leaf side, and for

determination of translaminar activity to the adaxial side. At 2 DAT, plants were inoculated with *P. viticola* by spraying a spore suspension containing 1.2 x 10^5 sporangia/ml to the abaxial side of the leaves. Plants were incubated in a dew chamber for 6 d at 21/18°C day/night and 100% r.h. until evaluation. Percent infected leaf area was evaluated separately in the treated leaf segment (local activity) or segments towards leaf tip (acropetal activity) or leaf base (basipetal activity). Results represent mean values of 5 replicates and are presented as percent efficacy relative to the disease levels on untreated plants.

Bioassays to determine rainfastness

Grape plants (cv. Riesling x Sylvaner) were grown from grafted rootstocks in containers under outdoor conditions. Before the trial started, they were transferred into a glasshouse and maintained at 18°C until shoots had 8-9 leaves (BBCH 18-19). Plants were sprayed to maximum retention with approximately 40 ml spray per plant containing a 250 g per litre SC formulation of mandipropamid at a concentration of 15 g a.i./100 litres using a spray cabinet with a turntable and air assisted nozzles. Three plants in separate pots were used as replicates in the experiment.

Pre-germinated potato seeds (cv. Bintje) were sown into containers in a mixture of fertilized field soil and a peat/sand substrate and grown for 21 d in the glasshouse at 25/20°C and 50/75% r.h. day/night. Plants were sprayed with mandipropamid 250 SC at a rate of 150 g a.i./ha and a spray volume of 500 litres/ha using a hand-held sprayer. Four pots of 5 litres soil volume each containing 5 potato seeds were used as replicates.

Twenty mm simulated rain was applied over a 15 min period to the plants by an overhead irrigation system equipped with TEEJET XR 11008VK flatfan nozzles 0.1, 1, 3, 6 or 24 h after fungicide application. In addition, a variant without exposure to rain was included ("no rain").

P. viticola inoculum was harvested from infected leaves and 2 DAT each grape plant was inoculated with 10 ml of a suspension containing 1 x 10^5 sporangia/ml using a DeVilbis sprayer. Plants were incubated in a dew chamber for 5 d, followed by 9 d at 20°C and 70% r.h. Percent infected leaf area per plant was evaluated 16 DAT.

P. infestans sporangia were harvested from 10 d old cultures on potato dextrose agar. 200 ml of sporangial suspension containing 20,000 sporangia/ml were applied per container 1 DAT. Plants were covered with plastic bags for 24 h to allow infection. Further incubation continued at 20°C and 70% r.h. for 8 d. Percent infected leaf area per container was evaluated 9 DAT.

Results are based on mean values of 3 (grapes) or 4 (potatoes) replicates, respectively, and are presented as percent efficacy relative to disease levels on untreated plants.

RESULTS

Phosphorimage analysis of vine leaves following application of 14C mandipropamid showed that there was significant local redistribution around the points of absorption into the leaf, but

no acropetal or basipetal translocation was observed as shown in Figure 1 (typical example from 14 DAT variant).
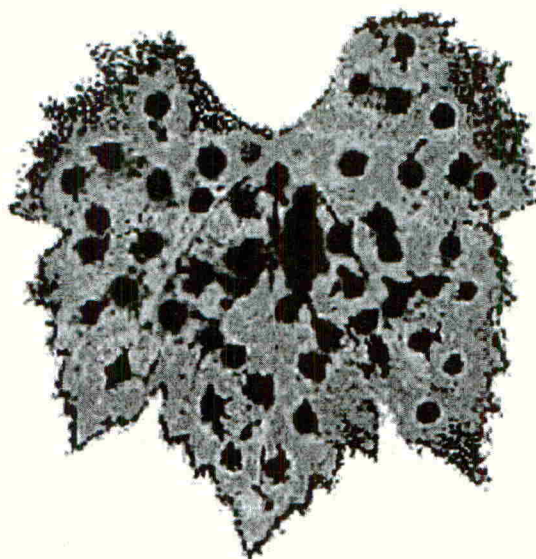

Figure 1.   Behaviour of mandipropamid on vine leaves under field conditions.
              Phosphor image after removal of surface deposit with acetonitrile, 14 DAT.
              Dark spots represent highest concentration of radioactivity

This pattern of local uptake and limited distribution around the application sites was confirmed in a bioassay with application of mandipropamid in a central zone across grape leaves (Table 1). High levels of activity against *P. viticola* were found in the leaf segment around droplet application (local activity), but not in zones above or below, i.e. there was no significant acropetal or basipetal distribution. However, strong translaminar activity was observed on the leaf surface opposite to the application site.

Table 1.   Translocation of mandipropamid in grape leaves as indicated by activity against *P. viticola* (percent efficacy)

mg a.i./litre	Treatment and inoculation on abaxial side of leaf			Treatment on adaxial side, inoculation on abaxial side of leaf: Translaminar activity		
	basipetal	local	acropetal	basipetal	translaminar	acropetal
200	0	100	0	0	100	11
60	0	100	0	0	100	0
20	0	99	0	0	100	0
6	0	98	0	0	95	0

Quantitative recoveries from potato leaves in water, chloroform or by extraction of foliar tissue showed a slow decline over time paralleled by a consequent rise in recoveries from the epicuticular wax and foliar fractions as mandipropamid migrated from the leaf surface into the leaf tissue (Table 2).

Table 2.   Distribution of mandipropamid (as % of recovered) in potato and grape leaves

Fraction	Potato			Grape		
	Sample time after application					
	1.5 h	3 d	7 d	1.5 h	5 d	10 d
(1) Leaf surface (water wash)	61.9	54.9	38.2	67.2	40.2	36.7
(2) Bound on surface and in wax (organic solvent wash)	33.2	36.4	49.0	31.5	58.4	62.1
(3) Leaf extract	4.9	8.7	12.8	1.3	1.4	1.3
Sum of fractions (2) and (3)	38.1	45.1	61.8	32.8	59.8	63.4

Movement of mandipropamid into the wax of potato leaves and its binding on the leaf surface was rapid. At the first assessment, 1.5 h after spraying, 38% of the applied mandipropamid was in the wax, bound on the surface or in the extracted plant tissue. This figure increased to 45% after 3 d and 62% after 7 d. The amount of mandipropamid in the wax had exceeded that on the leaf surface after approximately 6 d.

Similarly, movement of mandipropamid into the wax of grapevine leaves was also rapid. After 1.5 h, 33% of the applied mandipropamid was in the wax, bound on the surface or in the extract. After 3 d, the percentage of mandipropamid in the wax or extract was 60%, with 63% after 10 d. The amount of mandipropamid in the wax had exceeded that on the leaf surface after about 3 d.

Table 3.   Percent efficacy of mandipropamid against *P. viticola* on grapes (15 g a.i./litre) and against *P. infestans* on potatoes (150 g a.i./ha) following exposure of treated plants to simulated rainfall

Pathosystem	Timing of artificial rain (h after spray application)					
	No rain	0.1 h	1 h	3 h	6 h	24 h
P. viticola	100	89	97	100	100	100
P. infestans	99	53	94	95	95	99

Disease levels in check: 100% for *P. viticola*, 91% for *P. infestans*

Mandipropamid demonstrated excellent rainfastness on grape and potato leaves when tested against *P. viticola* and *P. infestans* after exposure to simulated rainfall (Table 3). Only the first timing of simulated rain immediately after application resulted in a significant reduction of fungicidal activity by mandipropamid compared to the efficacy observed on treated, but not rainwashed control plants (variant "no rain" in Table 3). A period without rain of 1 to 3 h after application was sufficient to achieve almost complete disease control.

CONCLUSIONS

Phosphor images of leaves treated with mandipropamid demonstrated some local redistribution of the compound around the application sites and limited uptake. The compound, however, is not significantly transported in basipetal or acropetal directions within the leaves. In a bioassay, it was shown that this local uptake and redistribution provided disease control in a zone of several mm around the application sites of droplets. These experiments also demonstrate excellent translaminar activity of mandipropamid. The uptake and distribution pattern differentiates mandipropamid from contact fungicides like mancozeb and systemic fungicides like phenylamides. The local uptake and redistribution is important to compensate for incomplete coverage of the leaf surface after foliar sprays under practical conditions and contributes to the curative activity observed with mandipropamid (Knauf-Beiter *et al.*, 2005).

Mandipropamid binds rapidly to the leaf surface of potato and grapevine leaves and moves into the wax layer. Consequently, the compound is protected from losses due to wash-off by rain. Efficacy trials with simulated rain demonstrate that the amounts of mandipropamid bound to the leaf surface after 1 to 3 h are sufficient to provide excellent disease control. Under practical conditions, this excellent rainfastness contributes to robust disease control in field trials in potatoes, grapes and vegetables (Huggenberger *et al.*, 2005).

ACKNOWLEDGEMENTS

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Site of action of mandipropamid in the infection cycle of target fungi

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ABSTRACT

Mandipropamid is a new fungicide for the control of foliar Oomycetes developed by Syngenta. The effect of mandipropamid in the infection cycles of target pathogens was intensively examined by light microscopy for *Phytophthora infestans* on potatoes and *Plasmopara viticola* on grapes. The compound was highly effective on cystospore and sporangial direct germination in *in vitro* assays. These effects were confirmed *in planta* following preventive foliar applications. Zoospore release from sporangia was not inhibited. In addition to the effect on cystospore germination, mandipropamid was also effective on mycelial growth and haustoria formation of both pathogens within the host tissue and on sporulation. The strong effect on cystospore germination is consistent with the excellent preventive efficacy of mandipropamid demonstrated in field and glasshouse experiments. The effects on mycelial growth and haustoria formation explain the curative activity observed against *P. viticola* and *P. infestans* when applied during the incubation period of the pathogens.

INTRODUCTION

Mandipropamid is a new fungicide developed by Syngenta for control of Oomycete diseases (Huggenberger *et al.* 2005). It is highly active against the economically important foliar Ooymcete pathogens. Its good persistence on the plant surface (Hermann *et al.* 2005) makes it highly suitable for preventive applications, but it also provides curative activity because of limited uptake into the host tissue. The present paper provides results on the site of action of mandipropamid in the infection cycle of *Phytophthora infestans* and *Plasmopara viticola in vitro*, on leaves and its efficacy after preventive and curative applications under glasshouse conditions.

MATERIAL AND METHODS

Mandipropamid was used as a suspension concentrate containing 25% active ingredient. Foliar applications were sprayed to maximum retention. Concentrations and application timings are indicated in the individual tables of results.

P. infestans was cultivated on rye dextrose agar. For the *in vitro* experiments on sporangial direct germination, the fungus was incubated for 14 d at 18°C in the dark, while for the experiments on zoospore release and cystospore germination the cultures were transferred to 15°C in the dark and used within the next 3 to 5 d. Sporangia were collected by rinsing the

petri dishes with precooled, bidistilled water and were incubated for 2 h at 6°C to induce zoospore release.

The differentiation of sporangia *in vitro* was examined in bidistilled water in mixture with fungicide solutions reaching the final concentrations as indicated in Table 1. Five µl drops of the suspensions were placed on glass microscope slides, covered by a cover slide which was sealed with paraffin for evaluation.

For the *in planta* experiments, potato cuttings, cv. Bintje and grapevine seedlings, cv. Gutedel were cultivated under glasshouse conditions to a 3-6 leaf stage. Sporangia suspensions of *P. infestans* at a density of 5000 sporangia/ml were incubated for 2 h at 6°C prior to inoculation. Sporangial suspensions of *P. viticola* at 5000 sporangia/ml were applied immediately after harvest. Inoculations were carried out on the lower leaf surfaces. The inoculated potatoes were incubated at 18°C and 100% r.h. until evaluation (first 24 h in the dark). For the effect on sporangial direct germination, potato plants were inoculated as described above, but incubated at 25°C and 100% r.h. until evaluation (first 24 h in the dark). The inoculated grapevines were incubated at 20/18°C and 100% r.h. for 5 - 6 d until evaluation.

Microscopy

Potato and grape leaf segments were stained with a droplet of 0.2% Uvitex 2B for observation under an UV microscope with epifluorescence illumination (filter combination 390-420/FT 425/LP 450). To determine the effect of mandipropamid on fungal growth stages within leaf tissue, grape leaf segments were stained with 0.05% aniline blue (Ortega *et al.* 1998). Potato leaf segments were stained in hot trypan blue in lactophenol (0.5%) followed by clearing with chloral hydrate.

For the application timing trials, grafted grapes (cv. Riesling x Sylvaner) were cultivated in the glasshouse at average temperatures of 18°C until growth stage BBCH 18-19. Tomatoes (cv. Roter Gnom) were cultivated in the glasshouse until growth stage BBCH 68-69. Treatments, inoculations and incubations were carried out as described by Hermann *et al.* (2005).

RESULTS

Effects on the cystospore germination *in vitro*

After incubation of sporangia of *P. infestans* in the presence of mandipropamid, the number of empty sporangia after 24 h did not change significantly (Table 1). The slight decrease in the treated samples was not rate dependent and it is assumed that it is not related to the primary site of action. However, a clear dose dependent effect was observed when evaluating the percentage of germinated cystospores which strongly decreased from 82 % in the untreated control to 0.5 % at 0.03 mg a.i./litre.

Table 1. Effect of mandipropamid on zoospore release and cystospore germination of *P. infestans in vitro* (24 h after incubation)

Rate	Sporangia		Cystospores	
(mg a.i./litre)	% empty	% full	% ungerminated	% germinated
0.8	82	18	100	0
0.16	81	19	99.7	0.3
0.03	63	37	99.5	0.5
0.006	71	29	76	24
Check	81	19	18	82

Effects on fungal development *in planta*

Table 2. Preventive effect of mandipropamid on the early infection stages of *P. infestans* on potato leaves

Rate (mg ai/litre)	% empty sporangia	% cystospore germination	% disease control
100	90	0	100
20	95	0.1	95
4	93	0.3	62
0.8	95	39	31
Check	92	88	(95% infection)

When applied preventively (1 d before inoculation), mandipropamid had no influence on zoospore release of *P. infestans* and *P. viticola* from the sporangia even if applied at high rates as indicated by the percentage of empty sporangia (Table 2, Table 3).

Table 3. Preventive effect of mandipropamid on the early infection stages of *P. viticola* on grape leaves

Rate (mg ai/litre)	% empty sporangia	% cystospore germination	% disease control
50	91	0	100
10	94	0.4	99
2	93	57	44
Check	95	99.9	(98% infection)

The inhibition of cystospore germination on the leaf surface showed a close correlation to disease development. At rates between 100 and 20 mg a.i./litre, mandipropamid suppressed cystospore germination of *P. infestans* and controlled disease development completely. Similar results were obtained for *P. viticola*. The high level of disease control at 50 and 10 mg a.i./litre is correlated very well with the 100% inhibition of cystospore germination.

Rates of mandipropamid between 100 and 4 mg a.i./litre inhibited the direct germination of sporangia completely (Table 4). The strong effect of mandipropamid is reflected in the control of disease symptoms.

Table 4. Preventive effect of mandipropamid on sporangial infections
of potatoes by *P. infestans*

Rate (mg a.i.(litre)	% sporangia germination	% disease control
100	0.3	100
20	0.5	100
4	0.5	96
0.8	3	74
check	19	(39% infection)

Following applications 1 to 2 d after inoculation mandipropamid strongly restricted mycelial growth; only a few haustoria were found and sporulation was suppressed. The haustoria of both *P. infestans* and *P. viticola* were also affected. The haustoria of *P. infestans* appeared twisted while the haustorial complex of *P. viticola* showed a strong autofluorescence. The reasons for these observations are unknown at present.

The activity on mycelial growth was evident within a limited time frame after inoculation (Table 5). Mandipropamid effectively stopped growth of *P. infestans* after 1 d curative treatment. It provided 91% control if applied at 250 mg a.i./litre. Treatment with 125 mg a.i./litre inhibited development of the disease by 70%. Mandipropamid was clearly less effective if applied 2 d curatively.

Table 5. Curative activity of mandipropamid against *P. infestans*
on potatoes and *P. viticola* on grapes

Rate	1 d curative	2 d curative
(mg ai/l)	% disease control of *P. infestans*	
250	91	36
125	70	19
62	63	10
31	25	5
Check (% infection)	(97)	(98)
	% disease control of *P. viticola*	
100	100	99
20	68	71
4	4	5
Check (% infection)	(97)	(98)

Against *P. viticola*, mandipropamid showed a better curative timing flexibility. If applied at 100 mg a.i./litre, it was highly effective following 1 and 2 d curative applications.

Application timing (glasshouse)

Mandipropamid (15 g a.i./100 litre), mandipropamid + mancozeb (12.5 + 150 g a.i./100 litre) and cymoxanil + mancozeb (12 + 139.5 g a.i./100 litre) were compared for their activity following preventive and curative applications against *P. viticola* on grapes (Table 6).

Preventive applications with mandipropamid-based treatments and with cymoxanil + mancozeb resulted in complete control. Mandipropamid-based treatments gave almost complete control as 1 d curative treatments slightly superior to cymoxanil + mancozeb. The ranking of the treatments was the same after 2 d curative application, but overall the activity decreased to 95 and 92%. With 3 d curative application, the activities of all products decreased.

Table 6. Preventive and curative activity of mandipropamid-based products against *P. viticola* on grafted grapes

Treatment	Rate	% efficacy			
	(g a.i./100 litre)	1 d preventive	1 d curative	2 d curative	3 d curative
Mandipropamid	15	100	100	95	64
Mandipropamid + mancozeb	12.5 + 50	100	99	93	61
Cymoxanil + mancozeb	12 + 139.5	100	96	93	59

% attack in check 89%

Mandipropamid showed excellent preventive activity against *P. infestans* on tomatoes (Table 7). Mandipropamid (15 g a.i./100 litre), cymoxanil + mancozeb (12 + 139.5 g a.i./100 litre) and cyazofamid (8 g a.i./100 litre) provided similar levels of efficacy. The activity of mandipropamid was similar (93%) if applied 1 or 2 d curatively. The efficacy of cymoxanil + mancozeb decreased to 96 or 76% if applied 1 or 2 d curatively. Cyazofamid did not provide useful curative activity.

Table 7. Preventive and curative activity of mandipropamid against *P. infestans* on tomatoes

Treatment	Rate	% efficacy		
	(g a.i./100 litre)	1 d preventive	1 d curative	2 d curative
Mandipropamid	15	100	93	93
Cymoxanil + mancozeb	12 + 139.5	100	96	76
Cyazofamid	8	97	50	15

% infection in check 42.5%

CONCLUSIONS

The present studies clearly demonstrate that cystospore and sporangial germination is the primary site of action of mandipropamid in the life-cycle of its target pathogens. This is different to many other Oomycete active fungicides such as phenylamides and strobilurins (Staub *et al.*, 1980; Godwin *et al.*, 1998), but is very similar to iprovalicarb (Jende *et al.*, 1999) and benthiavalicarb-isopropyl (Reuveni, 2003).

Application timing studies in the greenhouse demonstrate the excellent preventive activity of mandipropamid which can be explained by its strong effect on cystospore germination thus preventing the penetration of the pathogens into the host tissue. Mandipropamid also provides some curative activity as expressed by the effect on mycelial growth and haustoria formation.

The combination of these effects explains the highly reliable efficacy of mandipropamid-based treatments observed under field conditions (Huggenberger *et al.* 2005).

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The effect of oregano (*Origanum vulgare*) as an alternative soil-borne pathogen control agent, on soil organic matter biodegradation and other soil chemical properties

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ABSTRACT

Essential oils, from aromatic plants such as oregano, have been reported to be efficient in controlling soil-borne pathogens and nematodes and therefore, could be used as pest control agents permitted in organic farming. In the present study, the role of oregano on soil organic matter biodegradation was investigated together with its effect on mineral nitrogen and potassium and on organic phosphorus. Air dried oregano plant tissues at five rates (0, 100, 200, 300 and 400 mg/50 g soil) resulted in increases in mineral nitrogen forms and available potassium, with the higher rates of oregano, whereas organic carbon mineralisation and organic phosphorus were decreased. The addition of oregano at all rates resulted in a decrease in soil bacterial colonies.

INTRODUCTION

Oregano essential oil has been reported to be effective against soil-borne pathogens (Gravanis *et al.*, 2001) and nematodes (Gravanis *et al.*, 2004). Hence, oregano essential oil could be a promising means of controlling these pathogens and parasites in organic farming systems. The aim of this study was to determine if the application of oregano essential oil into the soil had an effect either on soil microflora or on soil chemical properties.

MATERIALS AND METHODS

Commercially, oregano essential oil is distilled from oregano plants. Preliminary experiments in our laboratory (unpublished data) have indicated that the oregano biotype that was used produces 5% essential oil after distillation of air dried plants. For practical reasons, instead of applying the oregano essential oil into the soil, it was decided to apply dry oregano plants that release the volatile essential oil under normal soil temperature conditions.

In this study, 13.3 g of a composting material containing 5 g of organic matter (Table 1), prepared by an organic olive farmer in the Larissa region (central Greece), was added to 50 g of air dried, light textured soil, that was poor in organic matter, but was derived from the same region.

Into 50 g of soil amended with this composting material, 0, 100, 200, 300 & 400 mg of air dried and well-milled oregano were added.

Table 1.   Soil and compost chemical properties

Material	Soil	Compost
Texture	Loamy sand	-
CEC (cmol/kg)	19	-
pH	8.1	7.44
Organic matter (%)	0.5	37.5
$CaCO_3$ (%)	8.6	1.1
Electrical conductivity	0.5	0.503
(1:x water extract) dS/m	(1:5)	(1:10)
N-total (mg/g)	1.54	6.9
P (μg/g)	232.0 (total),	1120.5 (total)
	16.2 (available)	
K	293 μg/g (exchangeable)	28.5 mg/g (total)

These treatments were maintained under two different conditions. The first was kept on the laboratory bench and the second in an incubator. In the incubator, the treatments were prepared in 5 replicates and kept at 28°C for a period of 15 weeks. During the first three weeks of the incubation period, the moisture was maintained at 2/3 of field capacity, but for the next three weeks the soils were left to dry. This process was repeated until the end of the incubation period. This methodology was followed, since Wu & Brookes (2005) reported that the alternation of drying and rewetting soil samples enhances mineralisation of both soil biomass organic matter and non-biomass organic matter.

At the end of the incubation period soil samples were analysed as follows:
- Organic carbon was analysed by chemical oxidation with N $K_2Cr_2O_7$ and titration of the remaining reagent with 0.5 N $FeSO_4$.
- Both ammonium and nitrate nitrogen were estimated by distillation in presence of MgO and Dewarda alloy, respectively.
- Organic phosphorus was measured after mineralisation by combustion of the soil sample and subtraction of the mineral phosphorus amounts, which had previously been estimated in the laboratory. The mineral amounts were extracted with N H_2SO_4 and measured by spectroscopy.
- Exchangeable forms of potassium were extracted with N CH_3COONH_4 in a flame photometer.

The amount of organic carbon mineralised at the end of the incubation period, was calculated by subtraction the contents in organic carbon found at the end of the incubation period, from the corresponding contents in the non-incubated samples that were kept on the laboratory bench. The calculated values of carbon mineralised, were referred to total soil organic matter that incubated as soil organic matter, plus composting material organic matter, plus oregano tissues. The soil organic carbon contents were transformed in organic matter contents by multiplying by 1.724, which is an experimental factor, referred by Hesse (1971).

For statistical analysis the *STATGRAPHICS plus 3.1* statistical package was used.

For investigating the effect of oregano on the soil microflora, a small amount of soil was spread onto PDA plates and incubated for two days at 25°C in darkness. The number of bacterial colonies formed were counted. For statistical analysis the *GenStat* 7th edition statistical package was used to draw a Boxplot graph.

RESULTS

The highest three rates of added oregano resulted in an increase in the pH (Figure 1a). The addition of oregano resulted in a lower decomposition of organic matter. The higher rate of the added oregano (400 mg) resulted in a reduction to 40% of soil organic matter compared to the soil samples without oregano (Figure 1b).

(a)   (b)
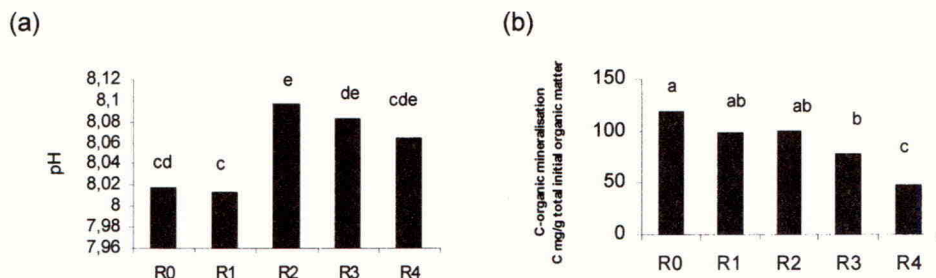
Figure 1.   pH of soil amendments (a), and C-organic mineralised (b) at the end of incubation period. R0, R1, R2, R3, R4: represent oregano rates 0, 100, 200, 300 & 400 mg, added in 50 g of soil, respectively. Columns with the same letter do not differ significantly according to LSD test (*P*=0.05).

(a)

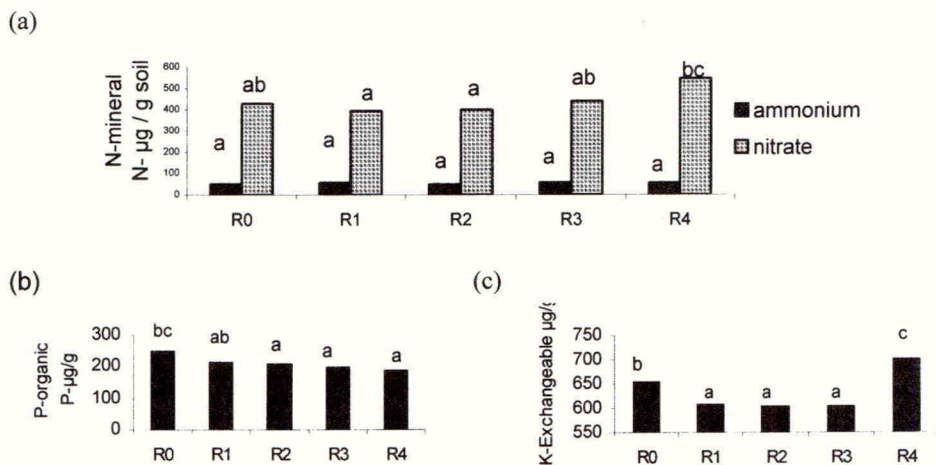(b)   (c)

Figure 2.   N-forms, at the end of the incubation period (a), P-organic (b) and K-exchangeable forms values (c), at the end of the incubation period.

to be higher with the high dose of oregano than the low dose and the untreated, although it was only significantly different from the lowest dose at the end of the incubation period (Figure 2a).

All rates of the added oregano decreased the organic phosphorus content (Figure 2b). The level of exchangeable forms of potassium was increased only at the highest rate of the added oregano (Figure 2c).

The addition of oregano resulted in significantly lower number of bacterial colonies that emerged on the PDA plates (Figure 3).
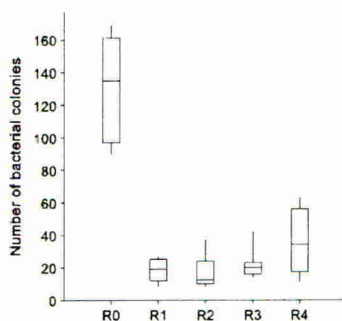
Figure 3.   Box plot for comparison of bacteria colonies emerged on PDA plates

CONCLUSIONS

The reduction of the number of the bacterial colonies formed, agrees with the work of Gravanis *et al.* (2001, 2004) who reported that oregano essential oil had a direct inhibitory effect on soil micro-organisms. Also the reduction of bacterial colonies following the addition of oregano indicates an indirect inhibitory effect on the soil, agreeing with the Chouliaras & Jacquin (1976) who have reported that a reduction of soil organic matter is related to soil microflora activity.

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Potential of phylloplane bacteria in the biological control of grey blight disease of tea (*Camellia sinensis*)

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ABSTRACT

Attempts were made to isolate and select efficient bacterial strains from the tea phylloplane. A total of 889 bacteria were isolated from young tea shoot samples collected from various tea districts of southern India. They were screened and 16 efficient strains were selected. Further selection, based on their ability to produce various cell wall lytic enzymes, reduced the number of interesting strains to six. Spore germination and glasshouse studies indicated that at least two of these strains were potential antagonists, which can be used as bio-control agents against grey blight pathogen *Pestalotiopsis theae*.

INTRODUCTION

Grey blight is an important foliar disease of tea (*Camellia sinensis*). The disease is caused by *Pestalotiopsis theae*. The disease has been reported from all the major tea growing countries of the world. The disease was known to exist in north-east India since 1872. The pathogen infects mature foliage, bare stalks and young shoots of the mature bushes. In the nursery, it causes stalk rot of vegetatively propagated plants. The crop loss accrued due to grey blight is estimated to be 17% during peak seasons. In the field and in the nursery, the disease is controlled by spraying either mancozeb or carbendazim. Increasingly, there is a desire to develop more ecofriendly methods of disease control. The success of phylloplane bacteria as biocontrol agents against foliar diseases is available (Jayapalgowdu & Balasubramanian, 1988). Phylloplane microbes of tea were screened for antagonism against brown blight disease and found that *Micrococcus luteus* is highly antagonistic (Chakraborty *et al.*, 1998). Commercial strains of *Trichoderma* and *Pseudomonas* were evaluated for their efficacy to control grey blight disease, but they were found to be ineffective (Sanjay, 2005). Hence attempts were made to isolate tea phylloplane bacteria and select effective strains for use as biocontrol agents in the control of this disease.

MATERIALS AND METHODS

Isolation of phylloplane bacteria

Phylloplane bacteria were isolated by a leaf washing technique. Pluckable shoots were collected from healthy tea bushes from different agro climatic zones of southern India. Two to three shoots (three leaves and a bud) were immersed in 50 ml of sterile distilled water and kept in a shaker at 150 rev/min for 15 min. The leaf washings thus obtained were serially diluted up to 10^{-8} dilutions and 0.1 ml from 10^{-5} to 10^{-8} dilutions was spread-plated on nutrient agar medium. The plates were incubated at room temperature for three days. The colonies that

appeared on the plates were selected, sub-cultured and purified. The purified isolates were screened for their antagonistic potential. The selected strains were identified as per Bergy's manual (Claus & Berkeley, 1986; Palleroni, 1984).

Screening for antagonism

In vitro screening was performed by dual plate assay. Mycelial plugs cut from a 4 day old PDA culture of *Pestalotiopsis theae* were macerated with a sterile spatula and spread uniformly on PDA plates, which were then spot-inoculated with 24 h old cultures of the bacterial strains. Four bacterial isolates at a time were screened in each plate. The plates were incubated at 30 ± 2 ºC for 3 days. Grading of antagonism was made by noting the zone of inhibition produced around the bacteria *viz* '-' no antagonism,'+' inhibition zone of < 0.5 cm, '++' zone 0.5 cm to 1.0 cm and '+++' zone >1.0 cm.

Production of cell wall lytic enzymes

Mineral salts medium (MSM) with various carbon sources was used to detect the production of cell wall lytic enzymes by the selected bacterial strains. The substrates used as carbon sources were chitin, casein, skim milk, starch, carboxy methyl cellulose and pectin. They were individually added in the MSM at a concentration of 0.1%, autoclaved and the molten cooled media were poured into sterile petri dishes. The bacterial strains were streaked on the plates and incubated at 30 ± 2 ºC for 3 days and observed for their growth. Utilization of the substrates was designated by '+' and '-'. Mycelium of *P. theae* was also tested as the sole carbon source.

Effect on spore germination

Pestalotiopsis spores were mixed with filter sterilized culture filtrate of the selected strains of phylloplane bacteria so as to have the final concentration of 1×10^{-5} spores/ml. The spore suspensions were incubated at 25ºC at 100% r.h. in cavity slides. The germination was represented on a percentage basis.

Glasshouse experiment

The potential of the selected bacterial strains for the control of *Pestalotiopsis* had been tested in the nursery. The plants were sprayed with 2-day old liquid culture of the respective bacterial strains. The plants (%) infected by *Pestalotiopsis* were noted on six month old plants. The fungicide mancozeb was kept as the standard treatment for comparison.

RESULTS AND DISCUSSION

Isolation and screening of phylloplane bacteria

A total of 889 bacterial strains were isolated and established as pure cultures. Among the isolates 21 were fluorescent pseudomonads. Testing of the bacterial strains for antagonism by dual plate assay revealed that among the other 868 strains 42 strains exhibited a zone of inhibition of + category, 24 of ++ and 16 of +++ category and the remainder showed no antagonism. All the 21 strains of fluorescent pseudomonads were mild in their antagonism to

Pestalotiopsis. The 16 strains which exhibited higher antagonism and which were selected for further studies were identified as Bacillus spp.

Production of cell wall lytic enzymes

The bacterial strains showed a wide variation in their substrate utilization. Among the 16 strains, only three (WPB 102, 104 and 109) were able to utilize all the test carbon sources and three others (APB 78, CPB 77, CPB 126) were able to utilize both chitin and pectin (Table 1). Based on this spectrum of substrate utilization, six strains were selected for testing as potential bio-control agents. The carbon source utilization ability of strains WPB 102 and WPB 104 were comparable to the type culture Bacillus subtilis.

Table 1. Substrate utilization by the selected bacterial strains

Strain	Substrates						
	Chitin	Casein	Skim milk	Starch	CMC	Pectin	Pestalotia mycelium
APB 78	+	+	-	-	-	+	+
CPB77	+	-	-	-	-	+	+
CPB126	+	-	-	-	-	+	+
TRB 3	-	-	-	-	-	-	+
ARB 9	-	+	+	-	-	-	+
MPB 94	-	+	-	-	-	+	+
MPB130	-	+	-	+	-	+	+
MPB136	-	+	+	-	+	+	+
MPB138	-	+	-	-	-	+	+
MPB139	-	+	+	-	-	+	+
MPB157	-	+	-	-	-	+	+
WPB5	-	-	-	-	-	-	+
WPB102	+	+	+	+	+	+	+
WPB 104	+	+	+	+	+	+	+
WPB109	+	+	+	+	+	+	+
NLB 12	+	+	+	+	+	+	+
B. sublitis	+	+	+	+	+	+	+
P. fluorescens	-	+	+	-	-	-	-

+ Utilized   - Not utilized

The selected bacterial strains were capable of suppressing the spore germination of Pestalotiopsis (Table 2). This could be due to the production of inhibitory substances by the bacteria. Suppression of spore germination is the first step in the biological control of fungal pathogens by bacterial antagonists.

Table 2. Effect of culture filtrate on the germination
of *Pestalotiopsis* spores (after 24 h)

Strain	Spore germination (%)
APB 78	14.6
CPB 77	17.3
MPB138	18.1
WPB104	12.2
WPB109	17.8
NLB 12	16.5
BS	17.0
PF	19.8
Control (Glucose sol.)	26.3

Biocontrol potential

Under glasshouse conditions, APB 78 and WPB 104 provided excellent control of the disease
(Table 3). The type cultures (BS- *Bacillus subtilis* and PF- *Pseudomonas fluorescens*) failed to
protect the plants from the disease satisfactorily. This further confirms the superiority of the
indigenous strains.

Table 3. Potential of selected bacteria on the control
of *Pestalotiopsis* on nursery plants

Strain	Infected plants (%)
APB 78	17.0
CPB 77	40.7
MPB138	41.7
WPB104	18.9
WPB109	40.7
NLB 12	30.5
BS	45.0
PF	45.5
Standard (mancozeb)	10.0
Control (untreated)	50.0

Bacterial biocontrol agents are advantageous due to their rapid growth rate, aggressive
colonization, easy handling and better survival. In the present study, the majority of the strains
obtained were gram positives, and they were most efficient in both the dual plate assay and in
producing lytic enzymes. By contrast, none of the 21 isolated fluorescent pseudomonads was
efficient in suppressing the growth of the pathogen. Similar observation was made by
Jeyalakshmi *et al*. (1998). In their studies, they screened several fungal and bacterial

antagonists against the fruit rot and dieback pathogen of chilly, *Colletotrichum capsici* and found that among the bacterial antagonists *B. subtilis* was superior to *P. fluorescens* in controlling the pathogen *in vitro* as well as pot culture experiments. Fluorescent pseudomonads are usually present in higher number in the soil as well as on phylloplane of most of crops. They are also are good biocontrol agents (Saxena *et al.*, 2000).

Production of lytic enzymes such as chitinases and ß-1,3-glucanases by certain forms of bacteria is the basis of biological control of some plant pathogens (Saxena *et al.*, 2000). In the lytic enzyme assay, the strains WPB 102, WPB 104 and WPB 109 utilized all the substrates tested. This indicated that they were capable of producing an array of lytic enzymes, which is the main mechanism of control in bacterial biocontrol agents. Moreover, these lytic enzymes would suppress the fungal growth at the stage of spore germination rather than in advanced stages (Jayapalgowdu & Balasubramanian 1988). These data indicated that the antagonists, which produce more lytic enzymes, do suppress the disease incidence to the maximum extent. Further, all the selected strains were *Bacillus* spp. having the added advantage of producing endospores. Endospores are tolerant to adverse conditions, allowing the organisms to survive well on the phylloplane. It has already been reported that *B. subtilis* isolated from tea phylloplane effectively controlled black rot disease of tea caused by *Corticium invisum* Barthakur *et al.* (1993). Recently, Ji & Wilson (2003) reported that the efficacy as well as population size of bacterial biocontrol agents could be increased on the phylloplane by supplementing with carbon and nitrogen sources. Experiments are underway to improve the field performance of the selected bacterial strains by improving their efficacy and population.

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Control of spoilage and ochratoxin A (OTA) production in moist grain for animal feed using the biocontrol agent *Pichia anomala*

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ABSTRACT

Pichia anomala has been found to reduce mould growth and ochratoxin A (OTA) production. The major hurdle in production of commercial biocontrol agents (BCAs) has been the lack of production of appropriate formulations. Of particular importance is the conservation of viability and ecological competence after application. With this in mind, studies were conducted to develop formulations of *P. anomala* which would have these attributes. Studies with fluidised bed-drying examined several additives for conservation of viability and showed that cotton seed flour + skimmed milk was the best treatment. Yeast cell osmoprotection was also employed. The biocontrol efficacy of formulated *P. anomala* cells was tested at the laboratory scale and results showed that they inhibited mould growth and OTA production. Formulation additives were found to have no adverse effect on mould growth and OTA production. Furthermore, modified yeast cells with increased levels of trehalose and arabitol gave similar efficacy as fresh cells. A subsequent pilot scale study, using malfunctioning airtight silos containing moist grain, showed that addition of fresh cells or formulated *P. anomala* cells both effectively controlled *Penicillium roqueforti*.

INTRODUCTION

For animal feed, airtight storage of the cereal grain in silos is an alternative energy-saving method compared to high temperature drying of temperate cereals. However, temporal fluctuation of carbon dioxide and oxygen levels do occur and this eventually leads to heavy mould growth and poor quality animal feed grain. *Pichia anomala* has been frequently isolated from airtight-stored grains and Björnberg & Schnürer (1993) first showed that strain J121 effectively reduced growth of *Penicillium roqueforti in vitro* in a dose-dependent manner. *P. anomala* was also shown to reduce ochratoxin A (OTA) accumulation in co-culture with *P. verrucosum*.

The major obstacle in the commercialisation of Biological Control Agent (BCA) products is the development of a shelf-life-stable formulated product that retains efficacy similar to that of fresh BCA cells. BCAs are living organisms and their economic production process, formulation, distribution and application are of great importance and require special considerations. Drying microorganisms enables preservation of the inoculum over a long period of time, maintaining high viability, and does not require cool temperatures during storage and distribution.

Fluidised bed-drying has been extensively used to manufacture active dry yeast on a large scale (Bayrock & Ingledew, 1997). Hallsworth & Magan (1995) showed that elevated concentrations of the dissacharide trehalose, in response to osmotic stress, in conidia of entomopathogenic fungi prolonged shelf-life. Mokiou & Magan (2002) showed that *P. anomala* cells intracellularly accumulated trehalose when exposed to water stress by addition of proline and NaCl to molasses media.

Ochratoxin A (OTA) is a class 2b carcinogen levels of which are controlled by EU legislation in a range of food raw materials and products, including cereals (Magan & Olsen, 2004). The origin of OTA in cool and temperate climates is generally attributed to *Penicillium verrucosum*, whereas in warm temperate and tropical zones, it is now commonly associated with *Aspergillus ochraceus* and the black aspergilli. Recent studies have identified the water and temperature requirements for growth and OTA production by *P. verrucosum* (Cairns-Fuller *et al.*, 2005). The objective of this study was to investigate the potential for controlling spoilage and ochratoxin A (OTA) production in moist grain for animal feed using ecologically competent formulations of the biocontrol agent *P. anomala*. Appropriate yeast formulations were produced by using the fluidised bed-drier after manipulating the endogenous solutes and using several additives and isotonic solutions.

MATERIALS AND METHODS

Microorganism, fermentation media and extraction of polyols and sugars

The microorganism used in this study was *Pichia anomala* (strain J121). Cane molasses-based medium made of cane molasses (40 g/litre) and urea (1.2 g/litre). Molasses was kindly supplied by UdL-IRTA, Lleida, Spain; pH 6.1/a_w 0.993-0.996. Modification of media a_w (0.98 and 0.96 levels) was made by the addition of proline. Extraction of polyols and sugars was done according to Mokiou & Magan (2002).

Fluidised bed-drying

P. anomala cells were washed twice in HPLC grade water or in NaCl 0.98 a_w isotonic solution and centrifuged. Carriers [corn meal (CM), cottonseed flour (CSF), and wheat starch (WS)] were added at a proportion 1:1 (w/w, *P. anomala* cells/carrier) and adjuvants (skimmed milk for CSF and WS and glycerol for CM) at 10% (w/w); the mixtures resulting were introduced in fluidised bed-dryer 350s (Burkard Manufacturing Co. Ltd, Hertfordshire, UK) and dried at 50°C for 20 min. Viability was assessed using the viablue stain.

Bioassays

Irradiated wheat grain with retained germinative capacity was modified by the addition of Sterile Distilled Water (SDW) to achieve 0.96 and 0.93 a_w levels. Inoculation concentration of 1 x 10^3 *P. verrucosum* (strain OTA11) and 1 x 10^5 Colony Forming Units (CFUs)/g grain of *P. anomala* were used. The biocontrol yeast and the mould were inoculated individually or in co-cultures. Grain in Petri dishes (3 replicates per treatment) were sampled after 1, 10, 20, 30 days. Treatments were kept with with isotonic glycerol solutions in plastic containers to maintain the required relative humidity at 25°C. At each sampling time, 1g of grain + 9 ml of SDW + Tween 20 + agar were added were shaken for 2 min. 0.1 ml of this and appropriate

serial dilutions were spread-plated on Nutrient Yeast Dextrose Agar (NYDA) modified to 0.93 a_w with PEG 200. Growth of the BCA and *P. verrucosum* was recorded as CFU/g. The rest of the sample was extracted for OTA.

Ochratoxin A (OTA) extraction and quantification

Extraction was in methanol for 24 h. cleaned up and analysed for OTA as detailed by Cairns-Fuller *et al.* (2005).

Statistical analysis

Data were analysed using Genstat software (Genstat 5th edition). Analysis of Variance (ANOVA Table) was used to compare different treatments. Percentage data was LOGIT transformed prior to statistical analysis. Statistical significance was judged at the $P<0.05$ level.

RESULTS

Intracellular sugar and polyol accumulation in *P. anomala* cells grown in molasses media

Modification of a_w of molasses medium by adding proline, and thus imposing *P. anomala* cells to different water stress levels (0.98 and 0.96 a_w) resulted in a change in accumulation/synthesis of sugars and sugar alcohols. In the unmodified molasses media, trehalose was found to be the predominant intracellular compatible solute followed by arabitol. In proline/0.98 a_w treatment, trehalose was found to be strongly intracellarly accumulated (61.24 mg/g f.w.) followed by arabitol (31.85 mg/g f.w.). Cells from proline 0.96 a_w treatment also accumulated high quantities of trehalose (48.36 mg/g f.w.) followed by arabitol (29.68 mg/g f.w.); however, glycerol was mostly synthesized (21.05 mg/g f.w.).

Fluidised bed drying of *P. anomala* cells with use of several additives and isotonic solutions

Figure 1 shows the effect of additives used as carriers (100% or 1:1 w/w) such as corn meal (CM), cottonseed flour (CSF), and wheat starch (WS) alone or with adjuvants (10% skimmed milk for CSF and WS and glycerol for CM) on *P. anomala* cell viability after drying at 50°C for 20 min using a fluidized bed-dryer. Viability of *P. anomala* formulation treatments significantly differed from the control treatment (unformulated cells). Significant differences between treatments were also noted. Of all the treatments checked, CSF:1+10%SM (w/w, additives/fresh cells) resulted in significantly increased final cell viability. Addition of several adjuvants to carriers resulted in increased or similar cell viability. Final cell moisture content of CSF:1+10%SM treatment was 4.3% (data not shown). Washing yeast cells with NaCl isotonic solution (osmoprotection) prior to drying resulted in the retention of the high intracellular accumulation of endogenous solutes. Results showed that there was a significant difference on cell viability among the different formulations, but no significant differences between water and isotonic washing treatments (data not shown).
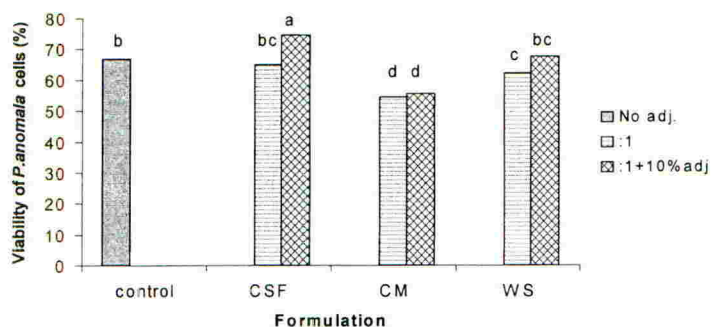
Figure 2. Effect of additives used as carriers (100% or 1:1 w/w) such as corn meal (CM), cottonseed flour (CSF), and wheat starch (WS) alone or with adjuvants (10% skimmed milk for CSF and WS and glycerol for CM) on *P. anomala* cell viability after drying at 50°C for 20 min using a fluidized bed-dryer. Control is untreated *P. anomala* cells. Different letters indicate statistical differences (P<0.05) between means.

Spoilage and Ochratoxin A production in moist grain

Effect of *P. anomala* on *P. verrucosum* growth in γ-irradiated wheat grain (0.93 a_w levels) at 25°C for 1, 10, 20 and 30 days is shown on Figure. 2. Mould growth was a result of 3 x 3 significant (P<0.05) interaction between different treatments used; wheat grain, a_w and time. 2 x 2 interactions and the main effects of each factor were also significant. *P. verrucosum* alone reached 4.5 x 10^6 CFUs/g at 0.93 a_w after 30 days starting from 1 x 10^3 CFUs/g one day after inoculation. When co-cultured with *P. anomala*, it reached a level of 3.2-3.75 x 10^5 CFUs/g with no statistical differences between the different treatments. However, there were statistical differences between treatments after 10 days. OTA accumulation was a result of a three-way significant (P<0.05) interaction between the different treatments used; wheat grain, a_w and time. Two- and one-way interactions were also significant. OTA was significantly accumulated after 30 days of inoculation for all treatments. When *P. verrucosum* grew alone, OTA accumulated after 30 days of inoculation to 28700 μg/kg. Co-culture with *P. anomala* resulted in similar OTA levels with C treatment, or significantly decreased in A, B and D.

DISCUSSION

This study is the first laboratory-scale investigation on biocontrol potential of formulated *P. anomala* cells against spoilage and OTA production in most grain. Manipulation of yeast cells prior to drying by enhanced endogenous solutes accumulation and use of additives and isotonic solutions was also shown to be important. *P. anomala* cells grown in molasses medium, which is cheap and readily available, accumulated trehalose in high amounts (Mokiou & Magan, 2002). When *P. anomala* cells were grown in molasses media modified with proline to 0.98 a_w, the amount of intracellularly accumulated trehalose was significantly increased. The disaccharide trehalose acts as a membrane-protecting agent for yeast cells during environmental stress conditions such as heat treatment, dehydration and freezing.
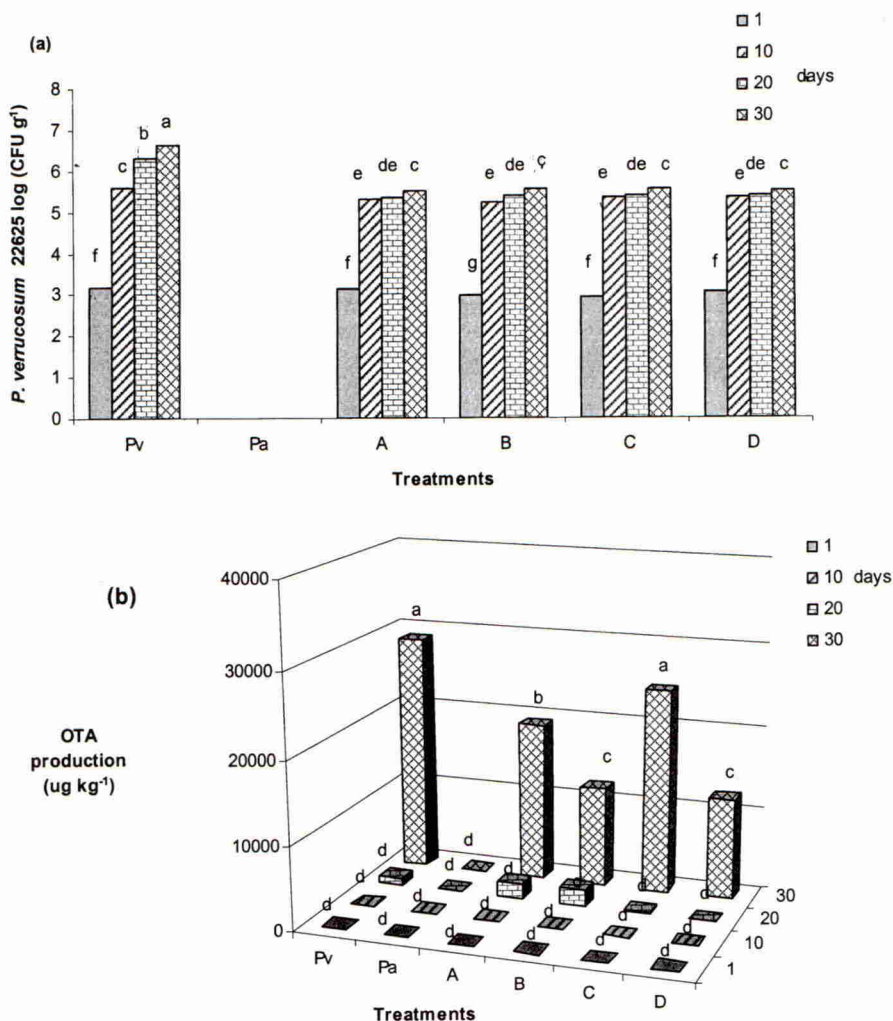
Figure 2. Effect of *P. anomala* on the growth/sporulation of *P. verrucosum* measured as (a) CFUs and (b) ochratoxin A (OTA), μg/kg, in co-cultures on wheat grain [0.93 a_w] at 25°C for up to 30 days. Different treatments were *P. verrucosum* alone (control), *P. anomala* unmodified fresh cells alone, *P. anomala* proline modified fresh cells washed with NaCl isotonic solution (A), *P. anomala* unmodified cells dried as cottonseed flour: 1 + 10% skimmed milk formulation (B), *P. anomala* proline modified cells dried as cottonseed flour:1 + 10% skimmed milk formulation washed with water (C), *P. anomala* proline modified cells dried as cottonseed flour: 1 + 10% skimmed milk formulation washed with NaCl isotonic solution (D). A, B, C, D treatments were co-cultures with *P. verrucosum*. Different letters indicate statistical (P<0.05) differences between means.

Biosynthesis and accumulation of trehalose by yeast cells correlates with increased survival following dehydration or freezing (Beker & Rapoport, 1987). The proposed mechanism of

desiccation protection by trehalose is known as the "water replacement hypothesis", whereby trehalose replaces water molecules in the membranes and forms hydrogen bonds with the phospholipids, thus preventing collapse of the membrane upon water removal (Crowe et al., 1987).

P. anomala cells reduced populations of P. verrucosum with no significant differences between treatments after 30 days of co-culture in wheat. Some differences were noticed after 10 days, possibly due to better yeast colonisation and growth on wheat during the early stages in the bioassay. A decrease in mould populations coincided with a decrease in OTA production. Osmoprotected dried cells reduced OTA production. The isotonic solution itself had an influence on OTA production but had no differential influence on fungal populations. Use of sterile grain might have had an effect on ochratoxin production. In this study, it was also shown that formulation additives had no adverse effect on mould growth and OTA production. Pilot scale study, using malfunctioning airtight silos where moist grain was stored, showed that addition of fresh paste and formulated P. anomala J121 cells effectively controlled P. roqueforti with no significant difference in mould biocontrol between them (Druverfors et al., 2005).

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