ECOFRIENDLY SYNERGISTS FOR INSECTICIDE FORMULATIONS (ECOSYN): *IN VITRO* CHARACTERISATION OF INTERACTIONS WITH DETOXIFYING ENZYMES.

UNIVERSITÀ CATTOLICA del Sacro Cuore

APRES LABS

MICHELA PANINI*, GRAHAM MOORES#

* Università Cattolica del Sacro Cuore, Piacenza (Italy); michela.panini@unicatt.it

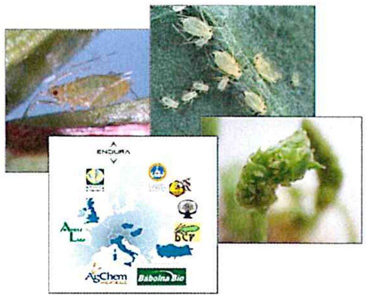
ApresLabs Ltd, Harpenden, Herts (UK); graham.moores@ApresLabs.co.uk

OVERVIEW

Synergists are non-toxic compounds that enhance the efficacy of insecticides. They inhibit enzyme system(s) of insects that metabolise or sequester insecticide molecules; as a result, insect sensitivity increases with the possibility to overcome or delay metabolic resistance conferred by increased levels of detoxifying enzymes.

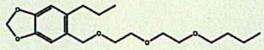
Piperonyl butoxide (PBO) is a well-known synergist capable of interacting with phase 1 metabolic enzymes, esterases and P450s. A structure activity relationship (SAR) was conducted with around 30 PBO analogues against FE4 and CYP6CY3 from resistant *Myzus persicae,* to identify key chemical moieties responsible for interactions against these enzymes, and synthesise new potent inhibitors.

Similar studies have been performed with *Bemisia tabaci* and can be applied to any pest insect where the enzymes responsible for resistance have been identified.



ESTERASES

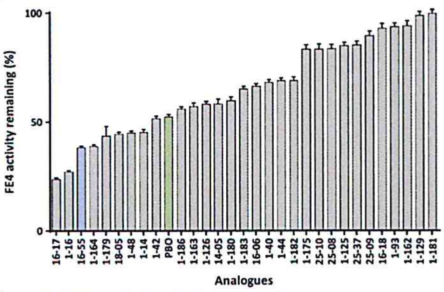
Resistant-associated FE4 esterase was purified from aphids supplied by Università Cattolica del Sacro Cuore, Department of Sustainable Crop production (Piacenza, Italy).

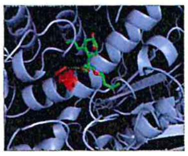


Piperonyl butoxide (PBO)

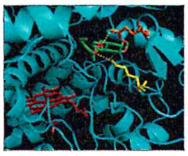
P450S

The recombinant P450 CYP6CY3 was provided by the Biological Chemistry and Crop Protection Department, Rothamsted Research (Harpenden, UK).

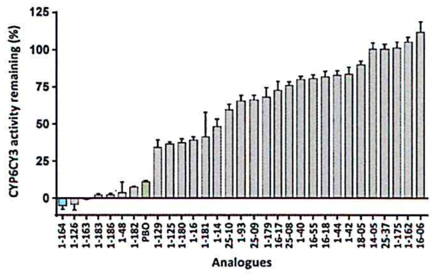




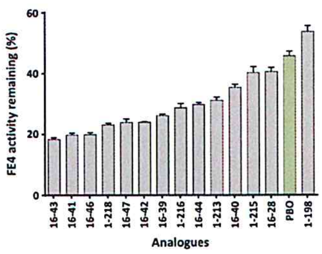
In silico model of PBO docked with FE4. Hydrogen bonds (dotted yellow lines) between PBO (green) and Arg-412 (red). PBO is placed just within the gorge of FE4, as was found previously with E4 (Philippou *et al.* 2011).

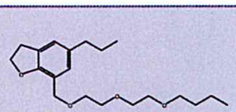


In silico model of PBO docked with CYP6CY3. Hydrogen bonds (dotted yellow lines) between PBO (green) and Gln-84 (orange), Lys-406 (yellow).



Enzyme inhibition studies, together with *in silico* modelling, identified the key regions of the synergists which were optimised to confer greater inhibition.





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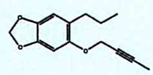
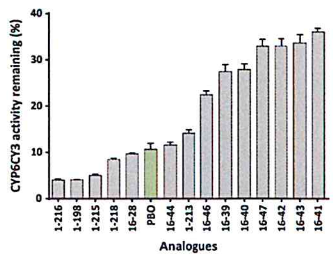
Analogue 16-55

On the basis of the observed results against esterase activity, putative novel analogue structures were then proposed starting from analogue 16-55.

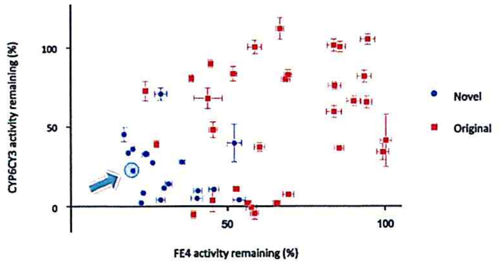


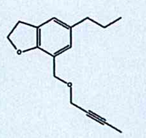
Analogue 1-164

On the basis of the observed results against oxidase activity, putative novel analogue structures were then proposed starting from analogue 1-164.



Suggestions of effective novel analogue structures were made and, together with constraints of synthetic pathways and commercial possibilities, novel analogues were synthesised. Upon receipt of the novel analogues, the programme of inhibition studies was repeated, thus creating a true iterative process.





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Analogue 16-46

A bi-variate plot shows that novel synergists synthesised as a result (blue) were predominantly more effective inhibitors.

ACKNOWLEDGEMENTS

The original and novel analogues were provided by Endura SpA (Bologna, Italy).

* Patent pending

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 605740

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Ecofriendly synergists for insecticide formulations (EcoSyn): *in vivo* evaluation of novel synergists against resistant pests

Introduction

A list of possible novel synergists was selected on the basis of *in vitro* assays and considering also costs and difficulties of chemical synthesis. The well known synergist piperonyl-butoxide (PBO) was included as a standard reference. Different combinations of pests, synergists, insecticides, application rates and types of bioassays were used.

Materials and Methods

Resistant *Myzus persicae* and *Bemisia tabaci* were collected in Northern Italy after insecticide applications field failures . *Meligethes aeneus* resistant population was collected in Poland whilst a susceptible one was collected in Northern Italy.
Laboratory bioassays were performed combining new synergists with commercial formulations of two pyrethroid (alpha-cypermethrin and tau-fluvalinate) and two neonicotinoid (imidacloprid and thiacloprid) insecticides.

Results and Discussion

In almost every case the mortality achieved applying the insecticide plus synergist was significantly higher than the mortality produced by the insecticide alone. Synergists used alone gave low mortality level comparable with untreated control.
In several cases performance improvements, in comparison with PBO, have been recorded and highest synergistic ratios were measured.

Poster authors

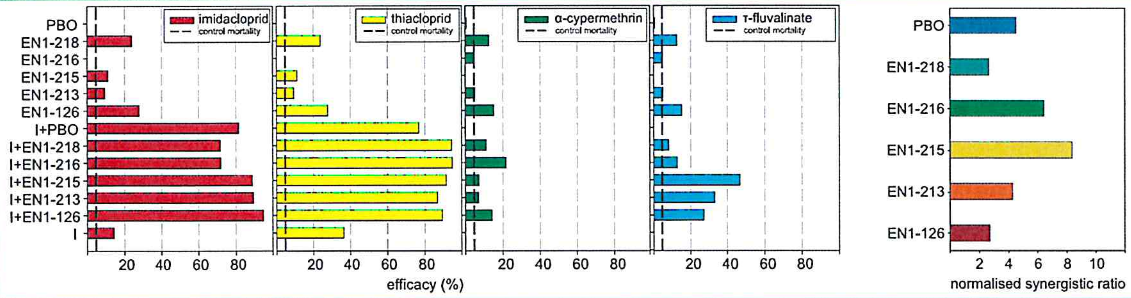
Emanuele Mazzoni, Matteo Anaclerio, Vincenzo Puggioni, Olga Chiesa, Valeria Todeschini, Michela Panini

Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy

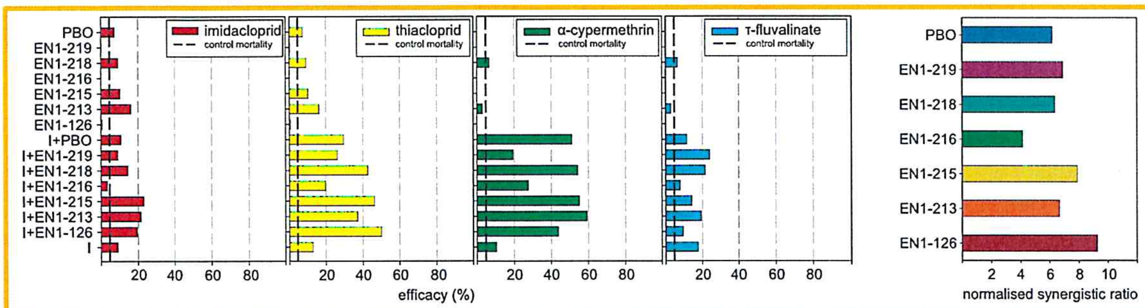
Corresponding author:
emanuele.mazzoni@unicatt.it

Acknowledgements

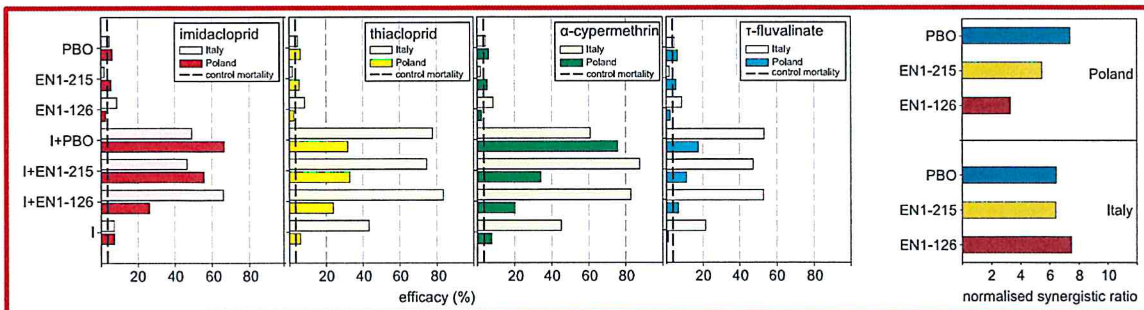
The authors are grateful to Pawe Węgorek, Joanna Zamojska and colleagues (Institute of Plant Protection, National Research Institute, Poznań, Poland) for providing resistant pollen beetle population.



Myzus persicae



Bemisia tabaci



Meligethes aeneus

Resistance 2015 - Rothamsted Research - 14th-16th September 2015

JÁNOS SZILÁGYI#, JÓZSEF SCHMIDT#, ZITA KÖVÉR#, LASZLÓ TAKÁCS#, MATTEO ANACLERIO*, VINCENZO PUGGIONI*, OLGA CHIESA*, MICHELA PANINI*, VALERIA TODESCHINI*, EMANUELE MAZZONI*

Bábolna Környezetbiológiai Központ Kft, Budapest, Hungary
*Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, Italy
szilagyi.janos@babolna-bio.com emanuele.mazzoni@unicatt.it

UNIVERSITÀ CATTOLICA del Sacro Cuore

Ecofriendly synergists for insecticide formulations (EcoSyn): *in vivo* and *in vitro* evaluation of novel synergists against *Musca domestica*

Introduction

In vivo assays were carried out to evaluate the performance against *Musca domestica* L. (Diptera, Muscidae) adults of novel potential synergists in combination with a pyrethroid insecticide.
The inhibitory effect of these new compounds was evaluated biochemically against enzymes involved in degradation of xenobiotics (oxidases and GSTs).

Materials and Methods

Bioassays
Contact tests using a glass surface sprayed with a commercial formulation of cypermethrin alone or in combination with the new synergists.
Biochemical analysis
Total activity and inhibition was determined using 7-ethoxycoumarin (7EC) for oxidases and the 1-chloro-2,4-dinitrobenzene (CDNB) for GSTs.

Results

Bioassays
All synergists, assayed *in vivo* against a moderately resistant population (Hun_A), showed a high level of efficacy and EN1-126 achieved the best performance (Figure 1).

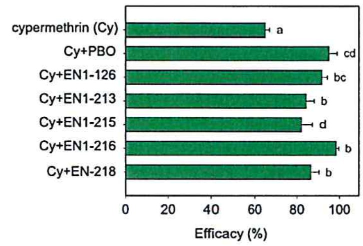


Figure 1 Efficacy (%; Abbott) of cypermethrin and cypermethrin plus synergists in residual bioassays.

Biochemical analysis
The Hun_A and Hun_B populations showed similar oxidase and GST activities, always higher than those of the susceptible reference strain S-WHO (Figure 2). PBO and novel synergists (except "EN16-55") significantly inhibited activity of oxidases. On the contrary only "EN16-55" inhibited GST activity (Figure 3).

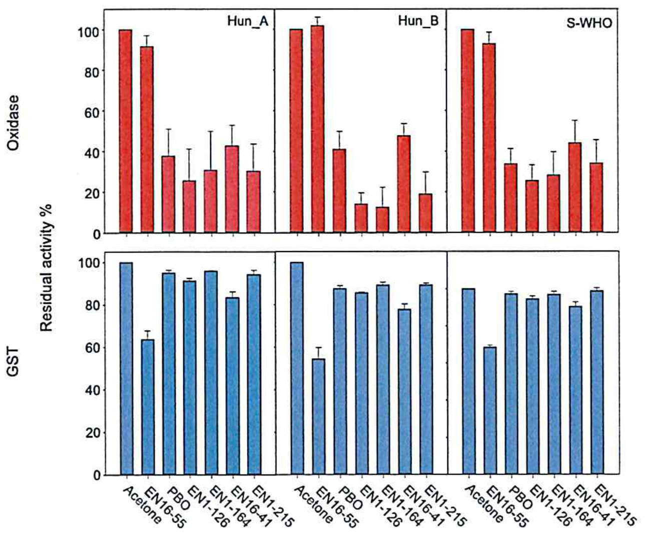


Figure 3 Biochemical analysis: A) total oxidase (red) and GST (blue) activities; B) oxidase (red) and GST (blue) inhibition assays.

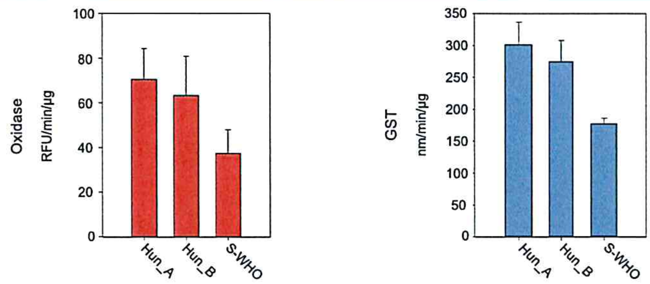


Figure 2 Biochemical analysis: total oxidase (red) and GST (blue) activities.

Discussion

Novel synergists increased significantly the efficacy of cypermethrin demonstrating the possibility to have product performing better than PBO. Different structures showed specificity towards different detoxyfication systems.

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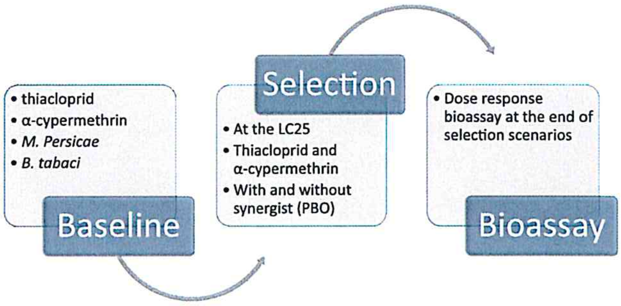
Ecofriendly synergists for insecticide formulations (EcoSyn): Evaluating the potential of synergist/insecticide applications to select for resistance compared with application of insecticide alone

Workflow



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Rothamsted Research Ltd
Harpenden (United Kingdom)

Università Cattolica del Sacro Cuore
Piacenza (Italy)

Materials and Methods

- Resistant *Myzus persicae* samples were collected in Italian orchards 2012-2013 after control failures with neonicotinoids/pyrethroids.
- A mixed field strain of *Bemisia tabaci* was collected in Crete.
- Bioassays were performed ahead and after insecticide selection using a leaf-dip bioassay. Pea seedlings were used for aphids and cotton leafs for whiteflies.
- The insecticide selection was carried out at the LC25 obtained from the baseline susceptibility.

M. persicae was selected for 12 generation whereas *B. tabaci* was selected for 5 generations.
The selection scenarios were:
> α-cypermethrin / α-cypermethrin + PBO (1 g L-1 for *Myzus* and 0.1 g L-1 for *Bemisia*)
> Thiacloprid / thiacloprid + PBO (1 g L-1 for *Myzus* and 0.1 g L-1 for *Bemisia*)

Results

M. persicae

- All four selection regimes resulted in an increase of resistance (see. Fig. 1 & 2).
- PBO did not prevent the development of resistance in any given regime, however, selection with thiacloprid+PBO and α-cypermethrin+PBO resulted in lower levels of resistance than selection with insecticide alone.

B. tabaci

- All four selection regimes resulted in a significant increase of resistance (see. Fig. 3 & 4).
- Selection with thiacloprid resulted in a greater shift in dose-response (>100 fold) compared to α-cypermethrin.
- PBO did not prevent the development of resistance in any given regime, however, however, selection with α-cypermethrin+PBO resulted in lower levels of resistance than selection with α-cypermethrin alone.

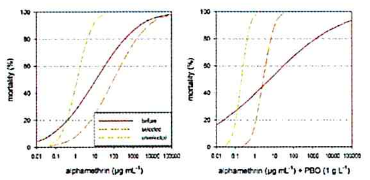

Fig. 1: Dose response curves for *M. persicae* against α-cypermethrin after selection with α-cypermethrin (left) or α-cypermethrin + PBO (right).

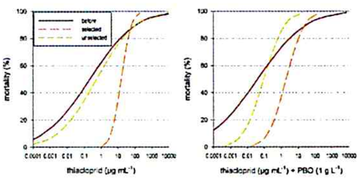

Fig. 2: Dose response curves for *M. persicae* against thiacloprid after selection with thiacloprid (left) or thiacloprid + PBO (right).

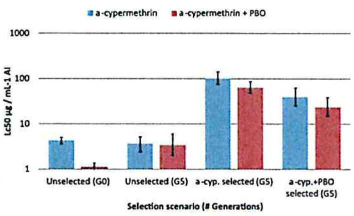

Fig. 3: LC50 values for *B. tabaci* after different selection scenarios using α-cypermethrin with and without PBO.

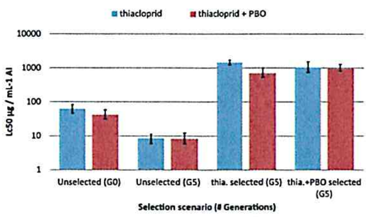

Fig. 4: LC50 values for *B. tabaci* after different selection scenarios using thiacloprid with and without PBO.

Poster authors

C.T. Zimmer[1]*, M. Panini[2], E. Roditakis[3], L.M. Field[1], E. Mazzoni[2], C. Bass[1]

[1]Rothamsted Research, Harpenden, Herts., UK

[2]Università Cattolica del Sacro Cuore, Italy

[3]Plant Protection Institute of Heraklion, Irákleion, Greece

*Corresponding author: christoph.zimmer@rothamsted.ac.uk

UNIVERSITÀ CATTOLICA del Sacro Cuore

ROTHAMSTED RESEARCH

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 605740

Ecofriendly synergists for insecticide formulations (EcoSyn): Improvements in the efficacy of pyrethroids in controlling pyrethroid-resistant pollen beetles, *Meligethes aeneus*, in spring oilseed rape

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Dewar Crop Protection Ltd
Bury St. Edmunds (United Kingdom)

Rothamsted Research Ltd
Harpenden (United Kingdom)

ApresLabs Ltd
Harpenden (United Kingdom)

Pangaea Agrochemicals
Cambridge (United Kingdom)

Introduction

The pollen beetle, *Meligethes aeneus*, has developed widespread metabolic resistance to most pyrethroid insecticides. Field studies were set up in 2014 and 2015 to explore the possibility that use of synergists, such as piperonyl butoxide (PBO), in conjunction with synthetic pyrethroids, could help overcome this resistance in pollen beetles in oilseed rape.

Materials and Methods

- Field trials were conducted in spring sown oilseed rape in 2014 and 2015 on a farm near Hatfield Heath, in Essex. Plots (6 x 6 m) were sprayed with treatments using a back pack sprayer delivering 200 L/ha through O2 nozzles at 2 bar pressure.
- Assessments of pollen beetle numbers were made 1, 3 and 6 days after application on 20 racemes (florets) per plot.
- In 2015 plots were harvested to determine effects of treatments on yield.

Results (2014)

- In 2014, there were 8 pollen beetles per raceme 1DAA.
- A PBO-based formulation (at 0.4 L/ha; Endura, Italy), a novel synergist EN1-126 (at 0.4 L/ha) and alpha-cypermethrin (applied as Fastac at 0.2 L/ha) had no effect on beetle numbers (Fig. 1).
- Tau-fluvalinate (applied as Mavrik at 0.2 L/ha) and pymetrozine (applied as Plenum at 0.15 kg/ha) gave significant reductions (74-75%).
- The addition of PBO and EN1-126 to alpha-cypermethrin gave significant improvement in control, to the level of pymetrozine.
- 4 DAA the crop was fully flowering, resulting in rapid re-infestation of plots; there were no effects of any treatment on beetle numbers.

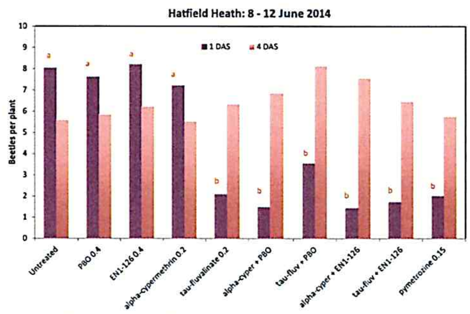

Fig. 1. Effect of pyrethroids with or without synergists on pollen beetles in spring oilseed rape 2014. On 1 DAS, columns with different letters are significantly different from each other at P<0.05 There were no significant differences 4 DAS

Results (2015)

- In 2015, there were 10 pollen beetles per raceme in untreated plots 1DAS.
- Lambda-cyhalothrin (applied as Hallmark Zeon at 0.075 L/ha) and alpha-cypermethrin (applied as Fastac at 0.2 L/ha) gave poor (17-41%) but significant control of pollen beetles 1 and 3 DAS, but not 6 DAS (Fig 2).
- The addition of Pangaea's proprietary PBO formulation to both pyrethroids improved control significantly 1 and 3 DAS, but only alpha-cypermethrin was enhanced 6 DAS.
- Pymetrozine (applied as Plenum at 0.15 kg/ha) gave excellent control (94-80%) throughout the assessment period.
- There was a substantial effect of the better treatments on the duration of flowering within the plots, and subsequent development of seed pods.
- This is likely to have had a significant effect on yield at harvest.

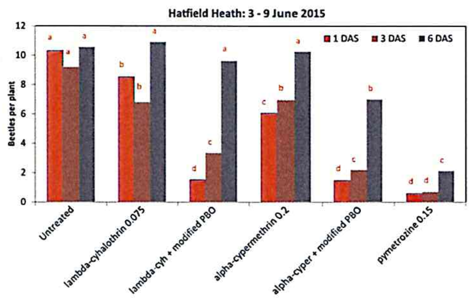

Fig. 2. Effect of pyrethroids with or without a synergist on pollen beetles in spring oilseed rape 2015. On the same date, columns with different letters are significantly different from each other at P<0.05

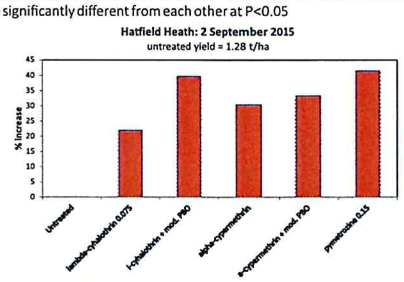

Fig. 3 Effect of pyrethroids with or without synergists on yield of spring oilseed rape 2015

Effects of pollen beetles on flowering of oilseed rape: pollen beetles feeding on oilseed rape buds

Blind stalks on oilseed rape flower due to pollen beetle attack

Hatfield Heath 2015
The yellow plots were where best control of pollen beetles was recorded

Discussion

- Addition of synergists PBO or EN1-126 to the pyrethroids alpha-cypermethrin and lambda-cyhalothrin can overcome the resistance of pollen beetles to these insecticides.
- The persistence of the effect does not last long for this pest (<6 days), but it is long enough for the crop to produce a viable yield.

Poster authors

Alan M. Dewar*, Graham Moores#, Michael J. Gifford, Mark Johnston****
* Dewar Crop Protection Ltd, Bury St Edmunds, UK
# ApresLabs, Research and Innovation Campus, Rothamsted, Harpenden, UK
**Pangaea Agrochemicals, Cambridge UK
Corresponding author:
alan@dewarcropprotection.ac.uk

Resistance 2015 - Rothamsted Research - 14th - 16th September 2015