# POSTER SESSION P5D

# POST-GRADUATE STUDENT POSTERS

Session Organiser: Professor Phil E Russell
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Poster Papers: P5D-1 to P5D-13

# Amino acid alterations in CYP51 contribute toward reduced triazole sensitivities in a UK field population of *Mycosphaerella graminicola*

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## ABSTRACT

*Mycosphaerella graminicola* is the causal agent of Septoria leaf blotch (SLB), an important foliar disease of wheat in Europe. Due to a lack of durable host resistance, and recent widespread emergence of resistance to strobilurin (QoI) fungicides, SLB control is largely dependent on triazoles; the efficacy of which has declined over the last five years. We measured the $EC_{50}$ values of isolates sampled from triazole-treated and untreated field plots in 2004 alongside a set of historical isolates. There were no significant differences in mean sensitivities of isolates from the 2004 plots; however the majority of isolates showed highly reduced sensitivities compared to the historical isolates. PCR-based assays developed to identify alterations in the target-encoding *CYP51* gene associated with reduced triazole sensitivity were used to further characterise the population of *M. graminicola* isolates. Results demonstrate that alterations commonly occur in combination and are widespread among the 2004 population but not among historical isolates, suggesting they contribute to the reduction in sensitivity.

## INTRODUCTION

*Mycosphaerella graminicola* is the most economically important foliar wheat pathogen in the UK (Hardwick *et al.* 2001). Due to a lack of durable host resistance disease control relies predominantly on the use of fungicides. Resistance to QoI fungicides was discovered in 2002 and is now widespread (Fraaije *et al.* 2003), consequently increasing reliance on triazoles. Concern over the possible development of resistance to triazoles prompted studies which have revealed a reduction in efficacy, particularly over the last five years (HGCA 2005). Four mechanisms of triazole resistance have been characterised in plant and human pathogenic fungi: reduced intracellular accumulation due to increased efflux pump activity, increased expression of the target CYP51 protein, modification of the ergosterol biosynthesis pathway and amino acid alterations in CYP51. We studied the presence of two CYP51 alterations in *M. graminicola* isolated from epoxiconazole-treated and untreated plots: a two amino acid deletion, of tyrosine and glycine, from codons 459 and 460, respectively (Cools *et al.* 2005), and a novel isoleucine to valine substitution at codon 381 (Cools, unpublished data). The equivalent codon to 381 in the structurally defined *Mycobacterium tuberculosis* CYP51 (L321) has been shown to exist within 4 Å of a heme-bound fluconazole molecule (Podust *et al.* 2001). In this study we have analysed a population of *M. graminicola* from treated and untreated plots. We report the widespread emergence of the two CYP51 alterations in the 2004 *M. graminicola* population and suggest this contributes toward the overall reduction in sensitivity to triazole fungicides.

## MATERIALS AND METHODS

### Sensitivity testing

ADAS experimental field site, Terrington, Norfolk, with eight plots of winter wheat (*Triticum aestivum* cv. Consort), four treated; with four quarter doses of Opus (active ingredient epoxiconazole, recommended dose 125 $gha^{-1}$), and four untreated, was sampled for *M. graminicola*. A total of 256 isolates were taken from individual lesions on leaf two. These were examined alongside nine historical reference isolates. Epoxiconazole sensitivities were tested with a method adapted from Pijls *et al.* (1994). Spore suspensions were made by growing each isolate on Czapek Dox plus 0.5% peptone extract agar, at 15°C, for six days. Spores were harvested and amended to a concentration of $2.5x10^5$ spores/ml. 100 µl of this solution was added to 100 µl double strength liquid Czapek Dox medium with 2% yeast extract in a 96 well microtitre plate amended with 12 final epoxiconazole concentrations (50µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.56 µg/ml, 0.78 µg/ml, 0.39 µg/ml, 0.195 µg/ml, 0.098 µg/ml, 0.049 µg/ml and 0 µg/ml). Plates were incubated in the dark at 23°C for 72 hours, after which optical densities at 630nm were taken with a Dynex MRX plate reader. Replicate readings were used to fit dose response regression curves and the $EC_{50}$ value (fungicide concentration at which 50% of growth is inhibited) of each isolate calculated.

### Detection of CYP51 alterations

PCR based assays (primers shown in Table 1) were developed to identify a point mutation and a six base pair deletion encoding a substitution (I381V) and a two amino acid deletion (ΔY459/G460), respectively. Spore suspensions of isolates for which an $EC_{50}$ value had been determined, were boiled at 110 °C for ten minutes and used as template DNA. A region of *CYP51* encompassing both alterations was amplified from each isolate using the primers F3BF and CYSTR (at 0.5µM) under the following conditions: 94°C for 2.30 min, followed by 40 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1.30 min and a final extension at 72°C for 9 min. A dilution (at $2x10^{-4}$) of this primary PCR product was used as a DNA template for both the following assays. To identify the presence of I381V a nested PCR reaction using the primers CYSTF and BF381REV (at 0.5µM) was carried out under the following conditions: 94°C for 2.30 min, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min and a final extension at 72°C for 9 min. The point mutation resulting in I381V introduces an additional BsrI recognition site and was identified when 2 µl PCR product was cut with 1 unit of BsrI at 65°C for two hours. Isolates containing the ΔY459/G460 deletion were distinguished with an allele-specific PCR assay with the forward primer DEL-T and the reverse primer UNIREV (at 0.2µM) under the following conditions: 94°C for 2.30 min, followed by 40 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30s and a final extension at 72°C for 4.30 min.

Table 1. Primer sequences used to identify mutations in CYP51 (3' - 5')

| Primer name | Sequence 3'-5' |
|---|---|
| F3BF | GTC ACA AGC AGA AGG CGT GCA GAT |
| CYSTF | GTT GCC ACG CGT GTC CCA C |
| BF381REV | CAA TGG AGG CAG TCG GGA AAG TGC |
| CYSTR | CCA CTT YAC TAC TGC CGG CGA |
| DEL-T | GAA AGA AGA CTA CGG CCT $T_a$ |
| UNIREV | CTC CCT CCT CTC CCA CT |

*a: allele specific nucleotide*

## RESULTS

Nine historical isolates were tested for epoxiconazole sensitivities and CYP51 alterations (Table 2). Results demonstrate the mean $EC_{50}$ was 0.058 µg/ml epoxiconazole. None of the isolates had alteration I381V but two of the more recent isolates have ΔY459/G460.

Table 2. Epoxiconazole sensitivities and CYP51 alterations in historical isolates.

| Isolate | Year | Location | $EC_{50}$ epoxiconazole (µg/ml) | ΔY459/G460 | I381V |
|---|---|---|---|---|---|
| ST1 | 1973 | Unknown | 0.025 | No | No |
| ST7 | 1973 | Hertfordshire | 0.056 | No | No |
| IPO323 | 1981 | Holland | 0.004 | No | No |
| S27 | 1993 | Somerset | 0.065 | No | No |
| ST16 | 1995 | Somerset | 0.040 | No | No |
| L952 | 1995 | Hertfordshire | 0.057 | Unknown | No |
| CTRL2 | 2001 | Hertfordshire | 0.100 | Yes | No |
| TWIST-B | 2002 | Hertfordshire | 0.080 | Yes | No |
| LARS6 | 2003 | Somerset | 0.062 | No | No |

Isolates obtained from ADAS plots were assessed for epoxiconazole sensitivities, 68 from untreated plots, and 97 from epoxiconazole treated plots (Fig.1). Analysis showed no significant differences in mean epoxiconazole sensitivities of *M. graminicola* from treated or untreated plots, and an overall mean $EC_{50}$ of 0.26 µg/ml. The 2004 population contained isolates with highly reduced sensitivities, including one with a 30 fold reduction when compared to the historical wild-type mean.

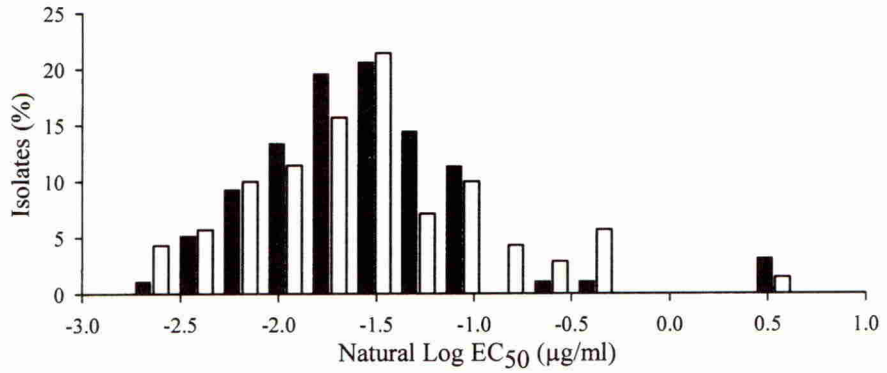


Figure 1. Distribution of $EC_{50}$ values of isolates obtained from epoxiconazole treated and untreated plots (black treated/ white untreated).

Table 2. $EC_{50}$ of Terrington isolates to epoxiconazole (µg/ml) and alterations in CYP51

| $EC_{50}$ (µg/ml) | Resistance Factor (compared to wt) | Number of Isolates | Isolates with ΔY459/G460 | Isolates with I381V | Isolates with combination |
|---|---|---|---|---|---|
| <0.1 | <2 | 2 | 1 | 1 | 1 |
| 0.1-0.3 | 2-5 | 39 | 23 | 25 | 21 |
| >0.3 | >5 (max=30) | 9 | 5 | 9 | 5 |

Fifty randomly selected Terrington isolates were assessed using PCR-based assays (Table 2). There was no evidence that treatment induced the selection of isolates with I381V or

ΔY459/G460 over the 2004 season. The majority of isolates with ΔY459/G460 also carried substitution I381V (26 out of 29). The presence of I381V or ΔY459/G460 was not significantly correlated with an increase in $EC_{50}$. There were large numbers of 2004 isolates with alterations compared to the historical isolates.

## DISCUSSION

We analysed $EC_{50}$ values of a population of *M. graminicola* from an experimental ADAS field trial in Terrington, Norfolk, alongside a set of historical isolates. No direct selection for isolates with reduced triazole sensitivities was evident over the 2004 season, but the mean $EC_{50}$ was 4.5 fold that of the historical isolates, suggesting an overall reduction in sensitivity. PCR-based assays were used to screen isolates and identify two amino acid alterations in CYP51: ΔY459/G460 and I381V. A reduction in sensitivity was not directly correlated with CYP51 alterations; however, results show that I381V and ΔY459/G460, while not common in the historical isolates, are widespread among the 2004 population. This may contribute to the overall reduction in sensitivity. Previous work with *Mycobacterium tuberculosis* showed the equivalent codon to 381 lies within 4 Å of heme-bound fluconazole, indicating that a change at this amino acid may alter affinity to azoles. A lack of any homologous region encompassing Y459/G460 in the structurally-defined *M. tuberculosis* CYP51 orthologue prevents prediction of the effect of this mutation on azole affinity but interestingly 94% of the Terrington isolates with ΔY459/G460 also had I381V. In *C. albicans* CYP51 alterations often occur in combination, exerting a greater impact on sensitivity than single mutations alone.

## ACKNOWLEDGEMENTS

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## REFERENCES

Cools H J; Lucas J A; Fraaije B A (2005). Molecular examination of *Septoria tritici* isolates with reduced sensitivities to triazoles. *Proceedings 14th International Reinhardsbrunn Symposium on Modern Fungicides and Antifungal Compounds.*

Fraaije B A; Lucas J A; Clark W S; Burnett F J (2003). QoI resistance development in populations of cereal pathogens in the UK. *Proceedings of the BCPC Conference - Crop Science & Technology.*

Hardwick N V; Jones D R; Slough J E (2001). Factors affecting diseases of winter wheat in England and Wales, 1989-98. *Plant Pathology*. **50:** 453-462.

HGCA (2005). *Wheat Disease Management Guide – 2005 Update*: London, UK, Home-Grown Cereals Authority.

Pijls C F N; Shaw M W; Parker A (1994). A rapid test to evaluate *in vitro* sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. *Plant Pathology*. **43:** 726-732.

Podust L M; Poulos T L; Waterman M R (2001). Crystal structure of cytochrome P450 14α-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *PNAS*. **98:** 3068-3073.

# Resistance to *Leptosphaeria maculans* in *Brassica napus* (oilseed rape) leaves (phoma leaf spot) and stems (canker)

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## ABSTRACT

The number of phoma leaf spot lesions formed in controlled environment experiments 8-21 days post inoculation with ascospores of *Leptosphaeria maculans* was consistently greater on winter oilseed rape cv. Darmor than on cv. Eurol. Similarly in winter oilseed rape field experiments in the 2003-2004 (Dec-Mar) and 2004-2005 (Oct-Dec) growing seasons cv. Darmor often had more lesions than cv. Eurol, but later in both seasons Eurol had more leaf lesions than Darmor. Eurol also developed more severe phoma stem cankers than Darmor at the end of both seasons. There was no difference in leaf size or leaf number between cultivars. No avirulence genes corresponding to resistance genes in these cultivars have been found in UK *L. maculans* populations.

## INTRODUCTION

Phoma stem canker is the most important disease of oilseed rape world-wide. Ascospores of the causal agent, *Leptosphaeria maculans*, infect the leaves of winter oilseed rape in autumn, causing leaf lesions. From these lesions the pathogen grows without symptoms down the petiole to reach the stem where, in summer, it causes stem cankers and resulting yield loss (West *et al.*, 2001). Cultivars of winter oilseed rape show a range of resistance/susceptibility to *L. maculans,* expressed both at the leaf stage and the stem stage. The resistance at the stem stage is measured by scoring stem canker in field plots in summer. Resistance at the leaf stage is scored by cotyledon reaction to inoculation with ascospores or conidia of *L. maculans*. This paper describes the development of leaf lesions in field and controlled environment conditions in two cultivars with differing 'field' resistance to *L. maculans* (assessed pre-harvest).

## MATERIALS AND METHODS

### Controlled environment experiments

*L. maculans* ascospore inoculum was obtained from naturally infected winter oilseed rape stem base debris collected after harvest at Rothamsted, UK and stored dry at -20°C until required. Plants were grown with one plant per pot (5cm diameter) and placed in seed trays. Plants were grown in a controlled environment cabinet ($15^0$C, 12 h light/12 h darkness, light intensity 210 μe $m^{-2}s^{-1}$) until each plant had two fully expanded leaves, with the third leaf just starting to expand (GS 1,3; Sylvester-Bradley & Makepeace, 1985). To inoculate plants with ascospores, six 2-3cm long pieces of stem base debris bearing mature pseudothecia were chosen at random and evenly attached to the underside of a tray lid with Vaseline (Chesebrough-Pond's Ltd, London). The pieces of stem were sprayed with distilled water

until run-off to induce release of ascospores and the lids with attached pseudothecia were placed over the trays with plants. The pieces of debris were removed after 2h; the inoculated plants were sprayed with distilled water and covered for 48h. Lesions were counted daily on 7 plants per cultivar, starting 8 days post inoculation. The experiment was repeated three times.

**Field experiments**

Plants were inoculated in autumn with crop debris from the previous season to provide a high ascospore inoculum concentration. Phoma leaf spot lesions were counted in the field on ten plants per plot of winter oilseed rape cvs Darmor (resistant) and Eurol (susceptible) over two growing seasons (2003-2004 and 2004-2005). There were three replicate plots of each cultivar arranged in randomised block design as part of a larger experiment with 42 cultivars. Lesion counts were done approximately weekly throughout the growing season. Basal stem lesions were assessed from late spring, every 2 weeks until harvest on a 0-4 scale (0 - no lesion, 1 - small lesion, 2 - large lesion, 3 - complete girdling of the stem, 4 - broken stem; Zhou *et al.*, 1999).

## RESULTS

In controlled environment conditions cv. Darmor developed more lesions than cv. Eurol in all three replicate experiments (Figure 1). In the winter oilseed rape field experiment cv. Eurol developed more phoma leaf spot lesions than cv. Darmor in both seasons, after a period (Dec/Mar 2003-2004 and Oct/Nov 2004-2005) when there was little difference between cultivars and Darmor often had more lesions than Eurol (Figure 2). Cultivars Eurol and Darmor had similar leaf areas and numbers of leaves in both controlled environment and field experiments, so this factor did not contribute to differences between these cultivars. Stem lesions in the field (Figure 2) were more severe on cv. Eurol in both seasons. Although cv. Darmor has the resistance gene *Rlm9* and cv. Eurol has *Rlm2*, there is no avirulence to these genes in UK *L. maculans* populations.

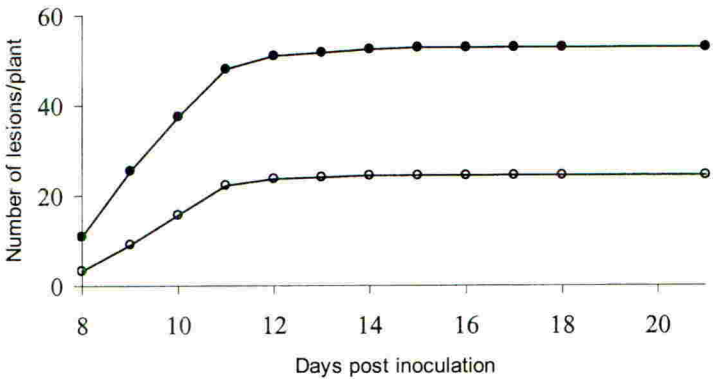


Figure 1. Numbers of phoma leaf spot lesions per plant on winter oilseed rape cultivars Eurol (o) and Darmor (●) 8-21 days post inoculation of plants (GS 1,3) with ascospores of *Leptosphaeria maculans* in controlled environments (15°C, alternating 12h light/12h dark). Each point represents the mean of 21 plants (SED=8.6, df=150)

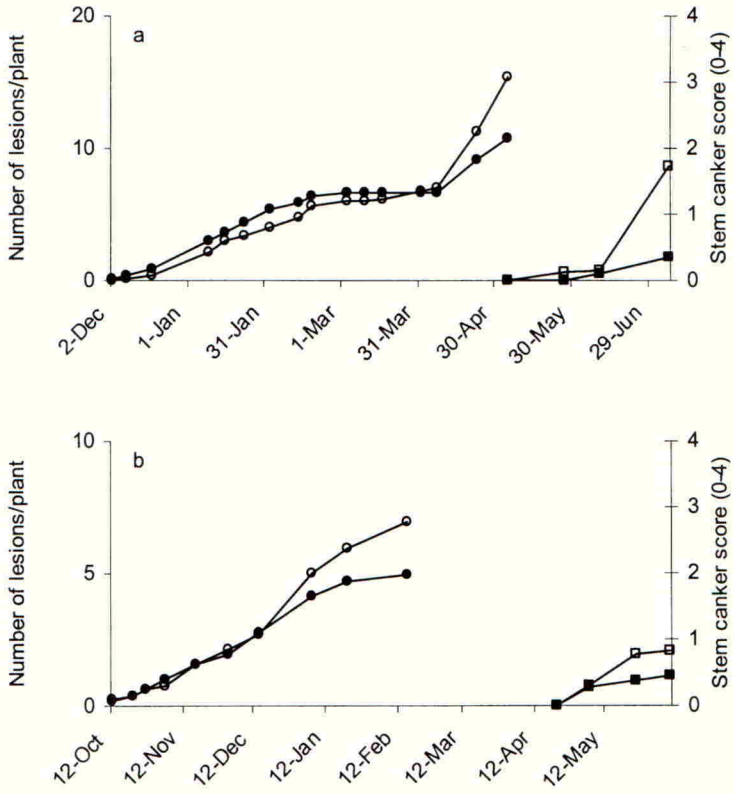


Figure 2. Cumulative numbers of phoma leaf spot lesions per plant on winter oilseed rape in the field at Rothamsted counted weekly from first lesion appearance in autumn until spring (○, Eurol; ●, Darmor) and mean stem canker scores for the same plants counted every 2 weeks until harvest (□, Eurol; ■, Darmor) on a scale of 0-4 (0, no lesion; 1, small lesion; 2, large lesion; 3, complete girdling of the stem; 4, broken stem; Zhou *et al.*, 1999). a: 2003/2004 season b: 2004/2005 season. Each point represents the mean of 30 plants.

(Stachowiak *et al.*, 2005). Naturally produced *L. maculans* inoculum could therefore infect these cultivars equally. The difference between cultivars in the field late in the leaf spotting phase of the disease might have resulted from a differential susceptibility to *L. biglobosa,* as this pathogen is thought to infect later in the season than *L. maculans* (West *et al.,* 2002). However no differential response between them was observed in controlled environment experiments with *L. biglobosa*. A systemic resistance reaction in Darmor induced by *L. maculans* reaching the stem might also explain the difference in leaf lesion formation after this event. Leaf lesions on lower leaves (and therefore early in the season) are responsible for the basal stem lesions formed in summer. The data show that the number of leaf lesions at this early stage was not directly linked to the severity of stem canker. This supports previous findings that the relationship between leaf lesions and stem canker is not strong (Sun *et al.,* 2000) and suggests that few lesions are required for stem canker formation.

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## REFERENCES

Stachowiak A; Olechnowicz J; Jedryczka M; Rouxel T; Balesdent M H; Happstadius I; Gladders P; Latunde-Dada A; Evans N (2005) Frequency of avirulence alleles in field populations of *Leptosphaeria maculans* in Europe. *European Journal of Plant Pathology* (in press)

Sun P; Fitt B D L; Gladders P; Welham S J (2000). Relationships between phoma leaf spot and development of stem canker (*Leptosphaeria maculans*) on winter oilseed rape (*Brassica napus*) in southern England. *Annals of Applied Biology* **137**, 113-25.

Sylvester-Bradley R; Makepeace R J (1985). Revision of a code for stages in development in oilseed rape (*Brassica napus* L.). *Aspects of Applied Biology: Field Trials Methods and Data Handling* **10**, 395-400.

West J S; Balesdent M H; Rouxel T; Narcy J P; Huang Y J; Roux J; Steed J M; Fitt B D L; Schmit J (2000). Colonisation of winter oilseed rape tissues by A/Tox(+) and B/Tox(0) *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathology* **51**, 311-21.

West J S; Kharbanda P D; Barbetti M J; Fitt B D L (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology* **50**, 10-27.

Zhou Y; Fitt B D L; Welham S J; Gladders P; Sansford C E; West J S (1999). Effects of severity and timing of stem canker (*Leptosphaeria maculans*) symptoms on yield of winter oilseed rape (*Brassica napus*) in the UK. *European Journal of Plant Pathology* **105**, 715-28.

# Overwintering *Podosphaera aphanis* as main source of inoculum in a second year Elsanta strawberry crop under Spanish tunnels and relative resistance of seven varieties

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## ABSTRACT

The spatial development of disease within a new planting of strawberries showed that *Podosphaera aphanis,* the cause of strawberry powdery mildew, can over winter within the crop. Latent infections were present in approximately 10% of the plants and covering the tunnels provided suitable conditions for symptom expression. Seven cultivars, untreated by fungicides, were compared for the development of powdery mildew. All of them developed symptoms of the disease. However, even in this environment, that favoured powdery mildew, Everest and Florence had less than 5% disease symptoms. This reduction in disease pressure may offer opportunities to reduce the amount of fungicide used.

## INTRODUCTION

Spanish tunnels are now used commonly in UK strawberry production, to improve crop yields and extend the cropping season. Covers are used to force the fruit at the start of the season, to reduce infection of the crop by *Botrytis cinerea* and to provide protection from rain damage. A field of strawberries can be harvested for two or three years. Usually the first harvest is taken 60 days after planting, the plants then produce the second year main crop and can be kept for a third year when they are forced to produce an early crop.

Strawberry powdery mildew is a threat to the economic sustainability of crops grown under protection. Temperatures over $13^0$C are required for sporulation (Peries, 1962) while the ideal temperature for growth is 20°C (Jhooty & McKeen, 1965). The industry is dependent on a few cultivars, which are mostly very susceptible to the disease. Good control of powdery mildew can be achieved using fungicides, but production protocols are placing limits on the products used, harvest intervals and allowable residues. In addition, growers rely on a limited range of fungicide active ingredients, placing enormous selection pressure on the pathogen population.

The source of inoculum that initiates primary infections in Spanish tunnels is unknown. British weather conditions are not favourable for pathogen growth over the winter months; but are not severe enough to kill it. The aim of the work described here is to improve understanding of how disease pressure develops during strawberry production. This knowledge will be used to develop crop management strategies that suppress inoculum pressures for the entire production cycle. In particular this report details work to discover the:

- Origin of initial crop infection,
- Amount of disease control achievable by deployment of varietal resistance.

## METHODS

Seven cultivars (Bolero, Elsanta, Everest, Florence, Rosie, Royal Sovereign and Symphony) were grown in a section of a commercially managed site, located near Mereworth, Kent. Cold stored bare root plants were planted the previous summer and arranged in a randomised block design of four replicates. Plots consisted of 20 plants (2 rows × 10 plants). Blocks were separated by 2 plants of cv Elsanta. These plants were scored for *P. aphanis* infection using the MAFF Strawberry Powdery Mildew Key 8.1.1. (1976), developed for use on Royal Sovereign. It uses presence or absence of leaf cupping and the amount of red blotching on the leaves to quantify disease severity.

One Spanish tunnel was planted the previous summer with 2248 Elsanta plants. These were scored for the presence or absence of red blotches on the upper surface of the leaves. There were four beds with two rows in each bed. Each row was 281 plants long. Spacing between the plants was 30cm; each row was separated by 15cm. 10 assessments were made between the 17 April and 11 May, 2004. The Spanish tunnels were covered on the 16 April, 2004. They were vented and irrigated according to normal commercial practice. Disease patterns were mapped and analyzed using ArcGis (ESRI Corporation, Redland California, USA), a geostatistical software system. The pattern analysis was done by the method of Average Nearest Neighbour Distance. This method measures the distance between each diseased plant and its nearest diseased neighbour. All the distances are then averaged. An expected distance is also calculated based on a hypothetical random pattern of the same incidence of diseased plants, covering the same area. A Nearest Neighbour Index is expressed as the ratio of the observed distance divided by the distance expected for the random pattern. If the index is less than 1 the pattern exhibits clustering and tends toward dispersion if the index is more than 1.

## RESULTS

All seven varieties grown in the trial showed low levels of disease (leaf cupping) within 13 cumulative hours of temperature $\geq 15^{\circ}C$ after covering the tunnel (Figure 1). Disease levels on the variety Royal Sovereign started to increase after 265 thermal hours $\geq 15^{\circ}C$ and continued to increase until the end of the experiment (17 July 2004, reaching 37% of plants diseased). Disease on Bolero, Elsanta, Rosie and Symphony started to increase after 531 thermal hours $\geq 15^{\circ}C$, levelling off after a further 243 thermal hours at $\geq 15^{\circ}C$ (at 6-12%). The level of disease on the more resistant varieties, Everest and Florence, remained at a constant low level (3-4%). The seven varieties that were grown in this trial could be classified into three categories (very susceptible, susceptible and moderately resistant) using area under disease progress curve (AUDPC) at a 95% confidence level (Figure 1).

Only four plants had any symptoms of powdery mildew when the tunnel of Elsanta was covered. Within 4 days 10% of the plants had symptoms (Figure. 2) and disease incidence grew logistically ($P<0.001$, $R^2=99.6$; Figure 2). At the first assessment the plants with powdery mildew symptoms were well separated and the spatial pattern was fully dispersed (Figure 3.). Within three days the number of infected plants had risen to 214 (9.5%) and the distribution showed clear signs of clustering. The likelihood of the pattern arising due to random chance was <10%. Midway through the assessments (assessment 5, 28 April) 1037 plants are infected (46%), the clustering of disease was very pronounced and unlikely to arise

from random chance (<1%). By the eighth assessment (5 May), 1677 plants had symptoms (75%) and the disease pattern was more uniform due to coalescence of the clusters.

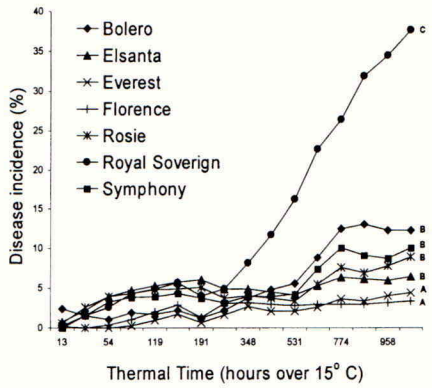


Figure 1. Disease progress on seven cultivars measured against thermal time. Letters indicate significant differences for comparison of AUDPC at the 95% confidence level.

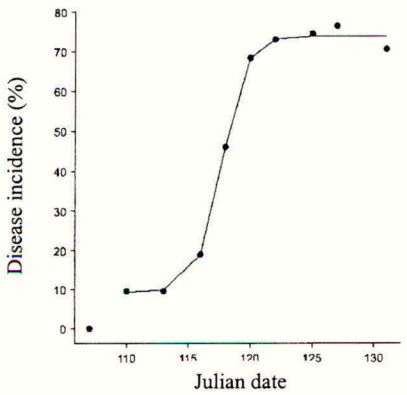


Figure 2. Growth in disease incidence (percent plants with symptoms) after the tunnel was covered. The logistic curve is fitted to all points except the first observation, which was made on the day after the covers were put up.

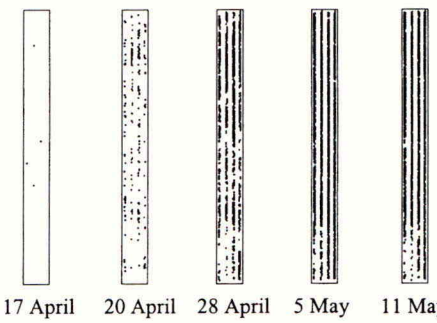


Figure 3. Location of individual plants that showed symptoms of *P. aphanis* within a tunnel over a period of 25 days (the figure is oriented north south as shown on the page).

## DISCUSSION

Low levels of powdery mildew were visible soon after the tunnels had been covered in the experiment comparing varietal resistance. All seven varieties developed symptoms of the disease. However, even in this high disease pressure environment, Everest and Florence had less than 5% of their leaf surface covered with red blotches. The dose of fungicide necessary to control an epidemic is a function of the amount of disease that would develop if the epidemic was left untreated (*cf* disease pressure). Therefore reduction in disease pressure from using more resistant cultivars offers opportunities to reduce the amount of fungicide applied, especially when the environment is suboptimal for disease development. Currently, however, growers do not have a wide choice of varieties which are acceptable to wholesalers and supermarkets and that have good resistance to powdery mildew.

Measuring the spatial development of disease within a second year commercial scale planting showed that inoculum can over winter within a crop of strawberry plants. Latent infections of mildew were present in approximately 10% of the plants and covering the tunnels provided suitable conditions for symptom expression. Provisional tests using a molecular diagnostic tool indicate that powdery mildew can be present in commercial planting stocks. There was time for an epidemic to develop on the planting stock the previous summer between the plants being planted in late July and the end of the season when the condition would not be favourable for the disease. This would suggest that the inoculum managed to overwinter successfully on about 10% of the plants.

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## REFERENCES

Jhooty J S; McKeen W E (1965). Studies on powdery mildew of strawberry caused by *Sphaerotheca macularis*. *Phytopathology* **55**, 281-285.

Ministry of Agriculture, Fisheries and Food (1976). Strawberry powdery mildew key no. 8.1.1.

Peries O S (1962). Studies on strawberry mildew, caused by *Sphaerotheca macularis* (Wallr. Ex Fries) Jaczewski I. Biology of the fungus. *Annals of Applied Biology,* **50**, 211-224

# Study of bermundagrass (*Cynodon dactylon* L) allelopathy to cotton (*Gossypium hirsutum* L) when growing in a nutrient solution. A new approach

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## ABSTRACT

The influence of bermundagrass to cotton growth was studied in the research site of the Agricultural University of Athens, in summer 2003. A hydroponic system was employed to create an artificial environment, in which allelopathy could be separated from resource competition. At the end of the experiment, cotton growth was clearly inhibited when plants were growing adjacent to bermundagrass. This is in agreement with results previously presented where cotton growth was inhibited when plants were growing in soil.

It appears that hydroponics is a very suitable method for allelopathy research, especially when taking into account that plant growth in the nutrient solution required significantly less time than in soil.

## INTRODUCTION

Allelopathy is a research field that receives growing interest. A considerable amount of research effort has been given to plant-plant interactions. Nevertheless even today there is a strong debate within the scientific community about to what extent allelopathy can be studied separately from plant competition. This is mainly based on several cases that growth inhibition was first attributed to allelopathy, but after a more thorough study other causes were identified to be responsible for growth problems (Romeo & Weidenhamer, 1999).

In this experiment, hydroponics was employed to study the interference between *G. hirsutum* and *C. dactylon.* Artificial environments appear to be useful to study interactions between plants, as competition can be better manipulated and separated from allelopathy. This study was also undertaken to confirm adverse effects of bermundagrass to cotton plants, as this effect has already been shown to happen when plants are growing in soil.

## MATERIALS AND METHODS

The experiment was conducted at the research site of the Agricultural University of Athens, between May-August 2003. The hydroponic system consisted of two pairs of sinks (volume/sink: 400litres) with an open upper surface. Sinks were made from polyester with painted outside surfaces and they were placed at 1m above ground. Two cylindrical plastic containers (volume/container: 200litres) the first at the ground level and the second at 1.4 m above ground, were connected with each pair of sinks, thus creating a closed circuit. Total

volume/pair of sinks was $1m^3$. A stable base of wood was fit in the upper surface of each sink. In each base, round holes (10 cm diameter) were made.

Cotton seeds (varieties Campo and Millenium) were sown in sand, in cylindrical tubes (height 30 cm, diameter 10 cm) on 26 May 2003. Emergence of seedlings was made 6-8 days after sowing. During the period of growth in tubes, cotton seedlings were watered at regular (3-4 days) intervals. No fertiliser was added while seedlings were growing in the tubes. Removal of seedlings to the sinks was made on 19 June 2003, with the development of the first leaf (stage 11, BBCH scale).

Only cotton seedlings (one seedling/place) were transferred to the first pair of sinks, which served as control. Cotton seedlings (two seedlings/place) as well as *C. dactylon* rooted cuttings were transferred to the second pair of sinks which served as treatment. Treatments were randomly allocated to plots as follows:

For Control sinks: A) 68 cotton seedlings (variety Campo) grown with no influence of *C. dactylon* and B) 68 cotton seedlings (variety Millenium) grown with no influence of *C. dactylon.* For Treatment sinks: C) 68 cotton seedlings (variety Campo) grown under the influence of *C. dactylon* and D) 68 cotton seedlings (variety Millenium) grown under the influence of *C. dactylon.*

In each pair of sinks the following chemicals were added: $Ca(NO_3)_2*4H_2O$: 760gr, $MgSO_4*7H_2O$: 400gr, $KNO_3$: 500gr, $NH_4H_2PO4$: 100gr, Fe chelate: 300gr, micronutrients: 50gr (Efthimiadis 1975). pH value of nutrient solution was recorded at 8 and EC value was recorded at 2mS/cm. Nutrient solution was continuously circulated with the aid of an electric pump. At the end of each day, sinks were refilled in order to replace water that was lost by evapotranspiration.

The experiment was completed on 2 August 2003, when the cotton plants were harvested (stage 51, BBCH scale). Stem diameter, number of buds, root dry matter, stem dry matter and leaf dry matter were then recorded. Additionally shoot:root ratio was calculated.

## RESULTS

At the end of the experiment, cotton plants were harvested. Characteristics measured, values recorded and percentage reduction for each characteristic are presented in Table 1 (for variety Campo) and Table 2 (for variety Millenium).

Table 1. Means and percentage reduction (compared to control) of plant characteristics for cotton plants (variety Campo) grown under the influence of *C. dactylon* in a nutrient solution

| Treatments | Stem DM (g) | Leaf DM (g) | Root DM (g) | Stem diameter (cm) | Number of buds | Shoot:root ratio |
|---|---|---|---|---|---|---|
| A | 2.97 | 3.92 | 1.66 | 0.74 | 13.3 | 4.36 |
| C | 1.48 | 1.81 | 1.07 | 0.50 | 10.1 | 3.50 |
| % reduction | 50.2 | 53.8 | 35.5 | 32.4 | 24.1 | 19.7 |

Table 1 shows that when cotton plants (variety Campo) were grown under the influence of *C. dactylon*, stem dry matter was reduced by 50.2%, leaf dry matter was reduced by 53.8%, root dry matter was reduced by 35.5%, stem diameter was reduced by 32.4%, number of buds was reduced by 24.1% and shoot:root ratio was reduced by 19.7%.

Table 2. Means and percentage reduction (compared to control) of plant characteristics for cotton plants (variety Millenium) grown under the influence of *C. dactylon* in a nutrient solution

| Treatments | Stem DM (g) | Leaf DM (g) | Root DM (g) | Stem diameter (cm) | Number of buds | Shoot:root ratio |
|---|---|---|---|---|---|---|
| B | 3.29 | 3.64 | 1.75 | 0.77 | 15.9 | 4.42 |
| D | 1.35 | 1.57 | 0.77 | 0.47 | 10.7 | 4.76 |
| % reduction | 59 | 56.9 | 56 | 39 | 32.7 | -7.7 |

Table 2 shows that when cotton plants (variety Millenium) were grown under the influence of *C. dactylon*, stem dry matter was reduced by 59%, leaf dry matter was reduced by 56.9%, root dry matter was reduced by 56%, stem diameter was reduced by 39%, number of buds was reduced by 32.7% and shoot:root ratio was increased by 7.7%.

Overall under the conditions of the experiment stem dry matter, leaf dry matter and root dry matter of cotton were mostly influenced by bermundagrass.

## DISCUSSION

Hydroponics appears to be very useful not only for commercial growth of plants, but for research purposes also. Previously, hydroponics was employed to study issues of plant physiology and plant nutrition (Yang *et al.*, 2002). During the course of such experiments special attention is required to detect negative symptoms on plant growth caused by root exudates or microorganism activity (Jung *et al.*, 2004).

In this study a hydroponic system was designed to study the interference of cotton by bermundagrass. Cotton and bermundagrass growth was successful in the nutrient solution. This was the first attempt recorded to grow bermundagrass in a nutrient solution, whereas for cotton there are previous such references for research purposes (Pline *et al.*, 2002). Growth of bermundagrass in the nutrient solution was very successful. During the course of the experiment weed plants appeared not to be stressed and vegetative production was high. Nevertheless weed plants exhibited allelopathy to cotton. This is in contrast with Reigosa *et al.*, (1999), who stressed that allelopathy develops when donor plants are under stress.

It has to be noted that allelochemicals activity was observed even though they were diluted in high volume. Probably dilution in the aqueous environment favoured allelochemicals activity. Biotic and abiotic factors that may cause alterations in allelochemicals toxicity (Dakshini *et al.*, 1999) are probably better controlled in such environment, while binding of chemical compounds in soil particles (Ito *et al.*, 1998) is also avoided.

In this experiment cotton was adversely influenced by bermundagrass. Both cotton varieties appeared to be susceptible to weed interference. All agronomic characteristics measured were

suppressed. No nutrient toxic or deficiency symptoms were developed to plants. These results provide strong confirmation of allelopathy interference by *C. dactylon* to cotton as they are in absolute accordance with results previously presented (Bouchagier & Efthimiadis, 2003), where growth inhibition of cotton when growing in soil was attributed to *C. dactylon* allelopathy also.

Overall it appears that hydroponics provide a practical, reliable, and time saving method to study allelopathy.

## ACKNOWLEDGEMENT

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## REFERENCES

Bouchagier P; Efthimiadis P (2003). Growth reduction of cotton (*Gossypium hirsutum*, L) caused by bermundagrass (*Cynodon dactylon*, L). A case of allelopathy. *Proceedings of the BCPC Conference-Pests & Diseases 2003*, **2**, 611-614.

Dakshini K M M; Foy C L; Inderjit (1999). Allelopathy: One component in a multifaceted approach to ecology. In: *Principles and Practices in Plant Ecology: Allelochemical Interactions*, eds Inderjit, K M M Dakshini & C L Foy, pp. 3-14. CRC Press, Boca Raton, Fl.

Efthimiadis P (1975). Contribution in plant improvement through the study of the root system of some selected varieties. *PhD Thesis*, Agricultural University of Athens, Greece.

Ito I; Kobayashi K; Yoneyama T (1998). Fate of dehydromatricaria ester added to soil and its implications for the allelopathic effect of *Solidago altissima* L. *Annals of Botany* **82**, 625-630.

Jung V; Olsson E; Caspersen S; Asp H; Jensén P; Alsanius B W (2004). Response of young hydroponically grown tomato plants to phenolic acids. *Scientia Horticulturae* **100**, 23-37.

Pline W A; Wilcut J W; Edmisten K L; Wells R (2002). Physiological and morphological response of glyphosate-resistant and non-glyphosate-resistant cotton seedlings to root-absorbed glyphosate. *Pesticide Biochemistry and Physiology* **73**, 48-58.

Reigosa M J; Sánchez-Moreiras A J; González L J (1999). Ecophysiologial approach in allelopathy. *Critical Reviews in Plant Sciences* **18**, 577-608.

Romeo J T; Weidenhamer J D (1999). Bioassays for allelopathy in terrestrial plants. In: *Methods in Chemical Ecology*, eds K F Haynes & J G Millar, pp. 179-211. Kluwer Academic Publishing, Boston.

Yang C M; Lee C N; Chou C H (2002). Effects of three allelopathic phenolics on chlorophyll accumulation of rice (*Oryza sativa*) seedlings: I. Inhibition of supply-orientation. *Botanical Bulletin of Academia Sinica 2002*, **43**, 299-304.

# The effects of conventional and conservation tillage systems on earthworm populations

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## ABSTRACT

Intensive cultivations affect earthworms directly through injury and fatalities and indirectly by changing their habitat, altering soil temperature, soil moisture and the availability of food. The encouragement of sustainable agriculture has resulted in increased interest and research into conservation tillage, a practice that allows earthworm populations to increase gradually in underpopulated soils. Deep-burrowing species (e.g. *Aporrectodea longa*) and mineral soil dwelling species (e.g. *Allolobophora chlorotica*) are favoured by conservation tillage, in contrast to conventional tillage, because of increased organic matter (on and in the soil) and decreased soil disturbance. This research is being carried out as part of the EU Life funded Soil and Water Protection (SOWAP) project. The primary hypothesis is that conservation tillage when compared with conventional tillage is beneficial to earthworm abundance. Preliminary results from demonstration sites in the United Kingdom and Belgium investigating the impact of different cultivation systems on earthworm populations, using mustard sampling and soil coring extraction techniques, are presented. Results indicate that conservation tillage provides soil environmental conditions which are more conducive to increased earthworm abundance than intense conventional tillage systems utilising the plough.

## INTRODUCTION

Earthworms are a vital constituent of the soil ecosystem and influence many soil properties and processes including structure, aeration and drainage. Intensification of tillage production systems has resulted in reduced earthworm abundance due to reduction in food supply, damage to burrow systems and direct injury (House & Parmelle, 1985; Curry *et al.*, 2002). The arrival of less intensive tillage cultivations such as conservation tillage provides environmental conditions that are beneficial to earthworm abundance (Fraser & Piercy, 1998). This paper presents data showing trends in earthworm abundance from SOWAP demonstration sites in the United Kingdom and Belgium.

## STUDY SITES AND METHODS

Sampling was carried out in autumn and spring after cultivations for winter sown crops over two cropping cycles at two newly established demonstration sites in the UK (Loddington, Leicestershire and Tivington, Somerset) and one demonstration site at Huldenberg, Belgium. Conservation tillage treatments were established alongside conventional tillage treatments utilising the plough, cultivating to a depth of 20 to 30 cm. Crop husbandry practices were the same across all treatments. At Loddington there were four demonstration plots comprising two forms of conservation tillage (Simba Solo disc cultivation (10 cm) plus mustard cover-crop and Simba solo disc cultivation) and two forms of conventional tillage (plough and plough plus Aqueel). There were three demonstration plots at Tivington consisting of two conservation tillage treatments (Discs (3 - 7.5 cm) and deep-tine (15 - 20 cm)) and conventional tillage. There were also three demonstration plots at Huldenberg with two conservation tillage treatments (Shallow tine (3 - 5 cm) and erosion plough (23 - 26 cm)) and conventional tillage.

Earthworm sampling was carried out using mustard extraction and soil coring. For each plot 6 mustard samples were taken; the intensity of the mustard sampling method meant that the 6 samples were divided into three paired samples. A 1 $m^2$ wooden quadrat divided in two constituted a paired sample. The location of the sample pairs was chosen at random and the quadrat was placed at each location. Two concentrations (60 g of mustard powder suspended in 20 l water (x 2) and 120 g in 20 l of water (x 2)) of mustard emulsion were applied over the quadrat in four consecutive 10 minute applications. Following the mustard extraction a core of dimensions 25 cm x 25 cm x 25 cm was taken from the centre of the 1 $m^2$ quadrat and sorted for earthworms. This sampling method was taken and adapted from an earthworm sampling method devised by Muys & Granval (2002). Soil coring consisted of taking soil cores of 785 $cm^3$ using a cylindrical soil auger (10 cm length x 10 cm width), in a stratified sampling design. The primary sampling point was 10 m from the top of each plot. Three parallel sampling lines were set up to form a grid, with three sampling points taken from each line, giving a total of 9 cores from each plot. The sampling lines were 10 m apart and each sampling point was 10 m from the next. All earthworms collected were preserved in 70% ethanol for identification.

## RESULTS

The total abundance and relative proportions of the ecological categories of earthworms collected within conservation and conventional tillage treatments using mustard extraction and soil coring from the first two sampling years of the SOWAP project, i.e. Autumn 2003 to Spring 2004 (Year 1) and Autumn 2004 to Spring 2005 (Year 2) are presented in Table 1.

At Loddington the conservation tillage treatments involving disc cultivation, (i.e. Simba Solo plus mustard cover-crop and Simba Solo) had greater total earthworm numbers than cultivations involving the plough. In the second experimental season sampling was only carried out in Spring 2005 due to delayed soil cultivations in autumn 2004, which resulted in lower total earthworm numbers. In Somerset the plough and disc treatments had similar, high total earthworm numbers which were higher than those in the deep-tine treatment when assessed by mustard extraction. By contrast, total earthworm numbers estimated from soil cores were greater in the conservation treatments in comparison to the plough treatment.

Table 1. The total abundance and relative proportions of the ecological categories of earthworms, expressed as a % of total earthworms, collected using mustard extraction and soil cores under different cultivation treatments in Somerset and Leicestershire in the UK and Huldenberg, Belgium from sampling seasons Autumn 2003 - Spring 2004 (Year 1) and Autumn 2004 - Spring 2005 (Year 2) of the SOWAP project.

| Ecological categories | **Loddington, Leicestershire, UK** Cultivation technique (plot size ~ 32 m * 70m) Sampled Autumn 2003, Spring 2004 (Year 1); & Spring 2005 (Year 2) | | | | | | | | **Tivington, Somerset, UK** Cultivation technique (plot size ~ 40 m * 50m) Sampled Autumn 2003, Spring 2004 (Year 1); Autumn 2004 & Spring 2005 (Year 2) | | | | | | **Huldenberg, Belgium** Cultivation technique (plot size ~ 32 m * 50m) Sampled Spring 2004 (Year 1); Autumn 2004 & Spring 2005 (Year 2) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plough (15-20 cm) | | Plough (15-20 cm) & Aqueel | | Simba Solo (10 cm) & cover crop | | Simba Solo (10 cm) | | Plough (15-20 cm) | | Disc (3-7.5 cm) | | Deep-tine (15-20 cm) | | Plough (25 cm) | | Erosion plough (23-26 cm) | | Shallow tine (3-6 cm) | |
| **YEAR 1** | M | SC | M | SC | M | SC | M | SC | M | SC | M | SC | M | SC | M | SC | M | SC | M | SC |
| Endogeic | 30.1 | 23.8 | 22.6 | 11.1 | 23.4 | 14.6 | 18.6 | 15.4 | 46.9 | 69.2 | 43.6 | 31.3 | 49.2 | 32.0 | 2.1 | - | - | - | 0.5 | - |
| Epigeic | 0.5 | - | - | - | 5.0 | - | 3.0 | 2.6 | - | - | 0.5 | 6.3 | 3.1 | - | 2.1 | - | - | - | - | - |
| Anecic | 3.4 | - | 7.8 | - | 3.5 | - | 4.0 | - | 0.6 | - | 5.3 | 3.1 | 2.1 | - | - | - | - | - | - | - |
| Juvenile epilobic | 40.2 | 33.3 | 53.9 | 66.7 | 40.2 | 64.6 | 42.1 | 46.2 | 50.3 | 15.4 | 44.3 | 37.5 | 36.1 | 44.0 | 4.3 | - | 3.5 | 13.3 | 5.0 | 25.0 |
| Juvenile tanylobic | 23.9 | 14.3 | 15.7 | 11.1 | 26.2 | 6.3 | 31.0 | 20.5 | 0.6 | - | 4.6 | - | 4.8 | - | 93.6 | 100.0 | 93.9 | 73.3 | 90.5 | 25.0 |
| Fragments | 1.9 | 28.6 | - | 11.1 | 1.8 | 14.6 | 1.4 | 15.4 | 1.5 | 15.4 | 1.8 | 21.9 | 4.8 | 24.0 | - | - | 2.6 | 13.3 | 4.5 | 50.0 |
| Sum total of earthworms | 209 | 21 | 102 | 9 | 401 | 48 | 371 | 39 | 469 | 13 | 436 | 32 | 291 | 25 | 47 | 1 | 115 | 15 | 221 | 4 |
| **YEAR 2** | | | | | | | | | | | | | | | | | | | | |
| Endogeic | 10.4 | 20.0 | 22.7 | 25.0 | 20.1 | 29.4 | 18.6 | 23.5 | 39.4 | 25.0 | 32.9 | 32.8 | 36.0 | 21.2 | - | - | 19.0 | 12.5 | 10.3 | 50.0 |
| Epigeic | 5.2 | - | 13.2 | - | 15.4 | - | 10.9 | - | 6.3 | 8.3 | 13.8 | 1.5 | 19.1 | 3.9 | - | - | - | - | 1.0 | - |
| Anecic | 9.1 | - | 11.3 | - | 3.1 | - | 5.5 | - | 1.4 | - | 1.0 | - | 2.3 | - | 8.7 | - | 20.3 | - | 13.4 | - |
| Juvenile epilobic | 32.5 | 60.0 | 37.7 | 25.0 | 44.4 | 52.9 | 40.5 | 70.6 | 46.2 | 52.8 | 48.3 | 47.8 | 33.1 | 55.8 | 26.1 | - | 29.1 | 12.5 | 29.9 | - |
| Juvenile tanylobic | 29.9 | - | 15.1 | 50.0 | 15.4 | - | 24.6 | - | 3.8 | 2.8 | 1.8 | - | 8.6 | 3.9 | 52.2 | - | 16.5 | 12.5 | 39.2 | - |
| Fragments | 13.0 | 20.0 | - | - | 1.5 | 17.7 | - | 5.9 | 3.0 | 11.1 | 2.3 | 17.9 | 0.8 | 15.4 | 13.0 | 100.0 | 15.2 | 62.5 | 4.1 | 50.0 |
| Sum total of earthworms | 77 | 5 | 53 | 4 | 259 | 17 | 220 | 17 | 665 | 36 | 785 | 67 | 486 | 52 | 23 | 2 | 79 | 8 | 97 | 6 |

M = mustard extraction; SC = Soil Core

Simba solo: a cultivator composed of predominately discs with tines.
Aqueel roller: elastometric micro-cellular polyurethane roller which contains indentations that reduce water and wind erosion.

In Belgium the conservation tillage cultivations had much greater total earthworm numbers than the conventional tillage treatment. In the second sampling season there was a decrease in total earthworm numbers across all treatments, probably reflecting seasonal variation. Mineral soil dwelling species also known as endogeic species, (*Ap. caliginosa*, *A. chlorotica* and *Ap. rosea*) and juveniles represented the major ecological categories recorded at the UK demonstration sites. The anecic species or deep burrowing species, *L. terrestris* and juveniles were relatively more abundant in Huldenberg in comparison to the UK sites, while endogeic species and species found in the litter layer (epigeics) were present to a lesser extent.

## DISCUSSION

The results show that endogeic species could withstand conventional tillage practices while, epigeic (*L. castaneus*, *L. festivus*, *L. rubellus*, and *Satchellius mammalis*) and anecic species (*Ap. longa* and *L. terrestris*) were less common in conventional than conservation tillage systems. Previous crop history may also determine earthworm species in a habitat and this will also be researched as part of the SOWAP project. The mustard solution proved an effective method for collecting all ecological categories of earthworms. The soil core sampling was effective at sampling epigeic and endogeic species, but not anecics, whose burrowing activities assist water infiltration and soil aeration. These preliminary research findings indicate that conservation tillage provides more favourable environmental conditions for endogeic, epigeic and anecic species than conventional tillage systems and a combination of factors including cover-crops and conservation tillage are important for the recovery of earthworm populations.

## ACKNOWLEDGMENTS

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## REFERENCES

Curry J P; Byrne D; Schmidt O (2002). Intensive cultivation can drastically reduce earthworm populations in arable land. *European Journal of Soil Biology* **38**, 127-130.

Fraser P M; Piercy J E (1998). The effects of cereal straw management practices on lumbricid earthworm populations. *Applied Soil Ecology* **9**, 369-373.

House G J; Parmelle R W (1985). Comparison of soil arthropods and earthworms from conventional and no-tillage agroecosystems. *Soil and Tillage Research* **5**, 351-360.

Muys B; Granval P (2002). Handleiding voor bemonsteren en identificeren van regenwormen (LUMBRICIDAE). Interdepartmental publication only, Leuven, Laboratorium voor Bos, Natuur en Landschap, KULeuven, 35pp.

# Response to individual compounds present in the headspace of *Phytophthora infestans* infected potato tubers using insect antennae as sensing elements

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**ABSTRACT**

Volatile organic compounds (VOCs) collected from potato tubers inoculated with *Phytophthora infestans* were analysed using gas chromatography – mass spectrometry (GC-MS). Examination of the volatile profiles showed 2-ethyl-1-hexanol, 1,3-dimethoxybenzene and 1-octen-3-ol as promising marker compounds for infected tubers. A sensor based on insect antennae was selected for possible use as an indicator of these compounds. Results show that the antenna of *Leptinotarsa decemlineata* is sensitive to three out of four compounds. The response of this sensor to individual compounds is discussed with relation to a practical application in which volatile mixtures must be monitored.

## INTRODUCTION

Diseased potato tubers can be detected through smell. This can be explained by the phenomenon that infestation of tubers results in the emission of a variety of VOCs. Monitoring of these VOCs can be used to develop a non-destructive technique for the selection and detection of infected potatoes (Lui *et al.* 2005).

Despite the development of ever more advanced analytical tools, it is worthwhile to study sensors present in nature, for example insect antennae. Antennae are highly sensitive to a variety of volatile compounds such as pheromones and other infochemicals. During this detection process, depolarisation of the olfactory neurons results in potential differences of a few mV between the base and tip of the antenna, which can be easily recorded resulting in a so called electroantennogram (EAG). Objective of this research was to test compounds present in the headspace of *P. infestans*-infected seed potatoes on the electrophysiological response of the insect antennae of Colorado beetle.

## MATERIAL AND METHODS

### Inoculation and incubation

Cultures of *Phytophthora infestans*, biotype IPO complex were produced on Agar at 22°C. Inoculum was prepared by scraping 48-h-old cultures with a needle and suspending them thereafter in sterile water. This suspension was adjusted to contain 20-50 zoospores/ml. Tubers of *Solanum tuberosum* cv. Bintje were wounded with a small spiked hammer (18 spikes, ø1 mm, 3 mm deep). The wound on each tuber was inoculated with 50$\mu$l of the spore suspension.

Control tubers were also wounded and inoculated with 50$\mu$l distilled water, which served as a control for any contamination by microbes which can grow on moist wounded tissue.

During the incubation period, tubers were stored under conditions present in potato stores (10°C, 90% RH, darkness and forced ventilation). These conditions were achieved with the controlled climate device displayed in Figure 1. It consisted of two temperature controlled water baths for cooling and a gas cylinder for air ventilation. The experiment consisted of two experimental groups: 1. infected-potato group; 2. control-potato group. Within each group, there were three replicates.

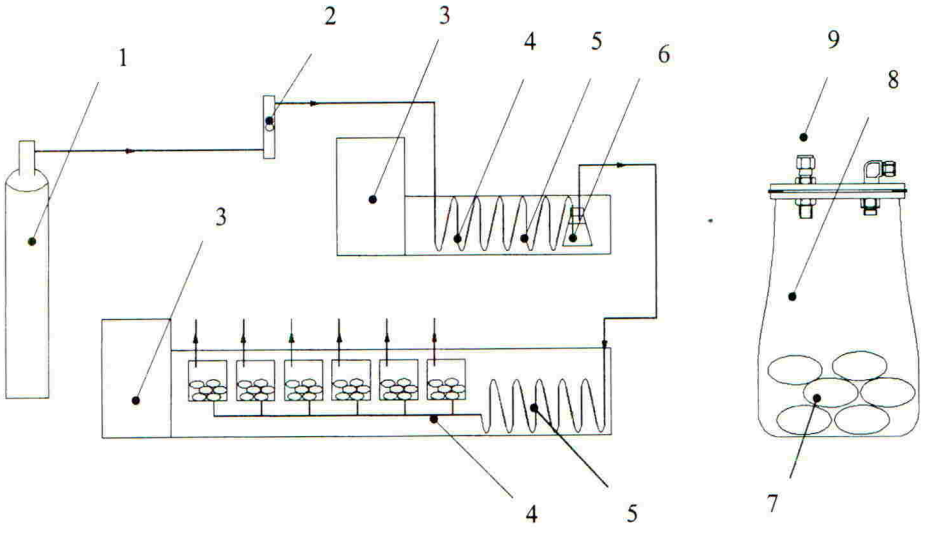


Figure 1. Diagram of the controlled climate device: 1 gas cylinder; 2 flow valve; 3 cooling units; 4 water baths; 5 radiators; 5 humidifying vessel; 6; 7 potatoes (1 kg); 8 headspace; 9 outlet

**Collection and identification of headspace volatiles**

After 40 days incubation period, traps filled with Tenax were placed at the outlets to concentrate the headspace above each of the tuber samples. Volatiles emanating from the tubers were collected simultaneously at a flow rate of 50 ml/min/trap for two hours. The collected volatiles were released from the traps in a thermal desorber and analysed using GC-MS. The MetAlign™ software package described by Laothawornkitkul, *et al.* (2005) was used to reveal the distinctive peaks in the chromatograms. Peaks specifically present in the volatiles from infected potato were identified by using the Wiley mass spectral library and the Wageningen Mass Spectral Database of Natural Products.

**EAG response of the Colorado beetle antennae**

Colorado beetle (*Leptinotarsa decemlineata* Say) were used in the experiments. The antenna was removed from the body and the distal segment was cut of. Then the excised antenna was

fixed between two glass electrodes filled with insect ringer solution. Compounds identified in the headspace of *P. infestans* infected potato were obtained from commercial source. These chemicals were dissolved in hexadecane and 25µl of each solution was pipetted on to a piece (6 cm x 0.5 cm) of filter paper and placed in a Pasteur pipette. The Pasteur pipette was attached to a stimulus controller and the pipette tip was inserted through a small hole in the glass tube, conducting a continuous airflow over the excised antenna. Since the antennal responses diminished in time, the responses to the test compounds are expressed as a percentage of the EAG response to the standard, cis-3-hexen-1-ol in hexadecane (1µl/ml). The antenna was stimulated with this standard before each measurement. All EAG equipment was obtained from Syntech, Hilversum, the Netherlands.

## RESULTS

### Collection and identification of headspace volatiles

Peak selection of MetAlign at 99% confidence interval revealed up to 20 specific compounds. Four of these compounds were selected for further testing based on commercial availability and presence of these compounds in the headspace of *P. infestans* infected potato according to literature. The volatile compounds selected and identified in the headspace of potatoes inoculated with *P. infestans* were: 1-octen-3-ol ($C_8H_{16}O$), 1,3-dimethoxybenzene($C_8H_{10}O_2$) and 2-ethyl-1-hexanol ($C_8H_{18}O$). These three compounds were subjected to the subsequent EAG response study.

### EAG response of the Colorado beetle antennae

Each compound tested gave an antennal response at concentration above $10^{-6}$ (v/v). This was mainly due to the chemicals solved because the solvent itself, hexadecane, did elicit relatively small measurable responses. Maximum excitation was elicited by the compound 1-octen-3-ol with a threshold of $10^{-5}$ (v/v). A lower response was observed for the compounds 2-ethyl-1-hexanol and 1,3-dimethoxybenzene as shown in Figure 2. Generally, it was found that the reproducibility between antennae was poor (sd < 20%) for each concentration using 10 antennae per compound.

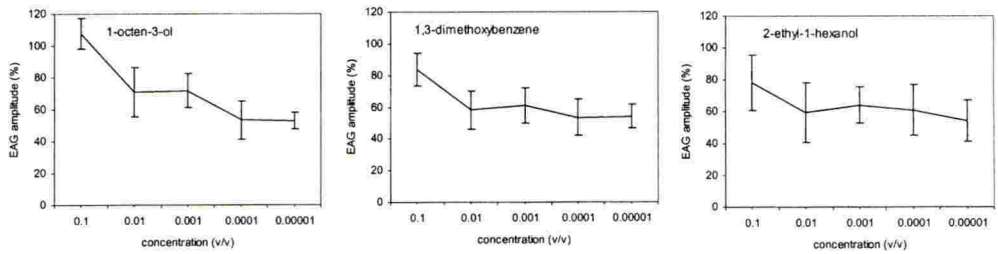


Figure 2. EAG dose-response curve for *Leptinotarsa decemblineata* to individual compounds present in the headspace of *P. infestans* infected potato. EAG amplitudes were normalised. (EAG response at $10^{-3}$ cis-3-hexen-1-ol = 100%)

## DISCUSSION

Current analytical techniques for volatile compounds are either time consuming (GC-MS), have a low sensitivity (electronic noses) or are characterised by a low number of identifiable compounds (laser based spectroscopy). It is therefore interesting to search for alternative sensors based on biological olfactory sensor organs. This was exploited by Schütz, *et al.* (1998) where highly sensitive insect antennae were used for detecting *P. infestans* infections in potato storage units. In that research a superposition technique was used to correct for interfering background volatiles that could occur in potato storage. This technique makes use of the assumption that the summation of the responses to individual compounds is equal to the response of their mixture. It is interesting to test this assumption because synergistic effects of volatile compounds on the response of the antennae of the stinkbug *Perillus bioculatus* is reported within this respect (Weißbecker *et al.*, 2000). For our next step we would like to test this theory on antennae of Colorado beetle. The compounds tested for this paper are useful in this respect because a response is clearly observed for each.

For this research, compounds were tested based on commercial availability. This despite the fact that complex terpenoids were identified in the headspace of infected potatoes. These compounds are likely to be more disease/spoilage specific. It could be valuable to obtain these compounds with extraction from cultures of *P. infestans* for further testing.

## ACKNOWLEDGEMENT

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## REFERENCES

Lui L H; Vikram A; Abu-Nada Y; Kushalappa A C; Raghavan G S V; Al-Mughrabi K (2005). Volatile Metabolic Profiling for Discrimination of Potato Tubers Inoculated with Dry and Soft Rot Pathogens. *American Journal of Potato Research* **82**, 1-8.

Schütz S; Weißbecker B; Koch U T; Hummel H E (1998). Detection of volatiles released by diseased potato tubers using a biosensor on the basis of intact insect antennae. *Biosensors & Bioelectronics* **14**, 221-228.

Laothawornkitkul J; van Bruggen A H C; Jansen R; Müller J; Lommen A (2005). Normalization, Baseline correction and Alignment of data to assess changes in volatile production during an infection of potato plants with *Phytophthora infestans*. *Proceedings of the BCPC Conference - Crop Science & Technology 2005.*

Weißbecker B; van Loon J A; Posthumus M A; Bouwmeester H. J; Dicke M (2000). Identification of volatile potato sesquiterpenoids and their olfactory detection by the two-spotted stinkbug *Perillus bioculatus*. *Journal of Chemical Ecology* **26(6),** 1433-1445.

# Normalization, baseline correction and alignment of data to assess changes in volatile production during infection of potato plants by *Phytophthora infestans*

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## ABSTRACT

One of the most time consuming steps in comparing chromatographic profiles is the manual identification of specific peaks in the paired chromatograms. Software has recently been developed to enhance this procedure. For this paper, results from manual peak selection performed during previous research were compared to the outcome of the "MetAlign™" software package. This confirmed the discrimination and identification of three volatile compounds related to *Phytophthora infestans* infected potato plants. Automation of the matching procedure allows the creation of field sensors based on commercially available field-portable gas chromatographic equipment for detection of the disease in the field.

## INTRODUCTION

The volatile organic compounds emitted by plants show a puzzling diversity. Stress factors can induce the production and emission of plant volatiles. Such factors can be: pathogen attack, insect herbivory, abnormal temperatures, drought, light, mechanical wounding, salinity and ozone exposure. These stress-induced compounds are especially interesting for agricultural engineers. Developing a sensor to monitor these volatiles in the field offers the possibility to detect the physiological status of crop plants as well as to identify the initial phase of pathogen and herbivore infections (Holopainen, 2004). In our previous research we studied the emission of volatiles from potato plants infected by the fungus *Phytophthora infestans* (Laothawornkitkul *et al.*, 2005). It was time consuming to assess the difference in chromatograms manually. For this paper, software was tested to automate the discrimination process and to compare the outcome with the manually performed discrimination.

## MATERIAL AND METHODS

### Plant materials and inoculation

Potato tubers, cv. Agria, were grown in a greenhouse at 20°C, 60% relative humidity and L16:D8 photo regime. Four-week-old plants (±20 cm high) were used in the experiment.

*P. infestans* (isolate No. 98014) was cultured in 9 cm diameter Petri dishes containing Rye Sucrose Agar. Cultures were incubated at 18°C and a L16:D8 photo regime for 7-10 days. Ten ml of cold distilled water (4°C) was added onto the pathogen colony. The sporangium suspension was incubated at 4°C for 2 hours to induce zoospore formation, and then filtered through 50 µm nylon mesh. The concentration of zoospores in the suspension was counted and adjusted to $1.8\times10^5$ zoospores $ml^{-1}$. The ventral leaf surfaces were spot-inoculated with 10 µl of the suspension per spot for a total of 10 spots per plant. Control plants were spotted by sterile distilled water. Inoculated and healthy plants were placed separately in 2.5-liter glass vessels. The vessels were placed in a growth chamber adjusted for volatile collection.

**Collection and identification of headspace volatiles**

There were 3 experimental groups in the experiment: 1. infected-plant group; 2. healthy-plant group, and 3. empty-vessels group. Within each group, there were 3 replications. The vessels were placed under light intensity of 300 µmol $m^{-2}$ $s^{-1}$, at temperatures of 18 °C at night and 20 °C during the day, with a 14L:10D photoperiod. For volatile sampling, traps filled with Tenax were placed at the air outlet of the vessels. Trapped volatiles were eluted from each trap by 3 ml of pentane:ether (4:1). Phenol was used as an internal standard. Then the samples were concentrated from 3 ml to 150 µl. Fifty µl of the concentrated samples was used for GC-MS analysis. Chromatograms from volatiles trapped 100 hours after inoculation were subjected to automatic data handling using MetAlign (www.metalign.nl; Vorst *et al*, 2005).

**Data handling**

The areas under each peak were calculated and standardized by dividing these by the area of the internal standard, phenol. To determine the volatile compounds differing between infected plants, control plants and empty vessels during each trapping period, the standardized peak areas were compared by the Kruskal Wallis nonparametric test for K independent samples in SPSS. The volatile compounds, which had significantly higher concentrations in the infected-plant group than in both control groups according to the Kruskal Wallis test, were subsequently subjected to paired comparisons by the Mann-Whitney U test. The degree of significance at 95% confidence was the criteria for peak selections throughout the analysis.

After optimizing the settings according to the specific chromatographic conditions, MetAlign is able to compare samples based on the ions detected in an unbiased and unsupervised manner by performing the following steps: (a) data smoothing by digital filters related to the average peak width, (b) estimation of local noise as a function of retention time and ion trace, (c) baseline correction of ion traces and introduction of a threshold to realise noise reduction, (d) calculation and storage of peak maximum amplitudes, (e) between chromatogram alignment using high S/N peaks common to all chromatograms, (f) iterative fine alignment by including an increasing number of landmark peaks with lower S/N and (g) filtering for significant differences at user-defined significance thresholds (here, 95% confidence using a t-test) and minimum x-fold ratios. The analysis was done in pairs, i.e. the infected-plant group vs. the control-plant group, and the infected plant group vs. empty-vessel group. The selected peaks were significantly present in the infected-plant group but not in the healthy-plant and empty-vessel groups.

Table 1. Settings in MetAlign

| Setting | Value |
|---|---|
| Peak slope factor | 0.5 |
| Peak threshold factor | 1 |
| Average peak width at half height | 5 |
| Scaling | Marker peak |
| Initial peak search criteria : maximum shift | 35 |
| Pre-align processing | Iterative |
| Significance percentage | 95 |
| Minimum ratio between means | 2 |
| Minimum S/N ratio | 10 |
| Either present in Group 1 or Group 2 | Yes |
| Select min. nr. Per peak set | 3 |

## RESULTS

By setting the same criteria for peak selection, i.e. 95% confidence and other settings in Table 1, the results of the manual inspection and MetAlign could be compared. Both methods gave the same results, namely four selected peaks as shown in Figure 1E. However, only three out of four significant peaks could be identified (Laothawornkitkul *et al.*, 2005). Therefore, automated matching of chromatograms using MetAlign confirmed the previous conclusion from manual and statistical comparisons.

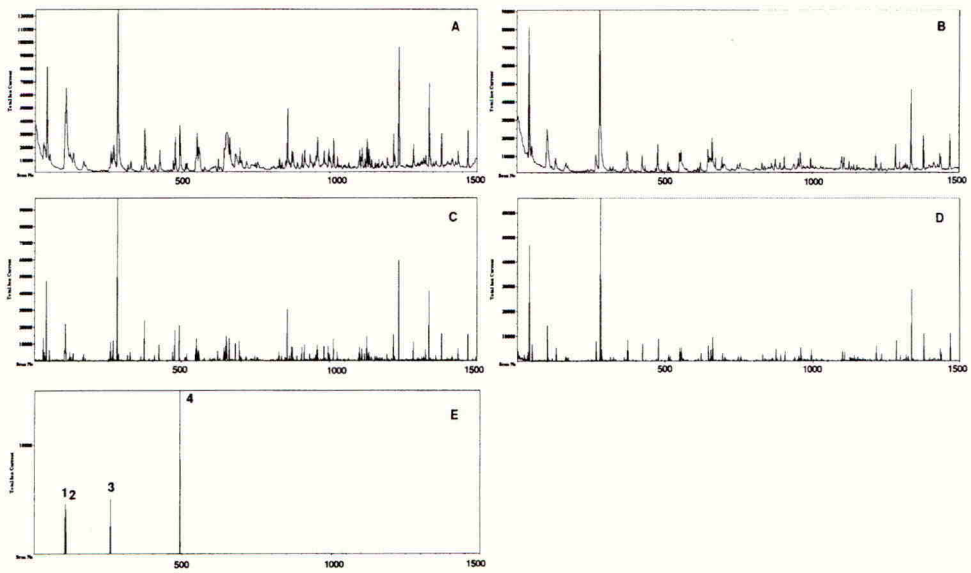


Figure 1. Typical GC-MS chromatogram of headspace sample from potato plant headspace. Shown is (A) Chromatogram from infected plant (B) Chromatogram from control plant. (C) MetAlign-assisted baseline correction of chromatograms from infected plant (D) and control plant. (E) Mass peaks that were significantly increased in volatiles from infected potato plants compared to uninoculated control plants and empty vessels.

## DISCUSSION

Manual inspection of GC-MS chromatograms has obvious difficulties in peak selection because of changes and drifts in peaks due to small and unavoidable differences in experimental conditions. Therefore the process requires tremendous time. In this paper, it is shown that the power of MetAlign is comparable to that of manual inspection in terms of results. However the principles of peak selection are different in both methodologies. Firstly, in manual inspection, the peak selection is based on peak area, while in MetAlign it is based on maximum amplitude of mass peak and detected ion. Secondly, the MetAlign program uses a parametric test, i.e. t-test, to select significant peaks while non-parametric tests can be selected when the data are analysed manually, which is an advantage when data are not normally distributed (e.g. our data set). The software performs the baseline correction- and peak alignment algorithm to correct for the amplitude and phase variation between the chromatograms. This is basically an extraction and ordering of the chemical information present in multiple GC-MS datasets. The results of MetAlign vary according to different settings. Therefore expert choices are critical along with the analysis process. Finally the software presents differential candidate peaks which in turn need experts to decide on subsequent actions dependent on the research questions.

Software can facilitate the analytical process particularly when the capacity and capability of modern GC-MS equipment increase allowing an increase in the number of samples to be analyzed. In the near future, automated peak detection becomes unavoidable when field-portable GC-MS equipment appears on the market (Smith *et al.*, 2004). This equipment in combination with data analyses software offers the possibility for automatic screening of volatiles for the purpose of disease- and other plant-stress detection in greenhouses or fields.

## ACKNOWLEDGEMENT

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## REFERENCES

Holopainen J K (2004). Multiple functions of inducible plant volatiles. *Trends in Plant Science* **9(11)**, 529-533.

Laothawornkitkul J; Jansen R; Smid H M; Bouwmeester H; Müller J; v. Bruggen A H C (2005). Collection and identification of volatiles from potato plants infected by *Phytophthora infestans* by *in situ* volatile trapping and GC-MS. *Phytopathology* (submitted).

Smith P A; Koch D; Hook G L; Erickson R P; Jackson Lepage C R; Wyatt H D M; Betsinger G; Eckenrode B A (2004). Detection of gas-phase chemical warfare agents using field-portable gas chromatography-mass spectrometry systems: instrument and sampling strategy considerations. *Trends in Analytical Chemistry* **23**, 296-306.

Vorst O; de Vos C H R; Lommen A; Staps R V; Visser R G F; Bino R J; Hall R D (2005). A non-directed approach to the differential analysis of multiple LC-MS-derived metabolic profiles. *Metabolomics* **1(2)**, 169-180.

# Wheat – *Phalaris* spp. competition/interference studies using an additive design series

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## ABSTRACT

A glasshouse experiment was conducted using an additive design to determine the approximate onset of competition between *Phalaris spp.* and wheat, the density of *Phalaris* plants necessary to reduce wheat yield significantly and to investigate the comparative aggressiveness of *Phalaris minor* and *Phalaris paradoxa* relative to spring wheat. Seed of *Phalaris* spp.were sown in tanks at densities between 0 and 400 /$m^2$ whereas the density of wheat cv. Axona was maintained constant (350/$m^2$). Wheat height was significantly reduced in the presence of *P. minor* 28 days after sowing, whereas such a reduction occurred one week earlier in competition with *P. paradoxa*. Wheat fresh weight was significantly reduced by *P.minor* at a seed density of 400/$m^2$ whereas a similar reduction occurred at 100 plants /$m^2$ in competition with *P. paradoxa*. Overall it was found that the number of leaves, dry weight, penetration of photosynthetically active radiation below the crop and weed canopy and dry weight of wheat ears decreased with the increasing weed density.

## INTRODUCTION

*Phalaris minor* Retz is a common grass-weed in India, mostly associated with winter cereals including wheat. It is an extremely competitive weed in cereal crops and can reduce wheat yield by 30% at a density of 150 plants/$m^2$ (Malik & Singh, 1995). *Phalaris paradoxa* L. is an annual grass of the Mediterranean region and has been known as a casual species in Britain for at least 100 years. It is recorded occasionally in cultivated and waste places in Britain (Hubbard, 1968).Fields on some farms were severely infested by *P. paradoxa* during the early 1980's and it seemed possible that this species could become a major weed in Britain (Thurley & Chancellor, 1985). Hence, it was considered appropriate to compare the competitive abilities of these two grass-weeds relative to wheat. This objective was accomplished using an additive design series experiment. In additive experiments two species are grown together, the density of one is maintained constant and that of the other is varied. The first species can be regarded as an indicator and experiments of this sort can be used to compare the relative aggressiveness of a group of species relative to the indicator (Harper, 1977). This type of design is comparable to a weed infested crop where crop density is maintained constant. The experiments reported here are crop – weed competition studies involving wheat-*Phalaris minor* and wheat- *Phalaris paradoxa* where wheat is considered the indicator species

## MATERIALS AND METHODS

Interference studies were conducted in tanks (45cm×28.5cm×30cm) maintained in a glasshouse in a randomized complete block design with four replicates per treatment. Five densities of *P. minor* and *P. paradoxa* (25, 50, 100, 200 and 400 plants/$m^2$) were sown in mixture with one density of wheat cv Axona (350 plants/$m^2$). Weed seeds were sown at a depth of 2mm and

wheat at 10mm in John Innes potting compost (pH-6.8, OM-7.3%; N-0.21%; P-35ppm and conductivity-$2.96\times10^{-3}$ siemens) and watered as necessary. Fresh and dry weights of the crop were taken from the central row of each tank excluding outer rows as discards, whereas fresh and dry weights of weeds were taken on a per tank basis. The responses of wheat to both species of *Phalaris* were statistically analysed using Genstat version 7.0.

## RESULTS AND DISCUSSION

*P. minor* significantly reduced the height of wheat 28 days after sowing (DAS) whereas for *P. paradoxa* this occurred 21 DAS (Tables 1, 2). Similar trends were observed for leaf number per plant (Figure1).

Table 1. Height , fresh and dry weight of wheat as affected by *Phalaris minor* density

| Treatments | Height (cm) | | | | Fresh weight (g) | Dry weight (g) |
|---|---|---|---|---|---|---|
| *P. minor*/m$^2$ | 21DAS | 28DAS | 35DAS | 42DAS | at harvest | at harvest |
| 0 | 10.2 | 18.73 | 29.02 | 29.74 | 20.85 | 10.12 |
| 25 | 10.35 | 18.24 | 27.54 | 28.52 | 20.49 | 10.12 |
| 50 | 10.41 | 17.02 | 27 | 27.78 | 20.02 | 10.54 |
| 100 | 10.35 | 16.05 | 25.98 | 26.71 | 19.28 | 10.19 |
| 200 | 10.2 | 16.19 | 25.66 | 25.9 | 17.94 | 9.65 |
| 400 | 9.71 | 15.72 | 25.6 | 25.38 | 13.42 | 6.01 |
| LSD (5%) | 1.08 | 0.96* | 2.07* | 1.64* | 3.62* | 2.47* |

Table 2. Height , fresh and dry weight of wheat as affected by *Phalaris paradoxa* density

| Treatments | Height (cm) | | | | Fresh weight (g) | Dry weight (g) |
|---|---|---|---|---|---|---|
| *P. paradoxa*/m$^2$ | 21DAS | 28DAS | 35DAS | 42DAS | at harvest | at harvest |
| 0 | 11.7 | 18.63 | 29.16 | 30.56 | 23.53 | 10.35 |
| 25 | 11.4 | 17.93 | 28.73 | 29.45 | 22.59 | 10.02 |
| 50 | 10.22 | 17.2 | 27.46 | 28.17 | 18.22 | 9.8 |
| 100 | 10.37 | 16.51 | 26.68 | 27.47 | 17.03 | 9.53 |
| 200 | 9.62 | 15.56 | 25.15 | 25.16 | 13.93 | 6.8 |
| 400 | 9.17 | 14.63 | 23.64 | 24.17 | 11.38 | 6.42 |
| LSD (5%) | 1.08* | 0.90* | 2.32* | 2.32* | 6.01* | 1.69* |

Wheat foliar fresh and dry weights were significantly reduced with increasing weed density. There was a significant reduction in crop fresh weight at a density of 400 *P. minor*/m$^2$ (Tables 1, 2), whereas a significant reduction occurred at 100 plants/m$^2$ for *P. paradoxa* which leads us to infer that *P. paradoxa* is relatively more competitive than *P. minor*. Thus % fresh weight of wheat was reduced by 35.5 % in competition with *P. minor* at a density of 400/m$^2$ and 41.1% in competition with *P. paradoxa* at only 200/m$^2$ (Figure 2 ) which further substantiate the aggressiveness of *P. paradoxa* relative to *P. minor*. These findings are in agreement with observations reported by Afentouli & Eleftherohorinos (1996) in which wheat grain yield was

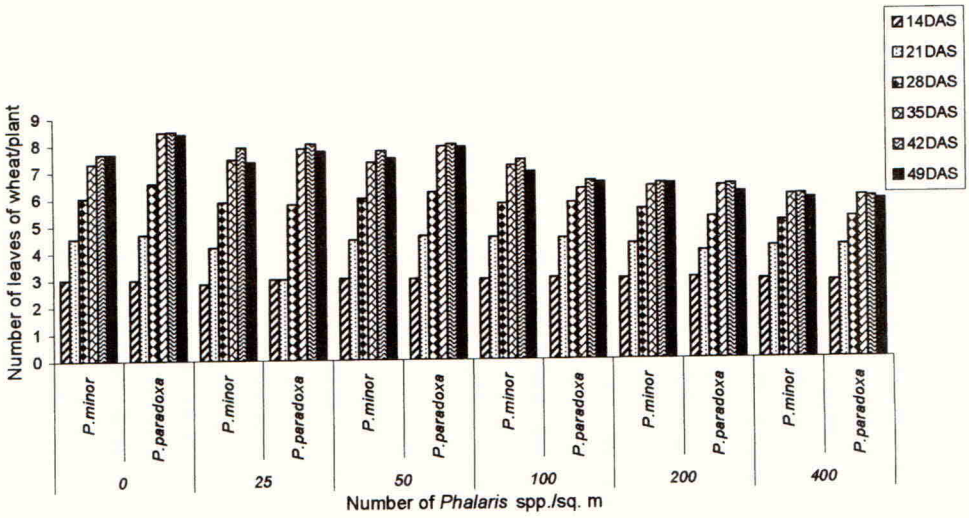


Figure 1. Number of leaves of wheat/plant as affected by *P. minor* and *P. paradoxa* densities.

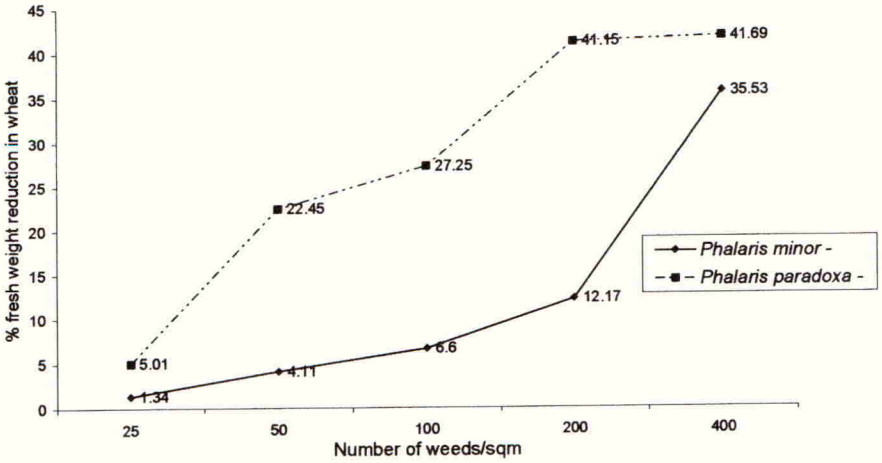


Figure 2. % fresh weight reduction in wheat as influenced by *P. minor* and *P. paradoxa*

reduced by 36 to 39 % in competition with *P. minor* at a density of 304 plants/$m^2$ . In a subsequent investigation they observed that at densities of 152 and 304 plants/$m^2$ *P.minor* reduced wheat yields by 32 and 42% respectively (Afentouli & Eleftherohorinos,1999). Likewise, Dhaliwal *et al.*, (1997) reported a 10% yield loss at densities of 60-70 plants/$m^2$ whereas yield loss exceeded 50% at 500 plants /$m^2$.

In a comparative study of competition between *Avena sterilis* L. and *P.minor* and several cultivars of winter barley, neither weed species had any significant effect on barley dry weight until after tillering (Dhima *et al.*,2000). However, whereas grain yield was reduced between 8-67% (depending on cultivar) by *A.sterilis* at a density of 120plants /$m^2$, the corresponding reduction for *P.minor* at 400plants/$m^2$ ranged from 1-55%. The findings of the present study clearly indicate that although *Phalaris*-wheat interference was mainly affected by weed density as growth rate of both *Phalaris* species was similar, Phalaris paradoxa is the stronger competitor.

These results differ somewhat from those of Afentouli & Eleftherohorinos (1996, 1999) who indicated dis-similar growth rates of *Phalaris brachystachys* Link. and P.minor but similar competitive ability. Thus it may be concluded that Phalaris paradoxa has potential for greater crop yield reduction than *P.minor* under a cool temperate climate experienced in the UK.

## ACKNOWLEDGEMENTS

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## REFERENCES

Afentouli, C G; Eleftherohorinos, I G (1996). Littleseed canary grass (*Phalaris minor*) and short spiked canary grass (*Phalaris brachystachys*) interference in wheat and barley. *Weed Science*.**44**,560-565.

Afentouli, CG; Eleftherohorinos, I G (1999) Competition between wheat and canarygrass and their response to herbicides. *Weed Science*, **47,** 55-61.

Dhaliwal, B K; Walia, V S Brar, L S (1997). Response of wheat to *Phalaris minor* Metz. population density. Proceedings 1997 Brighton Crop Protection Conference - Weeds, 1021-1024.

Dhima, K V; Eleftherohorinos,I G; Vasilakoglou,I B (2000). Interference between *Avena sterilis*, *Phalaris minor* and five barley cultivars. *Weed Research*.**40**, 549-559

Harper, J L (1977). *Population Biology of Plants. London*, Academic Press. 151-194

Hubbard, C E (1968). *Grasses*, 2nd edition.Penguin, London

Malik, R K; Singh S (1995). Littleseed canary grass (*Phalaris minor*) resistance to isoproturon in India. *Weed Technology*, **9**, 419-425

Thurley B; Chancellor R J(1985). Occurrence, distribution and spread of *Phalaris paradoxa* L. in Britain. *Annals of Applied Biology*, **107**, 79-86

# Effect of rapeseed oil ethoxylates on deposition and penetration behaviour of two selected calcium salt solutions

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## ABSTRACT

Two different calcium salts at five concentrations with and without addition of a rapeseed oil ethoxylate (RSO 5 EO) have been used to elucidate their influence on the structure and area of deposit, drying time of droplets and penetration rate of calcium. Isolated tomato fruit cuticles served as a model system. The initial spreading area of calcium droplets was significantly higher (up to three times) when adding RSO 5. Independent of the type of salt there was a homogeneous spreading of calcium in the circular deposit before and after droplet drying. The application of calcium chloride and calcium acetate without the addition of RSO 5 resulted in scattered residues, mainly at lower concentrations (0.01 and 0.05%). Increasing calcium concentration was paralleled by enhanced penetration of both calcium salts through the tomato fruit cuticle but did not directly correlate with penetration.

## INTRODUCTION

In pesticide application physicochemical parameters of the spray solution play a major role and affect atomisation of the spray liquid, impact droplet retention and spreading on the target surface and may influence subsequent biological processes (Downer *et al.*, 1999). Maximum deposition of a.i. is achieved by minimising losses caused by drift, rebound and run-off (Duncan *et al.*, 1999). A saturated liquid phase must exist between cuticle and salt residue in order to allow penetration. Consequently, humidity and point of deliquescence (POD) are major determinants for salt penetration (Schönherr, 2001). Former studies have shown that penetration rate is concentration driven (Knowles, 2001). The concentration of a.i. in the spray solution, deposition formation and area of residue are additional influencing factors (Bukovac *et al.*, 2003) as well as rewetting of dried residues (Knoche & Bukovac, 2001). By adding adjuvants to the pesticide formulation uptake of a.i. may be facilitated, e.g. caused by spreading or by extending the liquid phase of the spray solution (Bukovac *et al.*, 2003). In this study we hypothesized that increasing area of residues formed by individual droplets is related to a decrease of a.i. penetration due to reduced concentration of a.i. per unit area. In order to induce changes in droplet area a RSO with five ethylene oxide units in the hydrophilic chain was employed as a surfactant. Isolated tomato fruit cuticles were used as a model system for investigating relationships between residue formation and a.i. penetration.

## MATERIALS AND METHODS

### Isolation of plant cuticles and chemicals

Tomato fruits (*Lycopersicon esculentum* var. `Panovy´, S&G Kleve, Germany) were grown in a greenhouse without use of pesticides. Mature fruits, free of visual defects, were sampled and epidermal discs (1.8cm diameter) were excised. The discs were submerged in a mixture of pectinase (20g/litre), cellulase (8g/litre) and tri-natrium citric acid buffer (14.7g/litre) at 22°C room temperature as described elsewhere (Knoche *et al.*, 2000). Sodium acide (0.09g/litre) was added to prevent fungal and bacterial growth. After separation of cuticular membranes from the digested tissue the isolated cuticles were put in a borax buffer (2.01g/litre) for 24 hours and rinsed extensively in demineralised water. Calcium chloride ($CaCl_2 \times 2\ H_2O$) and calcium acetate ($(CH_3COO)_2Ca \times x\ H_2O$), respectively, was added to donor solutions to prepare final concentrations of 0.1% (0.025 mol Ca/litre), 0.5% (0.125mol Ca/litre), 1.0% (0.25mol Ca/litre), 1.5% (0.375mol Ca/litre) and 3.0% (0.75mol Ca/litre) weight by volume of pure calcium. Above named solutions were prepared without and with addition of rapeseed oil (RSO 5) at a concentration of 1g/litre.

### Calcium deposition

Individual calcium droplets (0.5µl) of above named solutions were placed on isolated tomato cuticles (n = 12) with a microsyringe. After droplet drying surfaces of cuticles were studied with an Environmental Scanning Electron Microscope (XL 30 ESEM, FEI-Phillips, Kassel, Germany). Droplet residues were analysed by x-ray-analysis of calcium atoms using the integrated EDAX system (energy dispersive analyses x-ray; GENESIS 4000). Micrographs were recorded and area of surface deposits measured employing the software `Optimas´ (Media Cybernetics Inc., USA; version 6.5 for windows).

### Penetration experiments

Isolated tomato fruit cuticles were mounted onto small stainless steel boxes filled with $1.35*10^{-3}$ litre of demineralised water as receiver solution. The abaxial cuticle surface was always in close contact with the water filled in the small boxes. A metal ring was put on the cuticle/box system to fix the cuticle. Afterwards the systems were cold stored (4°C) for 22 ± 0.5 hours and transferred to a climatic chamber with a temp. of 22 ± 1°C and a relative humidity of 75 ± 5%.

## RESULTS

### Characteristics of calcium spray deposits

Droplets of calcium solutions tended to spread when carefully placed onto the surface of tomato fruit cuticles. In few cases increase of calcium concentration was followed by changes in spread area if no rapeseed oil was added, however, there was no clear relationship. When increasing calcium amount per droplet by a factor of 5 or 10, respectively, a significant increase in total spread area was noticed. The addition of rapeseed oil ethoxylate at a concentration of 0.1% (m/V) distinctly increased droplet spreading (Figure 1). The area was 2 to 3 times larger as compared to unformulated calcium solutions.

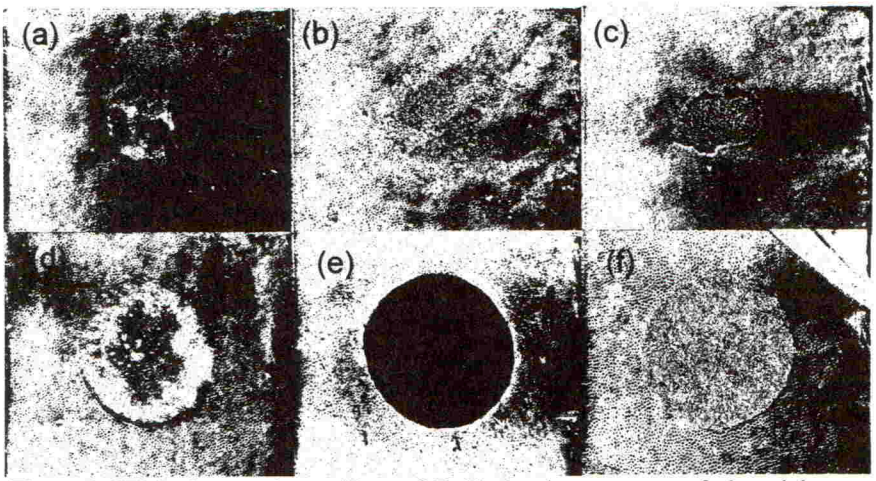


Figure 1. SEM micrographs; residues of $CaCl_2$ droplets on tomato fruit cuticles (a) 0.025mol, (b) 0.25mol, (c) 0.75mol $CaCl_2$/litre, (d) 0.025mol, (e) 0.25mol, (f) 0.75mol $CaCl_2$/litre, a – c no RSO 5 was added, d – f with addition of 0.1% (m/V) RSO 5

**Penetration**

The increase of the calcium concentration was associated with an increase of calcium uptake in all treatment groups independent of the calcium salt used and regardless if RSO 5 was added or not. However, there was no linear relationship; in general, a manifold increase in calcium concentration resulted in only small enhancement of calcium penetration (Figure 2).

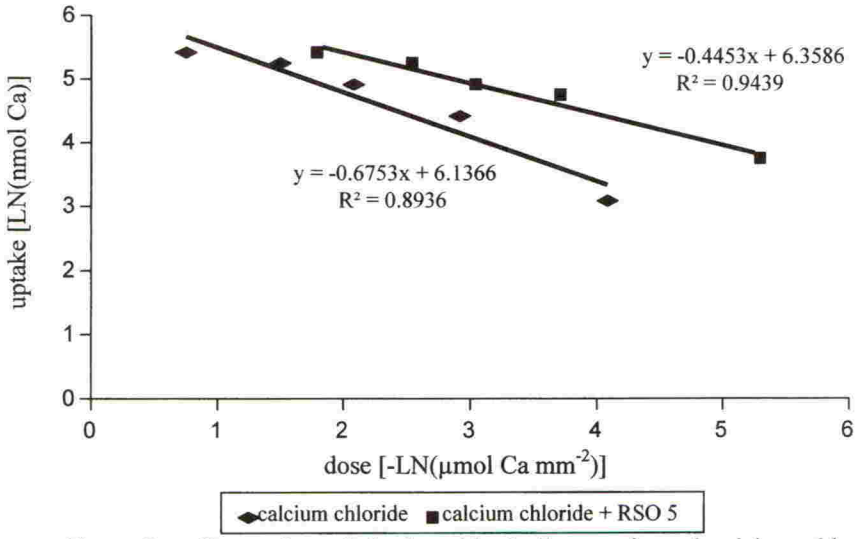


Figure 2. Regression of the logarithmically transformed calcium chloride uptake and calcium dose per unit initial spread area

Except for the highest calcium concentration applied penetration of calcium chloride salt was distinctly higher compared to calcium acetate form. This was also true for treatment groups

with RSO 5 addition. This addition, however, resulted in a very pronounced stimulative effect at the lower calcium concentration levels.

## DISCUSSION

Addition of rapeseed oil surfactant to calcium chloride and calcium acetate significantly improved droplet spreading. Increase of calcium dose is not necessarily correlated with an equivalent penetration effect. The logistic-kinetic penetration model of Watanabe (2002) reveals that no changes in the penetration rate factor (q) occur if active ingredient concentration is increased. Besides, an increase of contact area is not followed by changes in q, even though the total amount of penetration (A) is accompanied. In fact, the enhanced total penetration is related to larger initial spread area, whereas a lower impact is associated with the slope of the gradient. Even if applied at lower concentration higher uptake is accomplished when surfactants with good spreading properties are used. Consequently, following the model of Watanabe (2002), the rapeseed oil ethoxylates used with calcium chloride and calcium acetate should be regarded as spreaders and not as modifiers or activators.

## ACKNOWLEDGEMENTS

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## REFERENCES

Bukovac M J; Cooper J A; Whitmoyer R E; Brazee R D (2003). Pesticide delivery: Multiple role of adjuvants in foliar application of systemic compounds. In: *Pesticide Formulations and Delivery Systems: Meeting the Challenges of the Current Crop Protection Industry*, ASTM STP **1430**, pp. 91-107

Downer R A; Hall F R; Cooper J A; Fox R D (1999). Exploring the role of formulation and adjuvant chemistry in pesticide deposit formation. *Pesticide Formulations and Application Systems: Global Pest Control Formulations for the Next Millennium, 19th Volume*, ASTM STP **1373**, pp.29-40

Duncan A W; Holloway P J; Nigel M W (1999). Effects of some surfactants on foliar impaction and retention of monosize water droplets. *Pesticide Science* **55**, 343-389

Knoche M; Bukovac M J (2001). Finite dose studies: III. Effects of temperature, humidity and deposit manipulation on NAA penetration through isolated tomato fruit cuticles. *Pest Management Science* **57**, 737-742

Knoche M; Petracek P D; Bukovac M J (2001). Finite dose diffusion studies: I. Characterizing cuticular penetration in a model system using NAA and isolated tomato fruit cuticles. *Pest Management Science* **56**, 1005-1014

Knowles A (2001). Adjuvants for agrochemicals. *Pesticide Outlook*, 183-184

Schönherr J (2001). Cuticular penetration of calcium salts: effects of humidity, anions and adjuvants. *Journal of Plant Nutrition and Soil Science* **164**, 225-231

Watanabe T (2002). Transcuticular penetration of foliar-applied pesticides – its analysis by a logistic-kinetic penetration model. In: *Herbicide classes in development*, eds P Böger, K Wakabayashi , K Hirai, pp.319-340. Springer-Verlag, Berlin

# Enhancing rainfastness of contact fungicides with adjuvants

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## ABSTRACT

In our experiments we evaluated the effect of rapeseed (RSO), linseed (LSO) and soybean (SBO) oil ethoxylates as tank-mix-adjuvants on deposit formation and rainfastness of the contact fungicide mancozeb. Rainfastness of mancozeb alone was low, and the wash-off caused by a five millimetres simulated rain (5mm/h) could be reduced by adding selected RSO, LSO and SBO adjuvants to the spray solution. Enhancement of the rainfastness clearly depended on the number of ethylene oxide (EO) units in the hydrophilic chain. Improved rainfastness was achieved by adding the adjuvants RSO 5EO, LSO 10EO and SBO 10EO to the spray solutions.

## INTRODUCTION

Among all physicochemical, biochemical and metabolic processes that occur in the environment, rainfall has the greatest effect upon the residual activity of agrochemicals (McDowell *et al.*, 1987). The rainfall may affect the deposit characteristics by dilution, redistribution, physical removal, as well as by the extraction of pesticide from plant tissue (Thacker & Young, 1999). Due to cultivation characteristics, pest development rate and time availability, it is not always possible to apply pesticides when rain is unlikely. For these reasons agrochemicals must be rainfast, that is usually achieved with tank-mix-adjuvants (Kudsk *et al.*, 1991). In recent years a continuous trend towards development and use of adjuvants based on renewable resources (Green, 2000) like ethoxylated seed oils, has become apparent. In our studies we evaluated a new group of ethoxylated seed oils, namely rapeseed, linseed and soybean oil ethoxylates with differing ethoxylation grades for their ability to enhance the rainfastness of the contact fungicide mancozeb.

## MATERIALS AND METHODS

### Plant material and growth conditions

The experiments were conducted with 56-days old apple seedlings (*Malus domestica* Borkh.) raised from seed, in a growth chamber with constant temperature (20°C ± 1) and relative humidity (70% ± 5).

### Chemicals, fungicide application and rain simulation

The effect of rapeseed (RSO 5, RSO 10, RSO 20, RSO 60), linseed (LSO 10, LSO 0903, LSO 30, LSO 3003) and soybean (SBO 10, SBO 0903, SBO 30, SBO 3003) oil ethoxylates (Cognis® AgroSolution, Düsseldorf, Germany) at a concentration of 0.1% (wt/v) on the

rainfastness of the contact fungicide mancozeb [(manganese ethylene bis(dithiocarbamate) (polymeric) complex with zinc salt)] as Dithane Ultra WG 80% (Spiess-Urania Chemicals GmbH - Hamburg, Germany) was evaluated. The mancozeb concentration in the spray solution was 2.40g/litre a.i..The treatment solutions were applied with a Laboratory Pesticide Sprayer, equipped with a hollow cone, 80° nozzle (Lechler GmbH, Germany), placed 45cm above the plants top. Application was carried out at a speed of 6km/h and a pressure of 3 x $10^5$ Pascal, to give a volume of 390litres/ha. After a 4-h drying time, five millimeters heavy rain (5mm/h) was simulated with the B-LRS-2 rain simulator (Institute of Agricultural Engineering, University of Bonn, Germany). Untreated seedlings as well as treated but not rain-exposed seedlings served as control.

**Fungicide determination and deposit characteristics**

The second and third completely developed leaves without petiole from each seedling were taken for the residue determination. This was carried out by measuring the concentration of manganese atoms by Atomic Absorption Spectrometry (Perkin-Elmer Analyst 300 Wellesley, USA). Manganese atoms constitute about 17% of the mancozeb MW, and the concentration of the a.i. in the sample and on the leaves was calculated by a rule of three (Travis *et al.*, 1985). Fungicide residues were expressed on a fresh-weight (FW) basis. The characteristics of fungicide droplet deposits on the leaf surfaces were evaluated by scanning electron microscopy (XL 30 ESEM, FEI-Phillips, Kassel), in a low vacuum mode. Droplets (0.5µl) of the treatment solutions were applied with a microsyringe (Hamilton-Bonaduz, Switzerland) on the leaf surface and left to dry for 4h before rainfall simulation.

## RESULTS AND DISCUSSION

**Rapeseed oil ethoxylates**

The initial mancozeb concentration on the leaves was significantly reduced by addition of RSO-ethoxylates to the spray solutions (Figure 1).

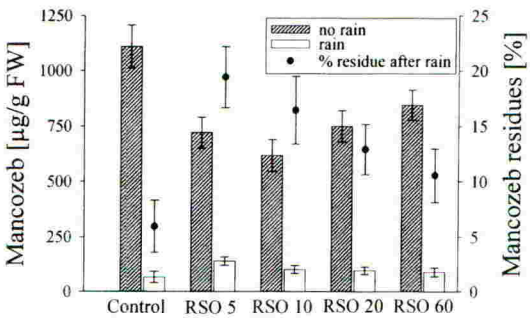


Figure 1. Effect of the addition of rapeseed oil ethoxylates to the spray solution on the retention and rainfastness of mancozeb.

Initial a.i. deposit in the control treatment was 1100µg/g FW. Addition of RSO-ethoxylates to the spray solution resulted in a reduction of mancozeb concentration on the leaf surfaces down to 650µg/g FW by the RSO 10. After the rain simulation, RSO-treatments had more a.i.

residues on the leaf surface, particularly by the addition of RSO 5 to the spray solution. Percentage of residues after the rain event (initial deposit was considered to be 100%) was 6% in the control treatment, and 19% with addition of ethoxylate RSO 5. The ethoxylates RSO 20 and RSO 60 enhanced the mancozeb rainfastness at a lower extent.

**Linseed oil ethoxylates**

The initial mancozeb concentration in the control treatment was 980µg/g FW, and due to addition of LSO ethoxylates the deposits were reduced to less than 800µg/g FW (Figure 2). The fungicide residues after 5mm heavy rain were significantly higher when LSO 10 or LSO 0903 were added to the spray solution. The percent of rainfast a.i. in the control treatment (7%) could be enhanced to 18% with addition of LSO 10 to the spray solution. The more hydrophilic adjuvant LSO 30 enhanced the rainfastness of mancozeb to a lower extent.

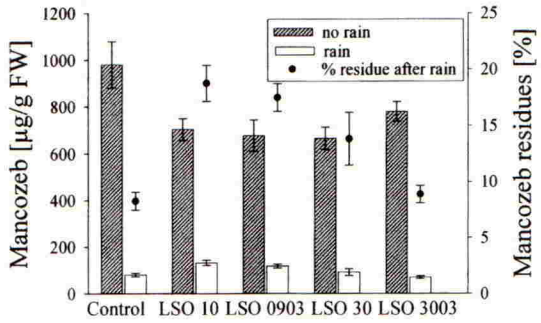


Figure 2. Effect of the addition of linseed oil ethoxylates to the spray solution on the retention and rainfastness of mancozeb.

**Soybean oil ethoxylates**

The initial fungicide concentration on the leaves was reduced by adding the SBO ethoxylates to the spray solution (Figure 3). Nevertheless, more residues resisted the rainfall when SBO 10 or SBO 0903 were added.

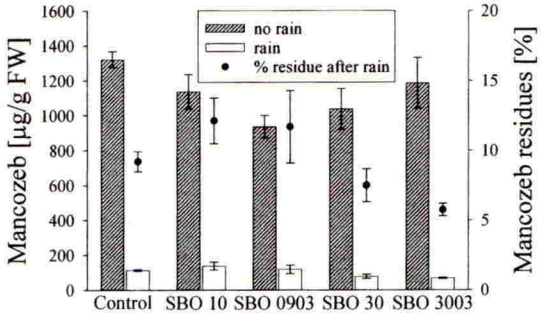


Figure 3. Retention and rainfastness of mancozeb as affected by the addition of soybean oil ethoxylates to the spray solution.

The percentage of fungicide on the leaves was enhanced from 8% in the control to about 13% following addition of SBO 10 and SBO 0903, while the adjuvants SBO 30 and SBO 3003 influenced the rainfastness of mancozeb in a negative form.

**Characterization of deposits**

As a rule, irrespective of treatment, mancozeb was not uniformly distributed inside the droplet area, and the residues were in crystalline form, mainly in little depressions in between cells and along the veins (Figure 4A).

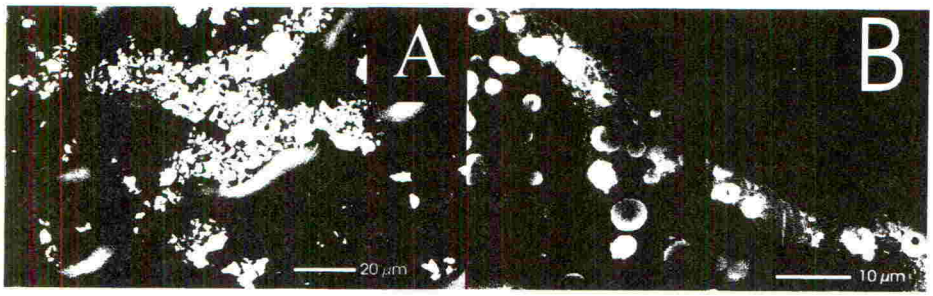


Figure 4. Mancozeb deposits on the adaxial surface of apple seedlings. A –crystals of the active ingredient deposited between leaf cells, before rain event. B – mancozeb forming rings and balls between and above the cells, after the rain event.

Rain markedly changed the deposit characteristics, but the alteration pattern was the same for all treatments. Fungicide residues were redistributed inside the original droplet area, while the presence of residues outside the droplet limits was not observed. A great part of the a.i. was still left in depressions between the cells, but mancozeb was also in a transparent hydrophobic layer over the cells. The remaining a.i. formed little balls or annuli (Figure 4B). The similarities between mancozeb deposits without or with seed oil ethoxylates addition in terms of form and structure of the deposits can be explained by the presence of adjuvants in the commercial formulation used in the experiments.

## REFERENCES

Green J M (2000). Adjuvant outlook for pesticides. *Pesticide Outlook* **11(5)**, 196-199.

Kudsk P; Mathiassen S K; Kirknel E (1991). Influence of formulations and adjuvants on the rainfastness of maneb and mancozeb on pea and potato. *Pesticide Science* **33**, 57-71.

McDowell L L; Willis G H; Southwick L M; Smith Jr S (1987). Fenvalerate wash-off from cotton plants by rainfall. *Pesticide Science* **21**, 83-92.

Thacker J R M; Young R D F (1999). The effects of six adjuvants on the rainfastness of chlorpyrifos formulated as an emulsifiable concentrate. *Pesticide Science - Extended Summaries of the IUPAC Conference* **55**, 198-200.

Travis J W; Sutton T B; Skroch W A (1985). A technique for determining the deposition of heavy metals in pesticides. *Phytopathology* **75(7)**, 783-785.

# Significance of apple leaf surface characteristics for retention and rainfastness of the fungicide mancozeb

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## ABSTRACT

It is not clear so far, whether alteration of leaf micromorphology and surface wax chemistry due to environmental factors, such as UV-B radiation, affects retention and rainfastness of applied pesticide solutions. In this study UV-B treated and untreated adaxial apple leaf surfaces were characterized in terms of the chemical composition, morphological surface structure and hydrophobicity by means of GC/MS, electron scanning microscopy (SEM) and contact angle measurement. Furthermore the retention and rainfastness of the fungicide mancozeb, was studied. After exposure to enhanced UV-B radiation (0.022 KW/m²) the total wax mass decreased and the chemical composition altered, whereas contact angle of applied water droplets did not change significantly compared to the untreated control. The alteration of the surface wax quantity and quality did not significantly affect rainfastness of the fungicide spray solution, whereas retention of a.i. increased 24 h after radiation with UV-B.

## INTRODUCTION

In all terrestrial plants the cuticle, consisting of a polymeric cutin matrix and soluble cuticular waxes, forms a protective coating on aerial parts preventing the plant from desiccation due to uncontrolled non-stomatal water loss and loss of organic and inorganic compounds by leaching. Cuticular waxes is a general term for complex mixtures of homologue series of long chain aliphatics (Riederer & Markstädter, 1996). The cuticle has a more or less rough cover of epicuticular wax (Barthlott, 1990), whereas intracuticular waxes are embedded in the polymer matrix. Retention and rainfastness of fungicides depend on the physical and chemical properties of the target surface as well as on the chemical properties of the applied spray. As described elsewhere (Barnes *et al.*, 1994) enhanced UV-B radiation may modify the chemical composition of surface wax and therefore also alter retention and rainfastness of applied fungicides. In order to understand the impact of enhanced UV-B radiation on the apple leaf surface, retention and rainfastness of the fungicide mancozeb were studied.

## MATERIAL AND METHODS

### Plant material and growth conditions

Plants of *Malus domestica* cv. `Golden Delicious` were raised in a growth chamber at constant temperature of 20°C ± 1°C and a relative humidity of 70% ± 5%. After 8 weeks the second completely developed leaf from the top was used for the experiments.

**UV-B radiation conditions**

UV-B radiation was provided by 9x100 W tubes of UV-B lamps (`Philips`, Germany), having an emission spectrum of 280nm-320nm. Level of irradiation was controlled by a precalibrated spectroradiometer (`Gröbel`, Karlsruhe, Germany). The dose of irradiance amounted to 0.022KW/$m^2$ applied for 150min. The samples were studied 0, 24 and 48 h after the irradiation with UV-B. Plants not exposed to UV-B radiation served as control.

**Wax extraction / microscopy**

The adaxial leaf side was immersed in chloroform for 20s, after adding the internal standard ($C_{24}$ alkane, Tetracosane). Then the samples were evaporated under a stream of nitrogen. Subsequently 20µl of pyridine (Merck, Germany) and 20µl BSTFA [(N,O-bis (trimethylsilyl) trifluoracetamide), Machery-Nagel, Germany] were added and the samples were incubated for 40min at 70°C. The specimens were analysed after adding 50µl of chloroform by GC-MS (5890 series II; HP, Avondale, PA).

The micromorphology of the leaf surfaces was studied by scanning electron microscope XL-30-ESEM (FEI-Philips, Kassel, Germany) under low vacuum conditions. The samples were examined uncoated at 4°C.

**Goniometry**

The hydrophobicity of adaxial apple leaves was assessed by drop shape analysis (Krüss G10, Germany), cutting a part from the central area of the leaf lamina avoiding the middle vein. Leaves were fixed to glass slides with double-sided adhesive tape. The contact angle of an applied drop of distilled water (volume 1µl) was measured.

**Fungicide application and rain simulation**

Mancozeb [(manganese ethylene bis(dithiocarbamate)(polymeric) complex with zinc salt] as Dithane Ultra WG 75% (Spiess-Urania Chemicals GmbH, Hamburg, Germany) was used for experiments. Fungicide concentration was 2.40g/litre a.i. and applied with a Laboratory Pesticide Sprayer, equipped with a hollow cone, 80° nozzles (Lechler GmbH, Germany) placed 45cm above the top of the plants. At a speed of 6km/h and a pressure of 3 x $10^5$ Pascal the application was accomplished. The total volume of the applied fungicide solution was 390 litres/ha. Exposure to heavy rain (5mm/h) was simulated 4h after fungicide application by means of a B-LRS-2 rain simulator (Institute of Agricultural Engineering, University of Bonn, Germany). Untreated as well as treated but not rain-exposed seedlings served as reference.

The rainfastness of mancozeb (17% of *Mr*) was evaluated by measuring the concentration of manganese atoms by Atomic Absorption Spectrometry (Perkin-Elmer Analyst 300 Wellesley, USA). The fungicide residues were related to fresh weight of leaves.

## RESULTS

The apolar wax compounds, extracted from the adaxial leaf surface, were identified as: triterpenes (oleanolic acid, ursolic acid), primary alcohols, fatty acids, alkanes and esters, each group having a certain chain length ranging from $C_{22}$ (fatty acids) until $C_{52}$ (esters). The total wax mass ranged from 0.34µg/cm² (control) to 0.51µg/cm² (24h) and decreased after 48h to

0.44µg/cm². Statistical analysis showed that no interaction between time of sampling and exposure to enhanced UV-B irradiation concerning the wax mass could be detected. Independent of time $C_{26}$ alcohol increased after enhanced UV-B radiation (Table 1).

Table 1. Effect of time after UV-B exposure and enhanced UV-B radiation on wax mass of apolar wax compounds, n=4, mean. Duncan, $P \leq 0.05$, ns=not significant

| Compound | Wax mass µg/cm² | | | | |
|---|---|---|---|---|---|
| | Sampling time after enhanced UV-B radiation | | | UV-B radiation | |
| | 0 | 24 | 48 | No | Yes |
| $C_{22}$ acid | $0.004^{ns}$ | $0.004^{ns}$ | $0.003^{ns}$ | $0^{b}$ | $0.004^{a}$ |
| $C_{24}$ alcohol | $0.004^{ns}$ | $0.006^{ns}$ | $0.004^{ns}$ | $0^{b}$ | $0.009^{a}$ |
| $C_{26}$ alcohol | $0.068^{b}$ | $0.099^{ab}$ | $0.109^{a}$ | $0.087^{ns}$ | $0.096^{ns}$ |
| acid | 0.044±0.008 | 0.039±0.009 | 0.031±0.009 | 0.032±0.008 | 0.044±0.008 |
| alcohol | 0.151±0.016 | 0.198±0.016 | 0.190±0.016 | 0.188±0.013 | 0.171±0.013 |
| alkane | 0.054±0.010 | 0.065±0.010 | 0.064±0.001 | 0.064±0.008 | 0.058±0.008 |
| triterpenes | 0.093±0.011 | 0.144±0.011 | 0.107±0.011 | 0.125±0.009 | 0.103±0.009 |
| ester | 0.036±0.006 | 0.043±0.006 | 0.045±0.006 | 0.038±0.005 | 0.045±0.005 |

Inbetween enhanced UV-B radiation and sampling no significant interaction could be detected with respect to the contact angle, ranging from 102° up to 104° in control plants and from 100° up to 103° in UV-B irradiated plants. The retention of mancozeb was significantly increased at 24h after UV-B radiation. The retention did not change at 48h after enhanced UV-B radiation. UV-B exposure of leaf surface did not significantly affect rainfastness of fungicide spray solution (Figure 1).

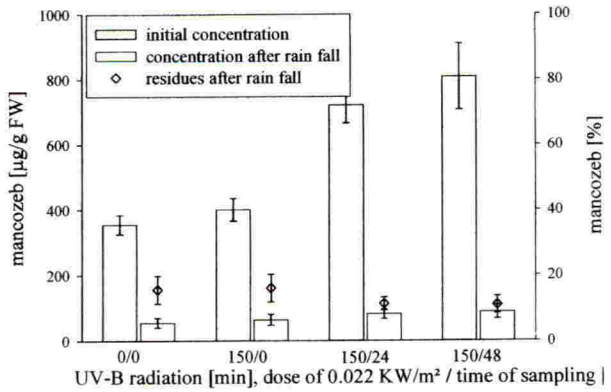


Figure 1. Effect of UV-B radiation on retention and rainfastness of mancozeb

Experiments concerning the morphological surface characteristics of the adaxial apple leaf, using SEM, displayed the typical puzzle-like epidermal cells showing a distinctive curvature of the periclinal cell walls. The control plants showed a high density of lamellae of the cuticle whereas the radiated surface had less lamellae. After enhanced UV-B radiation a significant alteration of the amorphous surface could not be shown (Figure 2).

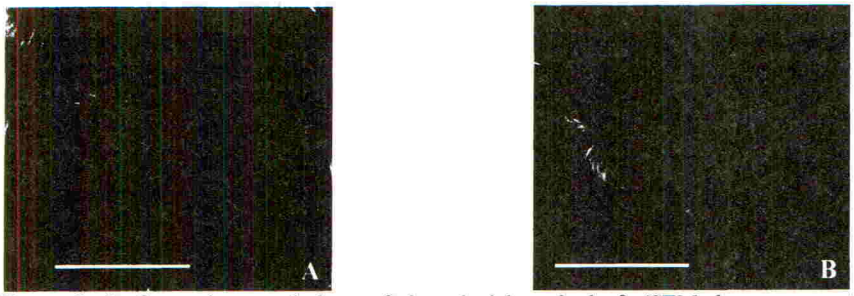

Figure 2. Surface micromorphology of the adaxial apple leaf. (SEM, bars represent 50µm) A: control; B: 24h after enhanced UV-B radiation

## DISCUSSION

UV-B radiation may influence chemical and physical characteristics of apple leaf surface and therefore retention and rainfastness of applied spray solution. The low level of extracted wax may be due to their growing in a controlled environment and the very young age of studied specimens. The impact of enhanced UV-B radiation on characteristics of apple leaves surfaces is a process occuring 48h after irradiation. Whereas 24h are decisive for retention of mancozeb, 48h plays a role concerning the alteration of single wax compounds. It was shown, that contact angles did not alter after enhanced UV-B treatment but reduced the lamellae of the surface like ontogenesis of apple leaves (Bringe *et al.*, 2005). There seems to be an interaction between changed environmental conditions (UV-B) and retention of mancozeb. Further investigations concerning UV-B radiation, time of sampling and surface wax chemistry should be done.

## REFERENCES

Barnes J D; Paul N; Percy K E; Broadbent P; McLaughlin C K; Wellburn A R (1994). Effects of UV-B radiation on wax biosynthesis. In: *Air Pollutants and the Leaf Cuticle, eds.* K E Percy, J N Cape, R Jagels & C Simpson**, pp.** 195-204 NATO ASI Series G **36** Heidelberg, Springer-Verlag.

Barthlott W (1990). Scanning electron microscopy of the epidermal surface in plants. In: *Scanning Electron Microscopy in Taxonomy and Functional Morphology.* ed. D Claugher Systematic Association Special Volume **41**, pp. 69-83. Clarendon Press, Oxford.

Bringe K; Schumacher C F A; Schmitz-Eiberger M; Steiner U; Oerke E-C (2005). Ontogenetic variation in chemical and physical characteristics of adaxial apple leaf surfaces. *Phytochemistry* (submitted).

Ensikat H J; Neinhuis C; Barthlott W (2000). Direct access to plant epicuticular wax crystals by a new mechanical isolation method. *International Journal Plant Science* **161**, 143-148.

Riederer M; Markstädter C (1996). Diffusion through cuticles: principles and models. In: *Plant cuticles: An integrated functional approach.* ed. G Kerstiens, pp. 189-200, BIOS Scientific Publishers, Oxford.

Schmitz M; Noga G (2000). Tocopherol and its potential for improving fruit quality in apple. *Acta Horticulturae* **527**, 111-117.

# Analysis of the molecular response to an etheramine surfactant application in *Arabidopsis* using microarrays

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## ABSTRACT

Herbicides provide growers with the means to chemically manage weeds and surfactants are often an effective way of improving the performance of these herbicides. However, the mode of action of surfactants at the molecular level is not well understood. As a preliminary step towards understanding the molecular mechanism of action of an etheramine surfactant, plant gene expression in response to foliar application of surfactant NUL1026 was analysed in *Arabidopsis thaliana* using the commercially available Affymetrix ATH1-121501 chip. One hundred and eighty-nine genes were found to be significantly up-regulated while 15 genes were significantly down-regulated 1 hour after plants were treated with 0.2% (v/v) of surfactant NUL1026. Functional category analysis of these genes revealed that the largest categories included metabolism, physiological processes, transport, protein metabolism, response to stimulus and transcription. Interestingly, microarray results showed the regulation of a number of genes encoding enzymes involved in the jasmonic acid biosynthesis pathway and also the *ACC* synthase gene for ethylene production were up-regulated indicating that treatment with surfactant NUL1026 affects the expression of a number of genes involved in the detoxification, senescence and signalling pathways.

## INTRODUCTION

Surfactants are used mainly with postemergence herbicides to help overcome the physical and biological barriers that prevent movement of the herbicide from the leaf surface to the interior of the cell (Holloway & Stock, 1990). By combining a surfactant that is highly effective with a particular herbicide, the rates of active ingredient used in weed control can sometimes be reduced.

However, surfactants themselves may be phytotoxic to plant tissue and may have their own intrinsic biological function. Hence, to further understand the molecular responses within a plant to surfactant application, this study reports global changes in gene expression in response to an etheramine surfactant, surfactant NUL1026, using the ATH1-121501 Affymetrix array. This approach has enabled the identification of putative surfactant responsive elements, which could potentially be instrumental in the development of new formulations that would be better targeted, offer more crop safety, allow for lower use rates, and hence be less stressful for the environment.

## MATERIALS AND METHODS

### Plant growth conditions and surfactant application

*Arabidopsis thaliana* ecotype Columbia seeds were surface-sterilised and grown for 15 days *in vitro* in Petri-dishes on 30 mL half strength Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with 1.5% (w/v) sucrose and solidified with 1% Phytagel (w/v). *A. thaliana* plants were sprayed with 0.2% (v/v) surfactant NUL1026 at a rate of 96 mL across 12 plates using the Potter spray tower at 40 kPa (Herron *et al.*, 1995). Control plants were sprayed with water.

### Target preparation, hybridisation and data analysis

Total RNA was extracted from 100 mg plant material after 1 hour post-spraying and isolated RNA samples were then processed as recommended by Affymetrix® (2002). All image and data analysis was performed using the Microarray Suite version 5.0 (MAS 5.0, Affymetrix®) while Micro DataBase version 3.0 (MicroDB 3.0) served as the interface. Data Mining Tool version 3.0 (DMT 3.0) was used for in depth data mining. Functions were assigned based on automatically derived functional categories by The Arabidopsis Information Resources (TAIR): http://www.arabidopsis.org/tools/bulk/go/index.jsp.

## RESULTS AND DISCUSSION

From a total of 22,810 genes present on the ATH1-121 501 array, 189 genes were induced by surfactant treatment while 15 genes were down-regulated in response to foliar treatment with surfactant NUL1026. In this investigation, *A. thaliana* leaves were sprayed with 0.2% (v/v) surfactant NUL1026 and a number of genes predicted to be involved in xenobiotic metabolism were regulated clearly indicating that this level of surfactant caused xenobiotic perturbation in plants.

The TAIR database classified those surfactant responsive genes into the following functional categories: metabolic processes, physiological processes, transport, transcription, protein metabolism, response to abiotic and biotic stimulus and signal transduction. In depth study of the 'response to abiotic and biotic stimulus' category revealed that a number of genes involved in the jasmonic acid (JA) biosynthesis and signaling pathways were up-regulated. JA, a plant signaling molecule plays a major role in a number of abiotic and biotic stresses but is best known for its role in wound responses (Creelman and Mulpuri, 2002).

Moreover, the expression level of the gene encoding the senescence associated family protein which also shows similarity to the senescence-associated protein 5, also increased following foliar application of surfactant NUL1026. It has been suggested that JA promotes leaf senescence by inducing the transcription of a number of senescence associated proteins genes (SAGs). Expression of SAGs is considered to be the driving force behind leaf senescence (Buchanan-Wollaston *et al.*, 2003).

Treatment with surfactant NUL1026 also enhanced the expression of the gene encoding a member of the 1-aminocyclopropane-1-carboxylate (*ACC*) synthase, *ACS6*, a rate-limiting enzyme in the ethylene biosynthesis pathway (Yang & Hoffman, 1984). Leaf senescence is

also promoted by ethylene and increase in transcripts of the senescence-associated protein gene may thus be also due to the ethylene produced. Besides, as shown by Sasaki *et al.* (2003), the JA pathways interact with the ethylene signal pathways in the expression of defence responses and O'Donnell *et al.* (1996) have reported that during wounding, ethylene and JA increase the level of each other in the plant and both compounds work in concert to regulate gene expression.

The *A. thaliana* molecular response following the application of adjuvant NUL1026 included the transcriptional up-regulation of transcriptional factors (TFs) and DNA binding proteins. Well-known categories of TFs were found to be surfactant-responsive and these included the plant specific WRKY TFs, known to be induced in response to pathogen, wounding and senescence (Eulgem *et al.*, 2000). In addition, our study also registered the transcriptional activation of the ethylene-response factors (ERFs), which belongs to the AP2 transcription factors. Of particular interest, was the up-regulation of the *ERF1* gene. This *ERF1* gene is considered as the convergent point between ethylene and JA pathways and represents a plausible downstream component of both these signaling pathways. Other ERFs have also been shown to be induced by JA and ethylene (Brown *et al.*, 2003) and likewise, treatment with NUL1026, triggered the up-regulation of *ERF5*, *ERF6* and *ERF2* genes. This cross-talking between the JA and ethylene pathways may amplify the plant response to surfactant NUL1026 by inducing the expression of a number of stress and defense response genes.

Microarray results provided strong indication that surfactant NUL1026 may be exerting its effects on plant tissues through a direct action on cell membrane. The genes that responded to surfactant NUL1026 are also induced by pathogen attack or wounding. This common response can be explained by the fact that in these events, the cell membrane is being targeted first. It appears that foliar application of surfactant NUL1026 may have had a severe impact since it seemed to have activated the biosynthesis of JA and ethylene, which in turn may have induced the transcription expression of the gene encoding senescence. Clearly, the results presented in this study show that application of surfactant NUL1026 may be having a suppressive effect on plant growth at the rates used in this experiment. The JA and ethylene biosynthesis, which might have arisen upon application of surfactant NUL1026, may potentially contribute to growth inhibition and eventual cell death by pesticides.

This study is the first step towards identifying those genes responsive to stimulation by surfactant NUL1026. Microarray analyses provided information on a range of putative "surfactant responsive" genes, including transcription factors and signaling components. Further examinations of these surfactant-regulated genes reflect the chemical xenobiotic nature of surfactant NUL1026, as a number of genes associated with Phases I- III detoxification were induced upon treatment with NUL1026. Besides, activation of these genes implies that surfactant NUL1026 may be penetrating inside the leaf tissues and consequently, this infiltration may reduce the resistance to diffusion of agrochemicals into the leaf. Due to increasing pressures to reduce agrochemicals usage, the search for surfactant products that perform very specific functions that complement pesticide chemistries has become crucial. Characterisation of the transcripts found to be responsive to surfactant NUL1026, not only provides an insight into gene function with respect to detoxification and stress responses but also provide an inventory of genes which can be used to develop more sophisticated surfactants that can affect the agrochemicals, the target, application and the environment simultaneously.

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## REFERENCES

Affymetrix (2002). GeneChip expression analysis technical manual. Affymetrix®, Santa Clara, California, USA.

Brown R L; Kazan K; Mcgrath K C; Maclean D J; Manners J M (2003). A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of *Arabidopsis*. *Plant Physiology* **132**, 1020-1032.

Buchanan-Wollaston V; Earl S; Harrison E; Mathas E; Navapour S; Page T; Pink D (2003). The molecular analysis of leaf senescence - a genomics approach. *Plant Biotechnology Journal* **1**, 3-22.

Creelman R A; Mulpuri R (2002). The Oxylipin Pathway in *Arabidopsis*. *The Arabidopsis Book*, 1-24.

Eulgem T; Rushton P J; Robarzek S; Somssich I E (2000). The *WRKY* superfamily of plant transcription factors. *Trends in Plant Science* **5**, 199-206.

Herron G A; Beattie G A C; Parkes R A; Barchia I (1995). Potter spray tower bioassay of selected Citrus pests to petroleum spray oil. *Journal of the Australian Entomological Society* **34**, 255-263.

Holloway P J; Stock D (1990). Factors affecting the activation of foliar uptake of agrochemicals by surfactants. In: *Industrial Applications of Surfactants II*. (ed. D R Karsa, 303-337, Special Publication No. 77, Royal Society of Chemistry, Cambridge, UK.

Murashige T; Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiology Plant* **15**, 473-497.

O'Donnell P J; Calvert C; Atzorn R; Wasternack C; Leyser H M O; Bowles D J (1996). Ethylene as a signal mediating the wound response of tomato plants. *Science* **274**, 1914-1917.

Sasaki A; Itoh H; Gomi K; Ueguchi-Tanaka M; Ishiyama K, Kobayashi M; Jeong D H; An G; Kitano H; Ashikari M; Matsuoka M (2003). Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. *Science* **299**, 1896-1898.

Yang S F; Hoffman N E (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology* **35**, 155-189.

# 3′ End sequencing and TPALi for the detection and classification of potyviruses causing mosaic disease of sugarcane

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## ABSTRACT

Sugarcane mosaic is an economically important disease of sugarcane worldwide and can be caused by at least four different potyviruses including sugar cane mosaic (SCMV) and maize dwarf mosaic viruses (MDMV). Accurate virus detection and identification is of key importance in any development programme to produce resistant sugarcane varieties. Technological advances in rapid sequencing of PCR products and the great increase in genomic sequence information available for potyviruses make it feasible to use bioinformatic approaches to aid identification and classification of unknown viruses. This paper describes a new method for the detection, identification and classification of potyviruses and their related strains from naturally infected plants using a combined approach of sequencing 3′ terminal PCR products and TOPALi phylogenetic analysis. The method was demonstrated using three naturally occurring and symptomatically different potyvirus isolates from Iranian sugarcane germplasm. Of the three viruses, two were identified as closely-related isolates of SCMV and one as MDMV and the results were consistent with serological analysis.

## INTRODUCTION

Sugarcane (*Saccharum* spp.) is a highly heterozygous clonally propagated crop providing about 65% of the world sugar production. Several different viruses induce mosaic symptoms in sugarcane, for example, *Sugarcane Mosaic Virus* (SCMV), *Maize dwarf mosaic virus* (MDMV), *Sorghum mosaic virus* (SrMV), and *Sugarcane streak mosaic virus* (SCSMV).

It has been shown that the highly variable untranslated regions (UTR) of potyviral genomes may be used as strain- and virus-specific probes for sensitive virus detection and differentiation, while the sequences can also be used for estimating close phylogenetic relationships (Frenkel *et al.*, 1992; Mc Kern *et al.*, 1992). Pappu *et al.* (1993) have developed a method for the group-specific PCR amplification of the 3′ end of potyvirus genomes. Marie-Jeanne *et al.* (2000) modified the method for the discrimination of *Poaceae* infecting potyviruses on the basis of the amplification of a highly conserved motif in the CP gene (between MVWCIEN and QMKAAA) and by enzymatic restriction of this fragment with *Alu*I and *Dde*I. The oligonucleotides used by Marie-Jeanne *et al.* (2000) were designed from the

alignment of sequences restricted to *Poaceae* potyviruses to minimize the degeneracy of primers since it has previously been shown that mismatches particularly at the 3′ termini of primers have a strong negative effect on the efficiency of the amplification reactions (Langeveld *et al.*, 1991). Although, this method differentiated *Poaceae* potyviruses, a disadvantage is that a single mutation is sufficient for an isolate to lose a restriction site and to inhibit isolate identification.

The work in this paper describes the phylogenetic analysis of 3′ terminal sequences derived by a modified PCR using degenerate primers and TOPALi software (Milne *et al.*, 2004) for the identification and classification of potyviruses causing mosaic disease of sugarcane in Iran.

## MATERIALS AND METHODS

### Virus source and maintenance

Isolates Sugarcane-Q86, Sugarcane-L66 and Sugarcane-CP68 were obtained from sugarcane plants grown in the Sugarcane Research Centre germplasm museum, Ahvaz, Iran. Previous studies showed that these three isolate were serologically different (Ghasemi *et al.*, 2002). All virus isolates were propagated in sorghum (*Sorghum bicolour var.* Mina) and held in an insect-proof glasshouse at 15-30°C with 10-16 h of illumination per day.

### Virus genome amplification, cloning and sequencing

Virus RNA extractions from leaf tissue were prepared using an mRNA capture kit following the manufacturer's recommendation (Roche, Germany). One tube RT-PCR using the forward primer, oligo 1n, with sequence 5′-ATGGTHTGGTGYATHGARAAYGG-3′ (H = A/C/T, Y = C/T, R= A/G, K = G/T) and reverse primer, oligo-dT mixture with sequences 5′-(21) T A-3′, 5′-(21) T C-3′, and 5′-(21) T G-3′ was applied as following conditions: one hour at 42°C, followed by 94°C for 5 min, then five cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 45s, 72°C for 3 min and finally 72°C for 30 min. Purified PCR products were ligated into the plasmid pGEM-T (pGEM-T Vector System I kit,Promega, USA) following the manufacturer's instructions and plasmid DNA propagated in *E.coli* DH5α.. Sequences from two independent clones were obtained for each virus isolate.

### Phylogenetic analysis

EMBL/GenBank/DDBJ accession numbers and sources of the viral DNA sequences used in the alignments are shown in Table 1. Sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997) with default parameters, no subsequent editing was required. Phylogenetic reconstructions were obtained by the Neighbor-Joining method using TOPALi software.

## RESULTS AND DISCUSSION

### PCR amplification, cloning, sequencing and determination of recombination events

Using the degenerate universal primer, Oligo1n as forward primer and a modified oligo dt as the reverse primer in PCR, a product of approximately 700 bp was obtained from cDNA prepared from RNA samples from each virus isolate, but not from total RNA preparations from healthy sugarcane (Figure 1, left). The nucleotide sequences of each of the PCR products contained, as expected, a 462 bp fragment corresponding to the C-terminal portion of the CP followed by a non-coding region of 231bp and a polyadenylated terminus.

Table 1. Viruses and accession numbers used in the phylogenetic trees

| Virus acronym* | Accession No. | Virus acronym | Accession No. | Virus acronym | Accession No. |
|---|---|---|---|---|---|
| SCMV-A | U57354 | MDMV-Hung | AJ542536 | MDMV-A | U07216 |
| SCMV-B | U57355 | MDMV-Bulg | AJ001691 | MDMV-B | D00949 |
| SCMV-D | U57356 | SrMV-H | U57358 | MDMV-Spin | AJ416633 |
| SCMV-E | U57357 | SrMV-I | U57359 | Sugarcane-Q86 | AY648298 |
| SCMV-Mx | AY195610 | SrMV-M | U57360 | Sugarcane -L66 | AY678443 |
| SCMV-Ger | X98165 | SrMV-CHNXi | AJ10197 | Sugarcane -CP68 | AY648951 |
| SCMV-Aba | AY434731 | SrMV-SCH | U07219 | PVY-O | AY512655 |
| SCMV-CHN | AJ297628 | JGMV-O | U07217 | JGMV-KS1 | U07218 |

* SCMV = Sugarcane mosaic virus; SrMV = Sorghum mosaic virus; MDMV = Maize dwarf mosaic virus; JGMV= Johanssongrass mosaic virus; Sugarcane-Q86 = A Potyvirus causing mosaic disease in sugarcane variety Q86 in Iran; Sugarcane-L66 = A Potyvirus causing mosaic disease in sugarcane variety L66 in Iran; Sugarcane-CP68 = A Potyvirus causing mosaic disease in sugarcane variety CP68-1119 in Iran; PVY- O = Potato virus Y isolate O.

**Phylogenetic analysis**

Phylogenetic analysis of the 691bp 3′ terminal region of the 26 potyvirus isolates was carried out using the TOPALi programme. The NJ tree (Figure 1, right) was reasonably well-resolved based on the interpretation of bootstrap support values of greater than or equal to 70%. Two Iranian sugarcane isolates (SC-Q86 and SC-CP68) together with 10 other SCMV isolates formed a major SCMV clade (100% support), with the other isolates split into four minor clades, a 4-isolate SrMV clade (100% support), a 5-isolate MDMV clade (100% support), a 2-isolate JGMV clade (100 % support), a ZeMV clade, and the SrMV-CHNXi appearing to be in a clade of its own. The third Iranian isolate SCMV-L66 clearly formed part of the MDMV clade.

Therefore, phylogenetic analysis of the 3' terminal sequences derived from PCR products has enabled the discrimination and classification of potyviruses causing sugarcane mosaic disease in naturally-infected sugarcane plants and the results were consistent with serological analysis. This combination of RT-PCR using degenerate primers and phylogenetic analysis may be useful for virus detection and discrimination in other potyvirus-host pathosystems. This method combines the advantages of the group-specific PCR detection method of Langeveld *et al.* (1991) with the strain-specific 3′ UTR hybridization/sequencing approach of Frenkel *et al.* (1992).

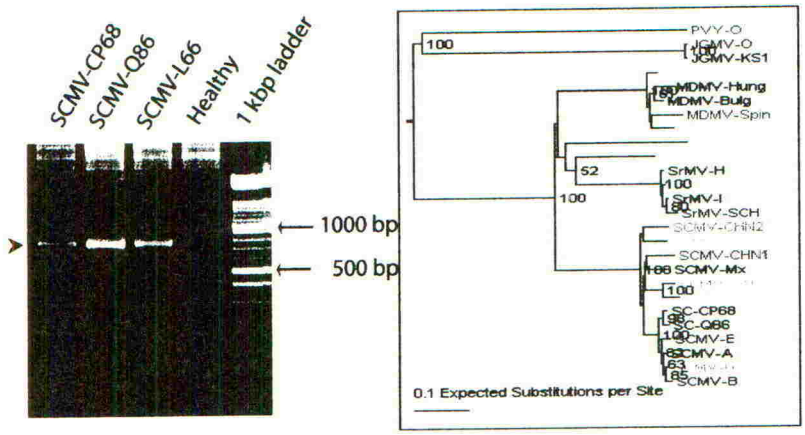


Figure 1. RT-PCR amplification of 700 bp 3′ end of Sugarcane isolates (left) Phylogenic status of Iranian *Poaceae* potyviruses (right)

## REFERENCES


Frenkel M J; Jilka J M; Shukla D D; Ward C W (1992). Differentiation of potyviruses and their strains by hybridization with the 3' non-coding region of the viral genome. *J. Virol. Methods* **36**, 51-62.

Ghasemi S; Taherkhani K; Izadpanah K (2002). Potyviruses causing mosaic in sugarcane in Khouzestan. *Proceeding of the 15th Iranian plant protection congress*. 7-11 Sept. 2002. Kermanshah, Iran.

Langeveld S A; Dore J-M; Memelink J; Derks A F L M; van der Vlugt C I M; Asjes C J; Bol J F (1991). Identification of potyviruses using the polymerase chain reaction with degenerate primers. *J. Gen. Virol.* **72**, 1531-1541.

Marie-Jeanne V; Ioos R; Peyre J; Alliot B; Signoret P (2000). Differentiation of Poaceae Potyviruses by Reverse Transcription-Polymerase Chain Reaction and Restriction Analysis. *J. Phytopathology* **148**, 141-151.

McKern N M; Ward C W; Shukla D D (1992). Strains of bean common mosaic virus consist of at least two distinct potyviruses. In: *Potyvirus Taxonomy*, ed. O W Barnett, *Achieves of Virology* **43**, suppl. 5. Springer, Vienna.

Milne I; Wright F; Rowe G; Marshall D F; Husmeier D; McGuire G (2004). TOPALi: Software for automatic identification of recombinant sequences within DNA multiple alignments. *Bioinformatics* **20**, 1806-1807.

Pappu S S; Brand R; Pappu H R; Rybicki E P; Gough K H; Frenkel M J; Niblett C L (1993). A polymerase chain reaction method adapted for selective amplification and cloning of 3' sequences of potyviral genomes: application to dasheen mosaic virus. *J. Virol. Methods*, **41**, 9-20.

Thompson J D; Gibson T J; Plewniak F; Jeanmougin F; Higgins D G (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acida Research* **24**, 4876-4882.