Project title:	Assessment of plant elicitors to induce resistance against head-rot in broccoli
Project number:	FV 378
Project leader:	Nicola Holden, SCRI
Report:	Annual report, Feb 2011
Previous report:	None
Key staff:	Nicola Holden Ian Toth Adrian Newton Dale Walters (SAC)
Location of project:	Scottish Crop Research Institute (Invergowrie, Dundee); Peacehill (Wormitt, Fife); Pitlochie (Kinross)
Industry Representative:	Alistair Ewan & Richard Haacker (East of Scotland Growers); David Martin (Plantsystems)
Date project commenced:	1st April 2010
Date project completed (or expected completion date):	31st March 2012

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

[Dr. Nicola Holden]
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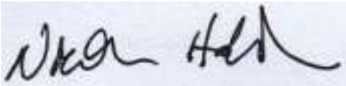
Signature .............   Date .....01/04/11................

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GROWER SUMMARY

Headline

On-going trials aim to determine whether head-rot in broccoli (calabrese) caused by opportunistic soil bacteria, can be controlled through the use of ‘elicitors’ to induce the plants own defence response.

Background

Head-rot in broccoli is a major cause of crop loss in the UK and abroad. It is caused by bacteria in the soil that are able to infect the plants under ‘ideal’ conditions, in particular high humidity. The bacteria are present in high numbers in soil and are ubiquitous, and because they will only infect plants under the most appropriate conditions, they are termed opportunistic pathogens. Current treatments rely on copper oxychloride (e.g. Cuprokylt), which act as a disinfectant on the plant. However, because the source of the bacteria is effectively limitless and the chemicals can be damaging to the plants and to the environment, finding suitable alternative treatments is a high priority. Plant defence elicitors do not have anti-microbial activity against fungi, bacteria or other pathogens, but instead they trigger a defensive reaction in the plant, making the plants more resistant to infection. Application of elicitors can be incorporated into a normal fungicide regime. Together these aspects make them an excellent candidate for treatment against head-rot in broccoli.

Summary of the project and main conclusions

A two-year project (2010 – 2012) was undertaken to assess whether elicitors can be used to protect broccoli against bacterial head-rot. Some of the elicitors have been used successfully against bacterial pathogens on other crops, for example Probenazole (Oryzmate) is a standard treatment against rice blast in Asia. Others have shown promise in experimental greenhouse trials, including BABA and Bion against head-rot in broccoli. Elicitors are potentially a good alternative to disinfectants for diseases such as head-rot where the bacteria that cause the disease are opportunistic pathogens, ubiquitous and present in high levels in the soil.

Methods

In year 1, broccoli transplants (vars. Marathon, Parthenon) were planted in mid April and mid July 2010 in replicated experimental sites (SCRI, Invergowrie and SAC, Pitlochie) for trials 1 & 2 respectively. In addition, a commercial site (Wormitt) was used to test licensed fungicides, where the transplants were planted in July 2010, for trial 3. The trial sites were approximately 100 m x 80 m, made up of six beds of treated plants, each bordered by 'guard' beds containing un-treated plants. In each treatment plot 20 plants were assessed for trials 1 and 2, and 60 plants assessed for trial 3. Each treatment plot was replicated three times. Assessment was carried out approximately 9 weeks after planting.

A group of five elicitors (BABA, Bion, *cis*-jasmone, Probenazole, Yea foliar) and three licensed fungicides with known or expected elicitor activity (Amistar, Justice, Flyer) were selected for the trial. Cuprokylt was included as a positive control in the commercial trial and all trials included a negative, untreated control. The elicitors were used either singly or in combination, with the aim of triggering multiple defensive pathways in the plant. Elicitors were applied three times in 10-day intervals; the first application was approximately one week before head initiation. A cocktail of head-rot bacteria was applied twice with a one-week interval and the first application was between the first and second elicitor application.

Laboratory experiments were carried out to determine the relative contribution of disease from three different head-rot bacteria species. The presence of head-rot bacteria on trial plants was detected using laboratory tests.

Results

Trials 1 & 2: Half the plants infected with head rot bacteria

- Trial 1 (April 2010): No significant effect on presence of head-rot symptoms was found with any of the treatments. However, plants that had been treated with bacteria were significantly more diseased than the untreated plants (this difference was only apparent at the SCRI site). Combinations containing Probenazole and Amistar appeared to provide some protection, while the combination of BABA and Bion resulted in the highest level of disease. The main issue from trial 1 was a lack of head-rot symptoms which made any statistical differentiation between the treatments difficult. Possible reasons for the lack of disease were particularly dry weather in June 2010 and low levels of nitrogen.

- Trial 2 (July 2010): Lack of disease symptoms meant that it was not possible to statistically distinguish any differences between the treatments (from both experimental sites). The relative lack of disease occurred despite increasing the concentration of added bacteria 100-fold. As in trial 1, the combination of BABA and Bion resulted in the highest number of diseased plants.

Trial 3: Plants not artificially infected with head rot bacteria

- Trial 3 (July 2010): The majority of treatments gave similar protection to Cuprokylt, with no significant differences between them. However, plots treated with Flyer had significantly greater numbers of diseased plants. Furthermore, the extent (spread) of head-rot symptoms was greater across the plants. This may be due to the changes in the total microbial population on the plants in the presence of the fungicide, which could in turn provide conditions that were more conducive for head-rot bacteria.

Laboratory analysis showed that one bacteria species, *Pseudomonas fluorescens*, was responsible for the greatest extent of disease in comparison to *Ps. marginalis* and *Pectobacterium carotovora*, although all three species were capable of causing typical head-rot symptoms on broccoli heads. It is of note that it was possible to detect head-rot bacteria from diseased and healthy plants in trial 3, the commercial site, where bacteria were not artificially added.

Main conclusions

- Head-rot bacteria are common and can be isolated from healthy plants.
- Disease occurred in the absence of artificially added bacteria.
- Greater levels of disease are required to differentiate between the treatments.
- Application of Flyer significantly increased the number of diseased plants.

Plans for Year 2

The transplants will be planted in late April 2011 and early July 2011 so that the trials are more likely to coincide with the climatic conditions that will induce head-rot. Irrigation will be applied to the trial and the level of nitrogen fertiliser increased. Although disease can occur in the absence of additional bacteria, we will add the same level of bacteria as in trial 1 to keep the conditions similar.

Financial benefits

At this point it is not possible to draw any conclusions about financial benefits as the trial is on-going.

Action points for growers

At this point it is not possible to recommend any action points as the trial is on-going.

SCIENCE SECTION

Introduction

Head rot is a disease of broccoli that causes a significant financial burden on growers. In the UK losses can cost the industry over £15 million per year. The disease (also called spear rot) is caused by bacterial infection of the plant and degradation of the plant tissue, resulted in soft rot (Harling & Sutton, 2002). The main causative organisms are *Pesudomonas fluorescens, Pseudomonas marginalis* and *Pectobacterium carotovorum*. All three bacterial species are soil borne and prevalent in the environment. They are not overt pathogens, but opportunistic and soft rot symptoms on the flowering tissue only occur when the environmental conditions are conducive, following damp, humid conditions.

Current treatment options for head-rot are limited to microcidal chemicals, principally those containing copper oxychloride. Treatments are generally applied as a prophylactic, when the weather conditions are expected to lead to high humidity in the crop. However, continued use of copper is finite given its phytotoxicity and toxicity in the general environment. Some European countries are now limiting its use. There are few other treatments that target the bacteria and some such as antibiotics are not permitted in the UK. Furthermore, the Pseudomonads in particular are especially adaptable to sub-lethal levels of microcides and there are reports of resistance to copper oxychloride.

It is not feasible to use a microcidal that will eliminate the bacteria from the crop sites because of their ubiquity. Therefore, it is necessary to find alternative treatment approaches. Such treatments include plant defence response elicitors that trigger induced resistance pathways. A range of compounds can elicit defence pathways and historically elicitors are based on natural compounds, such as chitosan (Walters *et al.*, 2005). Others mimic plant hormones (*cis*-jasmone) and some have been well characterised in terms of which defensive pathway they activate. Probenazole is used as a standard treatment for rice blast in Asia (Watanabe *et al.*, 1977) and greenhouse studies have suggested that BABA and Bion can reduce head-rot symptoms in broccoli (Pajot & Silue, 2005). A group of licensed fungicides contain strobilurins, which have proven elicitor activity {Herms, 2002 #138}; this class includes Amistar and Flyer.

A two-year project was set up to test a group of five elicitors and three fungicides with known or suspected elicitor activity against bacterial head-rot in broccoli.

Materials and methods

Experimental trials were duplicated at two sites (SCRI, Invergowrie and SAC, Pitlochie) and two trials were run in 2010: trial 1 from 28th April to 6th July and trial 2 from 28th July to 28th Oct. In addition, a third trial was set up at a commercial site (Wormitt) to test licensed fungicides, from 14th July to 6th Oct. Broccoli transplants (vars. Marathon, Parthenon) were planted on sites of approximately 100 m x 80 m, made up of six beds of treated plants, each bordered by 'guard' beds containing un-treated plants. In each treatment plot with the 'experimental' elicitors 20 plants were assessed, whereas 60 plants were assessed for the licensed fungicides, all treatments were replicated three times. Assessment was carried out approximately 9 weeks after planting (later for the autumn trials because of the cooler weather).

Five elicitors (BABA, Bion, *cis*-jasmone, Probenazole, Yea foliar) and three licensed fungicides with known or expected elicitor activity (Amistar, Justice, Flyer) were selected for the trial (Table 1). Cuprokylt was included as a positive control in the commercial trial. The elicitors were used either singly or in combination, with the aim of triggering multiple defensive pathways in the plant. Elicitors were applied three times, in 10-day intervals; the first application was approximately one week before head initiation. A cocktail of head-rot bacteria was applied twice, in a one-week interval, with the first application between the first and second elicitor application. In trial 1 bacteria were applied at a concentration of 10^4 cfu / ml, in trial 2 the concentration increased to 10^6 cfu / ml.

Laboratory experiments were carried out to determine the relative contribution of disease from three different head-rot bacteria species. The presence of head-rot bacteria on trial plants was detected by PCR amplification, using primers specific for variable regions in the 16S sequence of *Pectobacterium* (Toth *et al.*, 1999) or Pseudomonads (Spasenovski *et al.*, 2009) (although the PCR failed to detect *Ps. marginalis*).

Table 1: Elicitors used in the trial.

Elicitors used for trials 1 & 2	Concentrations used
BABA [a]	1 mM
Bion	1 mM
cis-jasmone [a]	3.2 mM
Yea foliar	0.3 % (v/v)
Probenazole [a b]	0.2 mM (trial 1), 1 mM (trial 2)
Amistar	1 L / ha
Elicitors used for trial 3	
Amistar	1 L / ha
Probenazol [a b]	1.0 mM
Justice	0.25 L / ha
Flyer	1 L / ha
Cuprokylt	5 kg / ha

All treatments were applied at a rate of 400 L / ha.

Activator-90 at 0.05 % (v/v) was added to all treatments.

Key: a = addition of 0.01 % Tween 20

b = addition of 1 % (v/v) acetone

Results

Experimental trials were set up at SCRI (Invergowrie, Tayside) and SAC (Pitlochie, Fife) to test elicitors: BABA, Bion, *cis*-jasmone, Probenazole, Yea foliar, Amistar against head-rot in broccoli. Half the plants in the trials were challenged with head-rot bacteria. Two sets of trials were run over the season in 2010, trials 1 and 2.

- Trial 1 (April 2010): No significant effect was found with any of the treatments, although those plants that had been treated with bacteria were significantly more diseased than the untreated plants (Fig. 1). The difference was not apparent at the SAC site because of lack of symptoms. Probenazole and Amistar and their combinations appeared to provide some protection, in contrast to the combination of BABA and Bion which gave the highest level of disease. The main issue from trial 1 was a lack of head-rot symptoms which made any statistical differentiation between the treatments difficult. Possible reasons for the lack of disease were a particularly dry June and low levels of nitrogen.

- Trial 2 (July 2010): A lack of disease symptoms meant that it was not possible to statistically distinguish any differences between the treatments (both experimental sites). This occurred despite increasing the concentration of added bacteria 100-fold (to 10^6 cfu/ml). As with trial 1, the combination of BABA and Bion resulted in the highest number of diseased plants (data not shown).

The effect of licensed fungicides, Amistar, Flyer and Justice, and Probenazole were compared to Cuprokylt on a commercial site (Wormitt, Fife). The plants were not artificially infected with head-rot bacteria, so that the trial relied on 'natural' infection of the plants.

- Trial 3 (July 2010): The majority of treatments gave similar protection to Cuprokylt, with no significant differences (Fig. 2). However, plots treated with Flyer had significantly higher numbers of diseased plants; in addition, the extent (spread) of head-rot was higher across the diseased plants.

Laboratory work was carried out to determine a 5-point scale of disease symptoms, where 0 = no symptoms and 4 = extensive spread of disease over > 60 % of the head/floret (Fig. 3). The analysis showed that one bacteria species, *Pseudomonas fluorescens*, was responsible for the greatest extent of disease in comparison to *Ps. marginalis* and *Pectobacterium carotovora*, although all three species were capable of causing typical head-rot symptoms on broccoli heads. It is of note that it was possible to detect head-rot bacteria from both diseased and healthy plants in the trial 3 (commercial site, with no added bacteria), using PCR amplification of specific DNA.

Figures

Figure 1: Average number of plants without head-rot symptoms for each of the 18 treatments, trial 1 at SCRI. The untreated control is # 18. Black arrows show Probenazole (#5) and Amistar (#6) and combinations containing either or both. Red arrows show Yea foliar (#4) and the combination of BABA + Bion (#7).

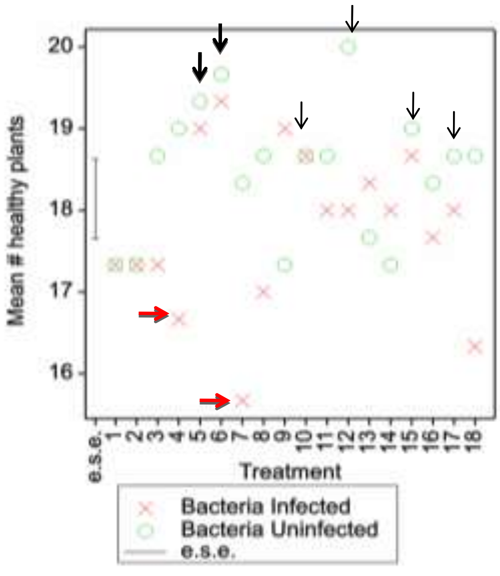


Figure 2: Average number of plants without head-rot symptoms for each of the 8 treatments, trial 3 at Wormitt. The untreated control is # 8, Cuprokylt is # 6. The red arrow shows Flyer (#4).

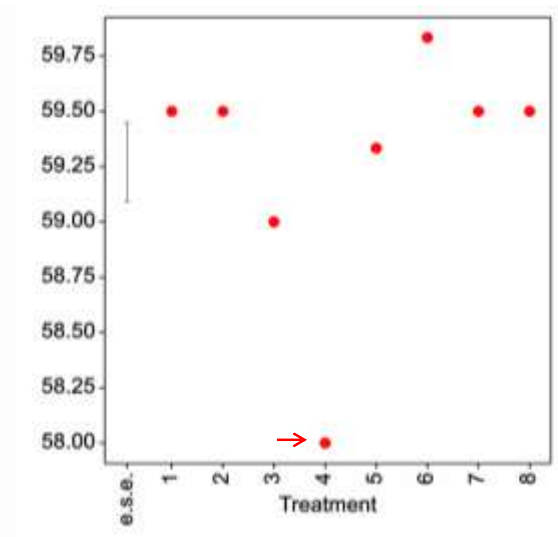
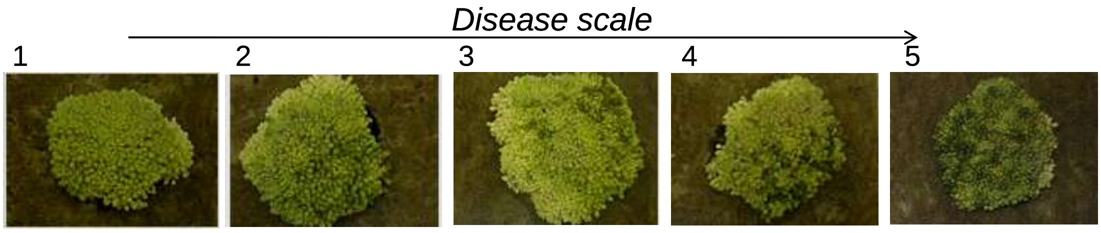


Figure 3: Disease assessment on detached broccoli florets, infected with a cocktail of head-rot bacteria. Disease scale showing degree of head-rot symptoms.



Discussion

Specific project objectives:

1. To determine the most effective elicitor(s), either singly or in combination, in protecting broccoli against head rot.

2. To determine whether licensed azoxystobin fungicides show protection against head rot in broccoli.

3. To determine the most effective formulations for applying this/these elicitors.

4. Publicise data and determine likelihood of Chemical Regulation Directorate (CRD) approval for use of elicitors on broccoli.

In the first year we have established the trials and tested five elicitors and three fungicides with known or expected elicitor activity. Unfortunately, it has not been possible to draw any conclusions that are statistically significant due to the low levels of disease in the experimental trials. In the commercial trial, greater levels of disease were observed and the majority of treatments (except Flyer) gave similar results to Cuprokylt. The year one results contribute to specific objectives 1 and 2.

In the commercial trial (trial 3), a significantly higher level of disease occurred on the plants treated with the fungicide Flyer. Although Flyer contains pyraclostobin, it may be that the fungicide altered the native population of microbes on the plants and creating conditions that allow for greater levels of colonisation by head-rot bacteria.

Addition of a cocktail of head-rot bacteria did result in a statistical increase in the number of diseased plants, although the differences were small, making any differences between elicitor treatments hard to distinguish. It was possible that the lack of disease symptoms were due to a particularly dry spell of weather in June 2010. We attempted to increase the likelihood of disease in the second experimental trial by increasing the concentration of added bacteria 100-fold. However, this had a negligible effect. It is of note that disease occurred in plants in the absence of artificially added bacteria (trial 3), although laboratory tests showed that all three species of bacteria used in the head-rot cocktail did cause symptomatic disease characteristic of head-rot on detached broccoli heads. This suggests that the environmental conditions rather than bacteria load have a greater impact on the incidence of disease.

For year two, two trials will be planted at the end of April 2010 and in early July in an attempt to encompass the weather conditions that are most likely to give rise to naturally-occurring head-rot. Irrigation will be applied on a regular basis to encourage damp, humid conditions and the concentration of nitrogen fertiliser increased to 250 kg / ha, since high levels are known to correlate with higher incidence of head-rot (Harling & Sutton, 2002). The trials will still include artificially added head-rot bacteria as their presence did result in a significant increase in the number of diseased plants (albeit small). The concentrations and formulations of the elicitors will be maintained in year 2 since these are based on either previous experimental data or standard formulations for licensed fungicides (specific objective 3). Trial sites will be restricted to SCRI, Invergowrie and Peacehill, Wormitt as no real disease was observed at Pitlochie, Fife. Experimental approval has been sought and awarded for Peacehill, Wormitt.

It was possible to detect head-rot bacteria on both healthy and symptomatic plants in the commercial trial, suggesting that the plants have a frequent of exposure to the bacteria. This re-iterates the point that any effect of microcidal treatments is likely to be short-lived and they will require repeated applications (as is the case for current use of Cuprokylt). Alternative treatments that induce the plant to defend itself are beneficial not just against head-rot bacteria, but may also confer broad-spectrum resistance against other opportunistic pathogens.

Conclusions

- Head-rot bacteria are common and can be isolated from healthy plants.
- Disease occurred in the absence of artificially added bacteria.
- Greater levels of disease are required to differentiate between the treatments.
- Application of Flyer significantly increased the number of diseased plants.

Knowledge and Technology Transfer

The year one results have been presented to the Brassica Growers Association research and development meeting, held at SCRI, Nov 2010.

The data was due to be presented at the meeting 'Innovative ideas in pest and weed control in field vegetables', run by the Association of Applied Biologist, held at Rothamsted in April 2011. Unfortunately the meeting has been cancelled due to lack of interest.

It is anticipated that the data will be presented at the CNPB meeting in 2012: Environmental Management & Crop Protection and published in the meeting proceedings (http://www.cpnb.org/).

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