Project title:	Assessment of plant elicitors to induce resistance against head-rot in broccoli
Project number:	FV 378
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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.

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GROWER SUMMARY

Headline

Experimental trials investigating the effect of plant elicitors on head rot in broccoli did not show any significant levels of control due to low levels of disease while a commercial trial showed that all the elicitors except Justice gave a similar level of head rot control to the fungicide Cuprokytl.

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 during periods of high humidity. The bacteria are common in soil and because they will only infect plants under the most conducive conditions, they are termed 'opportunistic' pathogens. Current treatments rely on copper oxychloride (e.g. ‘Cuprokylt’), which acts 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

Broccoli transplants (vars. Marathon, Parthenon) were planted in mid April and mid July 2010 and 2011, at replicated experimental sites (James Hutton Institute (JHI): Invergowrie and Scottish Agricultural College (SAC): Pitlochie, Peacehill, Kirkton Barns) for trials 1 to 4. In addition, a commercial site (Peacehill) was used to test licensed fungicides, where the transplants were planted in July 2010, for trial 5. 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 - 4, and 60 plants assessed for trial 5. Each treatment plot was replicated three times. Assessment was carried out approximately nine 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'; a.i. azoxystrobin, proquinazid and pyraclostrobin, respectively) were selected for the trial (Table 1). 'Cuprokylt' (a.i. copper oxychloride) 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.

Table 1: Treatments used in the trials.

Treatments in trials 1--4	**Concentrations used**
BABA [a] (Elicitor)	1 mM
Bion (Elicitor)	1 mM
cis-jasmone [a] (Elicitor)	3.2 mM
Yea foliar (Elicitor)	0.3 % (v/v)
Probenazole [a b] (Elicitor)	0.2 mM (trial 1), 1 mM (trial 2-4)
Amistar (Fungicide with elicitor activity)	1 L / ha
Treatments in trial 5 (commericial trial)	**Concentrations used**
Amistar (Fungicide with elicitor activity)	1 L / ha
Probenazole [a b] (Elicitor)	1.0 mM
Justice (Fungicide with elicitor activity)	0.25 L / ha
Flyer (Fungicide with elicitor activity)	1 L / ha
Cuprokylt (Fungicide)	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

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 (Figure 1).

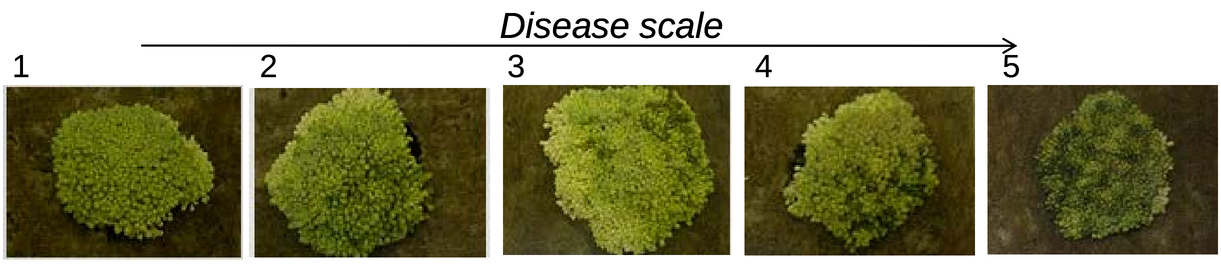

Figure 1: Disease assessment on detached broccoli florets, infected with a cocktail of head-rot bacteria. Disease scale showing degree of head-rot symptoms.

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.

In the commercial trial greater levels of disease were observed and treatments, including Probenazole, gave similar results to ‘Cuprokylt’. whilst application of ‘Justice’ significantly increased the number of diseased plants.

- It appears that plants have a frequent exposure to the infecting bacteria during the season, so the effect of elicitor treatments could be short-term requiring repeated applications

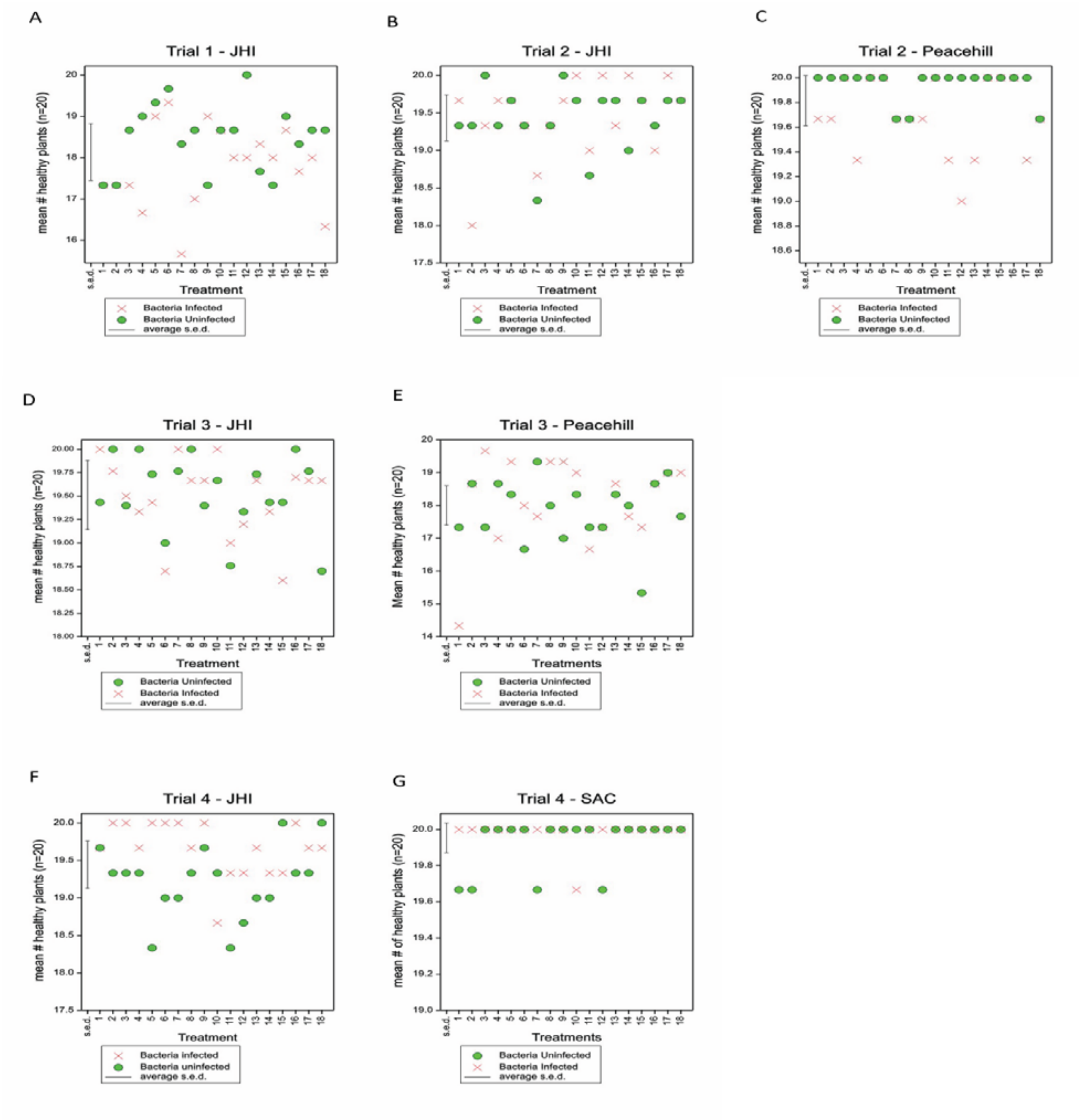

Figure 2: Graphs showing the mean numbers of healthy plants from each trial. Trials 1 - 4 from JH1 (A, B, D, F) and SAC sites (C, E, G). N.B. trial 1 at the SAC site yielded 100 % healthy plants (not shown).

- Trial 1 (April 2010): Application of bacteria to the trial run at JHI resulted in a significant increase in the number of diseased plants although no significant effects were seen for the treatments (Fig. 2A). Probenazole (#5) and Amistar (#6) and their combinations appeared to provide some protection, in contrast to the combination of BABA and Bion (#7) which gave the highest level of disease. No disease symptoms were observed on any plants at the SAC site (Pitlochie). 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). There was a significant increase in the number of diseased plants at the SAC site (Peacehill), but not at the JHI site (Fig. 2B, 3C). As with trial 1, the combination of BABA and Bion (#7) resulted in the highest number of diseased plants.
- Trial 3 (May 2011): An increased level of disease was observed at the SAC site (Peacehill) and there was a marginal effect with treatment type, which arose from an increased number of symptomatic plants treated with BABA (#1) and *cis* jasmone + Amistar (#15). On this occasion there was no effect with addition of bacteria. In addition, there were no significant effects with either treatment or application of bacteria at the JHI site.
- Trial 4: (July 2011): Only a low level of disease was observed for both trials with no significant differences between the treatment types or addition of bacteria.

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

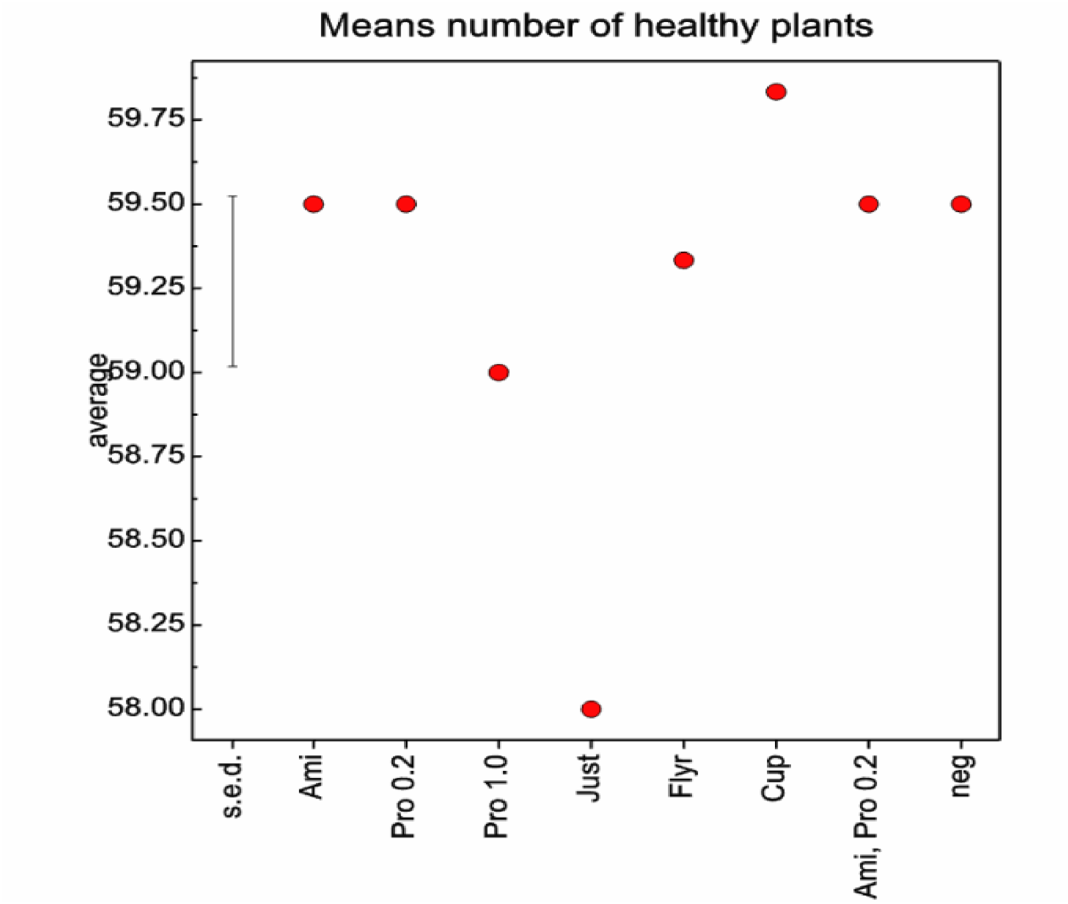

Figure 3: Average number of plants without head-rot symptoms for each of the eight treatments used on the commercial trial (Peacehill, 2010).

- Trial 5 (July 2010): The majority of treatments gave similar protection to Cuprokylt, with no significant differences (Fig. 3). However, plots treated with ‘Justice’ had significantly greater numbers of diseased plants; in addition, the extent (spread) of head-rot was greater across the diseased plants (not shown).

Laboratory analysis showed that one bacteria species, *Pseudomonas fluorescens* (Psf), was responsible for the greatest extent of disease in comparison to *Ps. marginalis* (Psm) and *Pectobacterium carotovorum* subsp*. carotovorum* (Pcc), 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.

Further work is required to definitively show a role for elicitors in control of head-rot bacterial in broccoli. While there is some evidence for a positive effect, some elicitor or fungicide treatments can increase the level of disease. One possibility is through the use of smaller-scale, protected trials.

Financial benefits

At this point it is not possible to accurately cost the elicitors since they are still experimental.

Action points for growers

No action points from this work at present

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, resulting in soft rot (Harling and Sutton 2002). The main causative organisms are *Pesudomonas fluorescens* (Psf)*, Pseudomonas marginalis* (Psm) and *Pectobacterium carotovorum subsp. carotovorum* (Pcc), formerly a member of the *Erwiniae* family. 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 and Silue 2005). Also a group of approved fungicides containing strobilurins, which have proven elicitor activity (Herms et al, 2002); 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

Two experimental trials per year were duplicated at two sites (James Hutton Institute (JHI): Invergowrie and Scottish Agricultural College (SAC): Pitlochie, Peacehill and Kirkton Barns), and run over two years, generating a total of eight trials. The trials were run from 28th April to 6th July /28th July to 28th Oct in 2010 and 17th May to 19th July /15th July to 17th Oct in 2011. An additional trial was set up at a commercial site (Wormit) to test licensed fungicides, from 14th July to 6th Oct 2010. 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. The trials were laid out in a split-plot design, generated in Genstat (VSN international). In each treatment plot with the 'experimental' elicitors 20 plants were assessed, whereas 60 plants were assessed for the approved 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 approved 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 and increased to 10^6 cfu / ml in subsequent trials.

The number of diseased plants were scored on the basis of incidence (+/- symptoms) and the extent of disease (5-point scale). The data was analysed in Genstat using a general ANOVA at a 5 % significance level. Means plots were also generated in Genstat.

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).

Table 1: Treatments used in the trials.

Treatments used for trials 1-4	Concentrations used
BABA [a] (Elicitor)	1 mM
Bion (Elicitor)	1 mM
cis-jasmone [a] (Elicitor)	3.2 mM
Yea foliar (Elicitor)	0.3 % (v/v)
Probenazole [a b] (Elicitor)	0.2 mM (trial 1), 1 mM (trial 2-4)
Amistar (Fungicide with elicitor activity)	1 L / ha
Elicitors used for trial 5 – commercial trial	**Concentrations used**
Amistar (Fungicide with elicitor activity)	1 L / ha
Probenazole [a b] (Elicitor)	1.0 mM
Justice (Fungicide with elicitor activity)	0.25 L / ha
Flyer (Fungicides with elicitor activity)	1 L / ha
Cuprokylt (Fungicide)	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

Table 2: Description of elicitor combinations in the trials, showing the numbers used in the graphs (Fig. 1).

Trial 1		Trials 2, 3, 4	
Treatments	**Number**	**Treatments**	**Number**
BABA	1	BABA	1
Bion	2	Bion	2
cis-jasmone	3	cis-jasmone	3
Yea foliar	4	Probenazole 0.2 mM	4
Probenazole 0.2 mM	5	Probenazole 1.0 mM	5
Amistar	6	Amistar	6
BABA, Bion	7	BABA, Bion	7
BABA, cis-jasmone	8	BABA, cis-jasmone	8
BABA, Yea	9	BABA, Probenazole 0.2	9
BABA, Probenazole 0.2	10	BABA, Amistar	10
Bion, cis-jasmone	11	Bion, cis-jasmone	11
Bion, Yea	12	Bion, Probenazole 0.2	12
Bion, Probenazole 0.2	13	Bion, Amistar	13
cis-jasmone, Yea	14	cis-jasmone, Probenazole 0.2	14
cis-jasmone, Probenazole 0.2	15	cis-jasmone, Amistar	15
Yea, Probenazole 0.2	16	Probenazole 0.2, Amistar	16
BABA, Bion, jasmone, Yea	17	BABA, jasmone, Probenazole 0.2, Amistar	17
no treatment control	18	no treatment control	18

Results

Experimental trials were set up at JHI (Invergowrie, Tayside) and SAC (Pitlochie, Peacehill, Kirkton Barns, all 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. The results of the trials are shown in Figure 1.

- Trial 1 (April 2010): Application of bacteria to the trial at JHI (Fig. 1A) resulted in a significant increase in the number of diseased plants (p = 0.047), although no significant effects were seen for the treatments. Probenazole (#5) and 'Amistar' (#6) and their combinations appeared to provide some protection, in contrast to the combination of BABA and Bion (#7) which gave the highest level of disease. No disease symptoms were observed on any plants at the SAC site (Pitlochie). 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). There was a significant increase in the number of diseased plants at the SAC site (Peacehill) (p = 0.008), but not at the JHI site (Fig. 1B, 1C). As with trial 1, the combination of BABA and Bion (#7) resulted in the highest number of diseased plants.
- Trial 3 (May 2011): An increased level of disease was observed at the SAC site (Peacehill). There was a significant effect with treatment type at the 10 % level (p = 0.055), although on this occasion there was no effect with addition of bacteria (p = 0.240). The treatment effect arose from the increased number of symptomatic plants treated with BABA (#1) and *cis* jasmone + 'Amistar' (#15). There were no significant effects with either treatment or application of bacteria at the JHI site.
- Trial 4: (July 2011): Only a low level of disease was observed for both trials with no significant differences between the treatment types or addition of bacteria.

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

- Trial 5 (July 2010): The majority of treatments gave similar protection to 'Cuprokylt', with no significant differences (Fig. 2). However, plots treated with 'Justice' had significantly greater numbers of diseased plants; in addition, the extent (spread) of head-rot was greater across the diseased plants (not shown).

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, Psf, was responsible for the greatest extent of disease in comparison to Psm and Pcc, although all three species were capable of causing typical head-rot symptoms on broccoli heads. Molecular detection of head-rot bacteria was optimised with laboratory infections of broccoli florets, using PCR primers that recognised DNA specific to Pcc or Psf and Psm (Fig. 4). 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 the specific PCR primers.

Figures

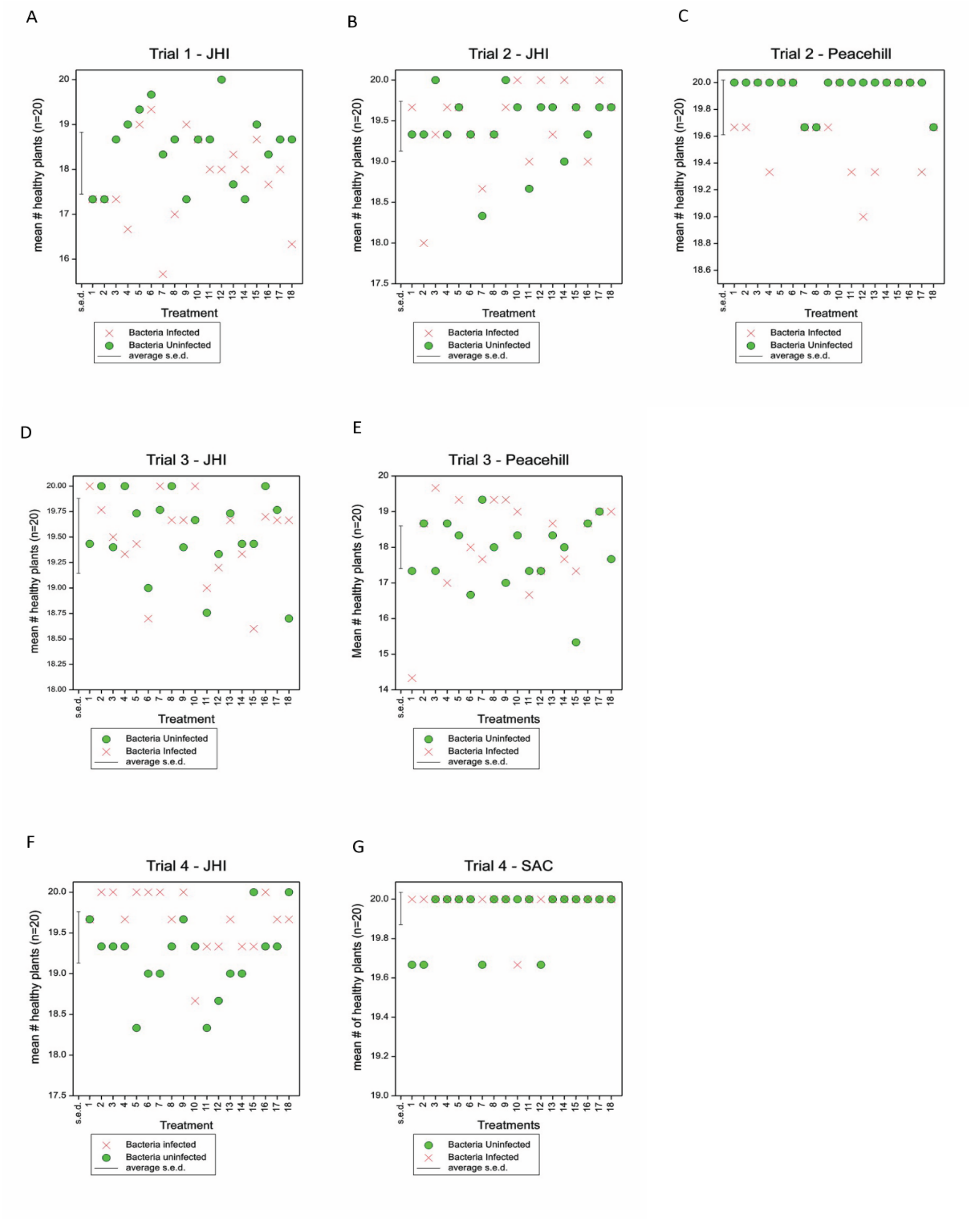

Figure 1: Graphs showing the mean numbers of healthy plants from each trial. Trials 1 - 4 from JH1 (A, B, D, F) and SAC sites (C, E, G). N.B. trial 1 at the SAC site yielded 100 % healthy plants (not shown).

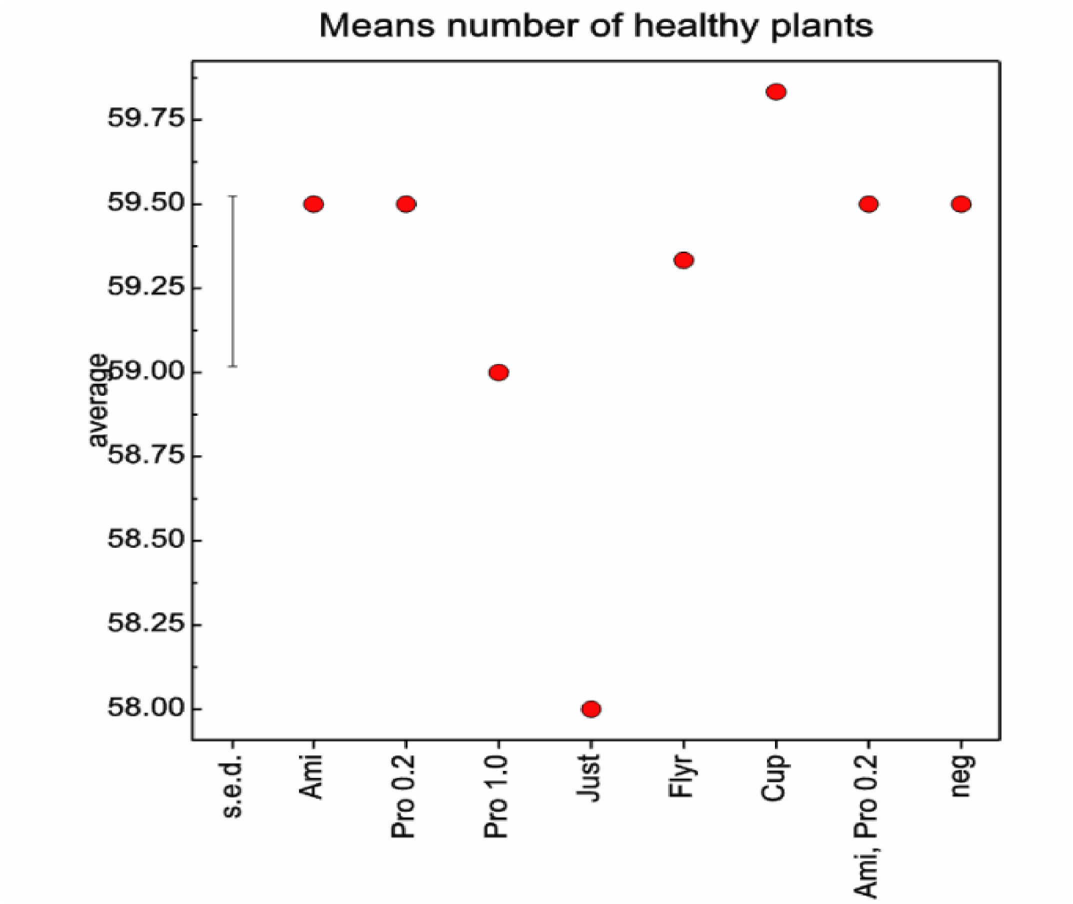

Figure 2: Average number of plants without head-rot symptoms for each of the eight treatments used on the commercial trial (Peacehill, 2010).

Key: Ami refers to Amistar; Pro to Probenozle at 0.2 mM or 1.0 mM; Just to 'Justice'; Flyr to 'Flyer'; Cup to 'Cuprokylt'; neg to the no treatment control.

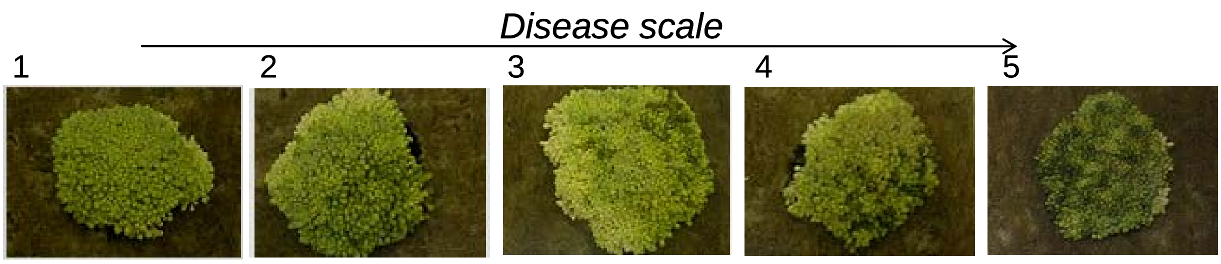

Figure 3: Disease assessment on detached broccoli florets, infected with a cocktail of head-rot bacteria. Disease scale showing degree of head-rot symptoms.

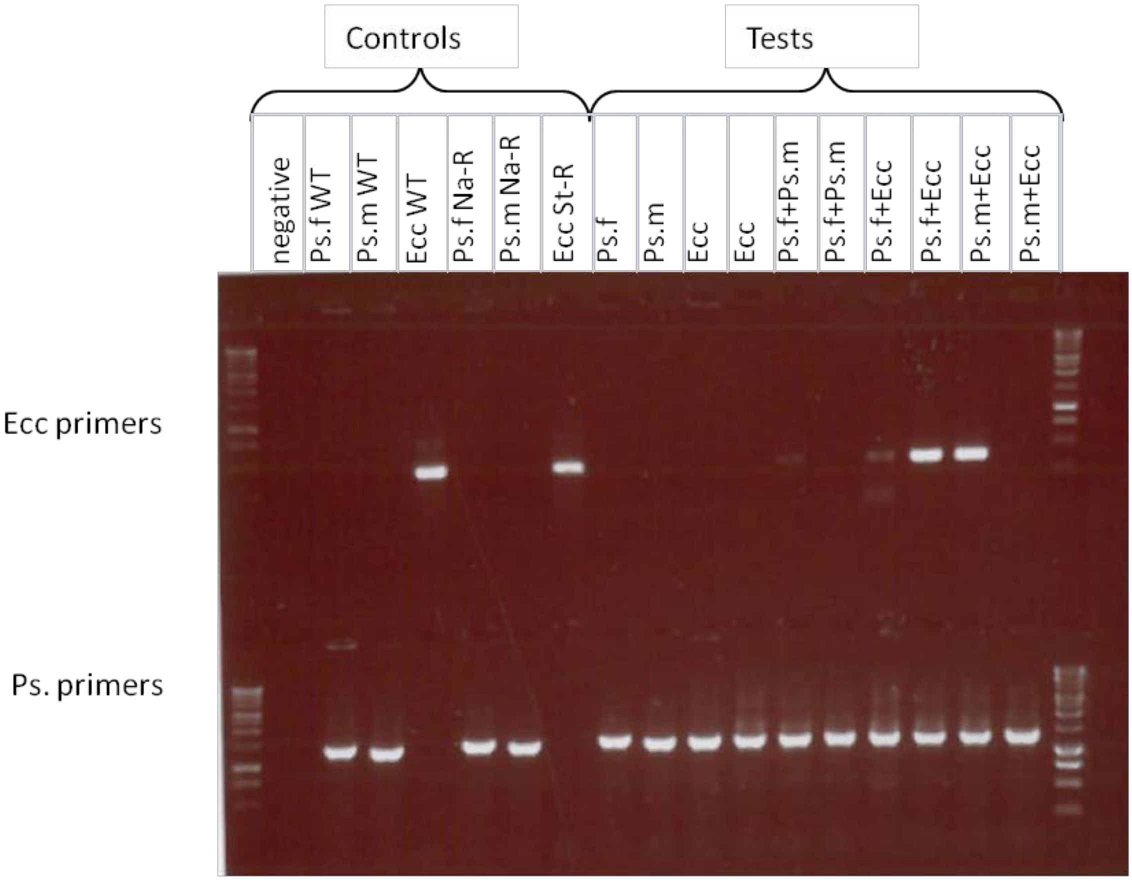

Figure 4: Molecular detection of head-rot bacteria. PCR amplification of head-rot bacteria from experimental infections. Head-rot bacteria were used to infected broccoli florets in different combinations and left for one week at room temperature before testing. Positive control *g*DNA was included to verify the primers.

Key: Ecc refers to Pcc. WT and Na-R refers to WT and nalidixic acid resistant variants of the isolates, respectively. DNA primers specific to Pcc (Ecc primers) and Psf or Psm (Ps. primers) were used.

Discussion

Field trials were established to test five elicitors and three fungicides with known or expected elicitor activity. It has not been possible to draw any statistically significant conclusions due to the low level of symptomatic disease in the experimental trials. Attempts were made to increase the disease level by increasing the bacterial inoculum 100-fold (trials 2 - 4), increasing the level of inorganic nitrogen to 250 kg / ha (trials 3 & 4) and increasing humidity by installing mist irrigation (trials 3 & 4). All of these measures are thought to increase the likelihood of development of head-rot symptoms (Harling and Sutton 2002).

Unfortunately the level of disease remained too low to show significant differences between the treatments. However, in the commercial trial (trial 5), greater levels of disease were observed and a significant negative effect was seen with ‘Justice’, whilst the other treatment, including Probenazole, gave similar results to ‘Cuprokylt’.

Data with the strobilurin-containing fungicides was not conclusive, although ‘Amistar’ did appear to provide some protection in trial 1 and in combination in trials 2, 3 and 4 (esp. treatment # 17). In addition, application of ‘Justice’ (proquinazid) in the commercial trial (trial 3) resulted in a significantly higher level of disease compared to the two strobilurins. It is possible that alterations in the native population of microbes on the plants created permissive conditions for increased levels of head-rot bacteria.

The formulation used to suspend the elicitors (and fungicides) resulted in a colloidal solution for the majority, which was sufficiently dispersed to apply as a foliar spray. Additional work would be required to determine the most efficient route of delivery of the elicitors, e.g. as a foliar spray or as a root soak. This is most likely to be influenced by the plant species and growth cycle.

Laboratory tests showed that all three species of bacteria used in the head-rot cocktail caused symptomatic disease characteristic of head-rot on detached broccoli heads. In addition, symptomatic disease occurred in plants in the absence of artificially added bacteria in trial 5. Together, this suggests that prevailing environmental conditions rather than bacterial load have a greater impact on the incidence of disease. It also suggests that the plants have a frequent exposure to the bacteria, which 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 may still be beneficial not just against head-rot bacteria, but may also confer broad-spectrum resistance against other opportunistic pathogens. Fundamental research on the mechanisms that underpin the defensive response would provide the level of detail that is required for targeted application of the elicitors. This is important for producers in terms of how elicitors may be applied, when to apply them and the kinds of protection that they are likely to deliver.

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 some treatments can significantly increase the number of affected plants.

Knowledge and Technology Transfer

Grower meetings:

The work was presented to the Brassica Growers Association annual conference in January 2012 (Lincoln, UK).

Scientific meetings:

Work on plant defense elicitors in relation this and another HDC-funded trial (FV 393) has been presented at the British Society for Plant Pathology meeting on small bioactive molecules in December 2011 (Cambridge, UK) as a poster. The work will also be presented at the Crop Protection of Northern Britain meeting in February (Dundee, UK) as a poster and as a paper in the conference proceedings.

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