Project title:	Reducing bacterial infection in seed onions through the use of plant elicitors
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Project leader:	Nicola J. Holden, The James Hutton Institute
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Date project completed (or expected completion date):	31/12/2011

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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.

Nicola Holden
Lead Researcher (Molecular Bacteriology)
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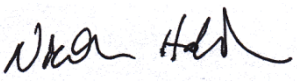
Signature ...  ..... Date .....31st Jan 2012.....

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CONTENTS

GROWER SUMMARY

Headline

Compounds that increase natural pathogen resistance mechanisms in plants have been shown to reduce infection of red onion bulbs by the bacterial pathogen *Burkholderia gladioli* pv. *Alliicola*

Background

Burkholderia gladioli pv. *alliicola* (Bga) is a bacterial pathogen causing rot in onion bulbs. The disease is particularly economically damaging in red varieties, which are heat-treated over the previous winter to prevent bolting. In the UK and Europe, losses of up to 40% have been recorded in store due to bacterial infection. Bga is believed to be the principle cause of bacterial rot in onions and is prevalent in the soil but can also be found on plant roots and in water. Infected sets are thought to be a source of re-infection in maturing bulbs. Infection occurs following wounding to the leaves of growing plants and is exacerbated by wet climatic conditions.

Compounds that induce natural plant defense pathways (elicitors) have been reported to provide protection against a number of pathogens, in a range of crop species. In Asia, some are used as standard applications for rice. The compounds can be mimics of natural plant hormones, or are present elsewhere in nature and act by inducing a genetic response in the plant that results in increased protection against microbial pathogens. Although they are not currently widespread, there is a growing body of experimental work with them, which has shown that they tend to work better in combination, most likely because multiple defense pathways are triggered.

Plant defensive systems effectively work two tiers, the first (basal) tier provides a broad level of protection against a range of different microbes, while the second is triggered when some of those microbes are able to overcome basal defense. Elicitors work by trigging various arms of the basal resistance and therefore are most suited to microbes that are termed opportunistic, i.e. those that can only cause symptomatic disease under optimal conditions. Bga is a typical opportunistic pathogen only able to infect onions through fresh wounds in the leaves that occur, for example, after hail-storms. It is likely to be present on

healthy, un-damaged plants, but not at high enough concentrations or in the right location on the plant, to cause disease.

Current treatment options for Bga (and other opportunistic pathogenic bacteria on plants) are restricted to treatments or fungicides that actively kill the bacteria, e.g. Cuprokylt (copper oxychloride).

This project was established to test whether compounds that strengthen the plants own defence against a broad range of opportunistic plant pathogens can also prevent or reduce the level of disease in onions caused by Bga. Treatments were chosen on the basis of their reported activity against a number of different pathogens in horticultural and combinable crops.

Summary of the project and main conclusions

Glasshouse trials were set-up to test elicitors on Bga infected onions between April and November 2011. The onions were infected with different levels of Bga (Figure 1). Onions (var. Red Baron) were grown from seed in compost, in individual pots and allowed to establish for 11 weeks before fungicides were applied.

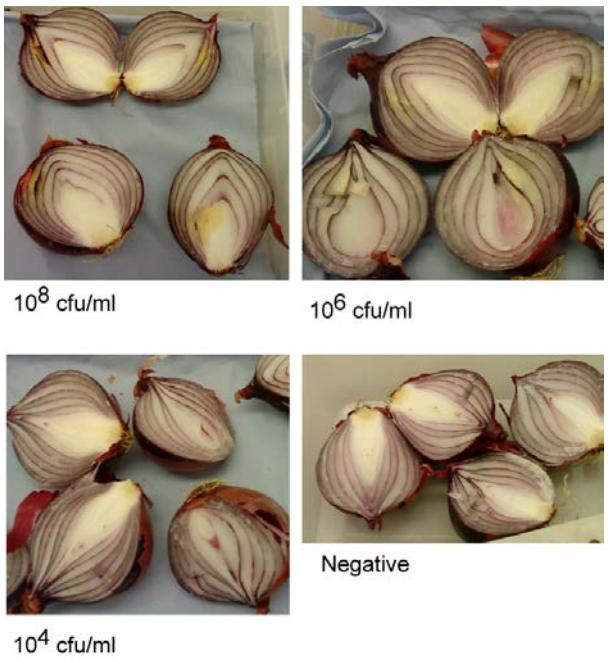

Figure 1. Photographs of artificially infected mature red onion bulbs, at different concentrations of inoculum. The values refer to the inoculum concentration and the uninfected control (Negative).

Seven treatment variations were tested, incorporated into a standard fungicide programme (Table 1). The elicitors were incorporated into applications 1, 3, 5 on an 18 day period.

The bulbs were harvested one week after the final treatment then stored in paper bags in cold storage (1-4^{o}C) for at least four weeks. The onions were then tested for disease levels.

Table 1. Treatment Programme

Application (Date)	Treatments				
	1 SFP	**2 Amistar**	**3 Unicur**	**4 Unicur + Cuprokylt**	**5, 6, or 7 Elicitors**
1 (30/05/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur + Cuprokylt	SFP + elicitor AB, AD, CD
2 (08/06/11)	Valbon, Switch	SFP	SFP	SFP + Unicur + Cuprokylt	SFP
3 (17/06/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur + Cuprokylt	SFP + elicitor AB, AD, CD
24/06/11	*Apply bacteria*	*Apply bacteria*	*Apply bacteria*	*Apply bacteria*	*Apply bacteria*
4 26/06/11)	Fubol Gold, Switch	SFP	SFP	SFP	SFP
5 (05/07/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur + Cuprokylt	SFP + elicitor AB, AD, CD
6 (14/07/11)	Valbon, Switch	SFP	SFP	SFP + Cuprokylt	SFP
7 23/07/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur	SFP + elicitor AB, AD, CD
8 (01/08/11)	Invader	SFP	SFP	SFP	SFP

SFP = Standard Fungicide Program.

Table 2. Fungicides and elicitors used in the trial

Fungicide	Concentration	Label	Treatment Number
Amistar	1 L / Ha	azoxy	2
Unicur	1.25 L / Ha	pro+fluox	3, 4
Cuprokylt	2 kg / Ha	cup oxy	4
Invader	2 kg / Ha	n/a	SFP
Valbon	1.6 kg / Ha	n/a	SFP
Switch	1 kg / Ha	n/a	SFP
Fubol Gold	1.9 kg / Ha	n/a	SFP

Elicitor combinations	Concentration	Label	Treatment Number
BABA + Bion	1 mM + 1 mM	AB	5
BABA + Probenazole	1 mM + 0.2 mM	AD	6
cis-jasmone + Probenazole	3.2 mM + 0.2 mM	CD	7
No treatment control	n/a	NTC	8

The addition of plant defensive elicitors into a standard fungicide programme resulted in a significant and large reduction in the incidence (and extent) of infection of red onions by Bga that was greater than standard treatments, opening the way to possible use of these elicitors in commercial onion production.

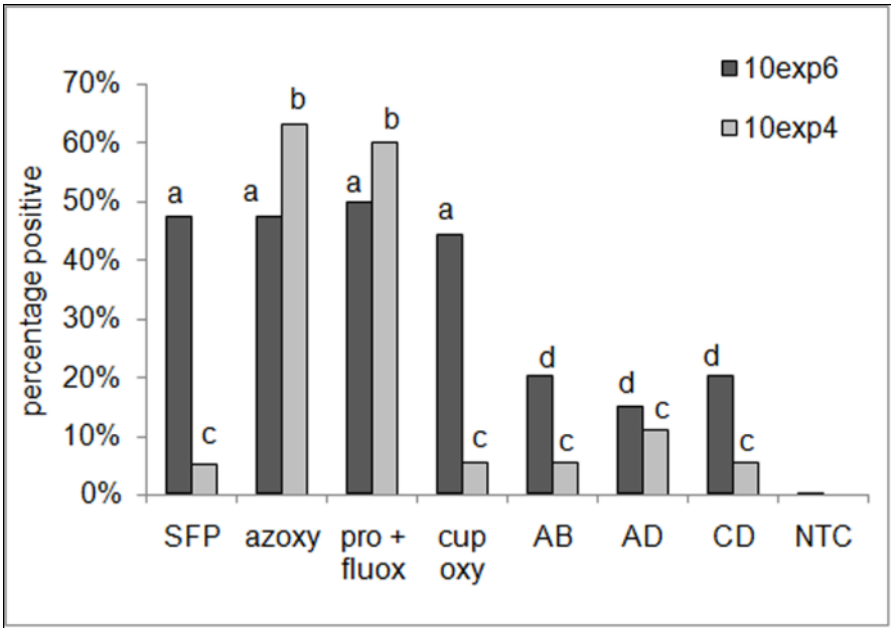

Figure 2. This bar chart shows the number of infected bulbs at two doses of bacterial inoculum. Dark grey is the higher dose and light grey is the lower dose.

Higher dose – columns marked D are significantly more effective in treating Bga than columns marked A.

Lower dose – columns marked C are significantly more effective in treating Bga than columns marked B.

Further work is now required to understand the underlying mechanisms of protection, which in turn, will provide information for a targeted use of the elicitors.

Financial benefits

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

Action points for growers

Incorporation of plant defense elicitors reduced the level and incidence of Bga infection in red onions. However, at this stage these compounds are experimental and not licensed for use in the UK.

SCIENCE SECTION

Introduction

Bacterial rot in onions is caused by opportunistic soil-borne bacteria, primarily *Burkholderia gladioli* pv. *alliicola* (Bga) (previously *Pseudomonas gladioli*) (Yabuuchi*, et al.*, 1992). *Burkholderia* can be commonly isolated from soil and plants; some isolates promote plant growth, some are phytopathogenic, while others are clinically important opportunistic human pathogens (Compant*, et al.*, 2008). Bga is thought to internalise into onion tissue following wounding on the leaves, caused by weather events, e.g. hail storms. The bacteria then migrate into the developing bulb where they proliferate between the bulb scales. Infection generally manifests in mature onion bulbs, following transplantation of infected sets. Current practices include a heat-treatment step, where harvested Rijnsburger onion sets are stored at 28 ^{o}C and 90 % humidity, for up to five months. These conditions represent optimal growth conditions for Bga and are most likely to be an important stage in development of disease.

Bga infection in onions is currently treated with microcidal chemicals, principally copper oxychloride. Treatments are generally applied as a prophylactic, incorporated into the fungicide programme. However, continued use of copper is likely to be limited given its phytotoxicity and toxicity in the general environment, and 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 *Burkholderiaceae* (like the pseudomonads) are very adaptable to sub-lethal levels of microcides and can develop resistance.

Use of microcides to eliminate the bacteria from the crop sites is not feasible because of their ubiquity. Therefore, alternative treatment approaches are required. 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 defense pathway they activate. Probenazole is used as a standard treatment for rice blast in Asia (Watanabe*, et al.*, 1977) and experimental work has been carried out on others, for example in broccoli (Pajot & Silue, 2005). Some licensed fungicides that contain strobilurins also have elicitor activity (Herms*, et al.*, 2002); including Amistar and Flyer.

A nine-month project was set up to test a group of four elicitors incorporated into a standard fungicide programme against bacterial rot in onion bulbs grown from seed.

Materials and methods

Glasshouse trials were set-up to test elicitors on Bga infected onions between April and November 2011. The glasshouse was maintained at 18 ^{o}C, 65 % humidity, on a 16 / 8 hour light/dark cycle. Onions (var. Red Baron) were grown from seed in compost, in individual 2 L pots and allowed to establish for 11 weeks before fungicides were applied.

A total of seven treatment variations were tested, incorporated into a standard fungicide programme (SFP) (Table 1). 20 replicate onions were used for each treatment test. Elicitors were incorporated into applications 1, 3, 5 on an 18-day period. Fungicide and elicitor (Table 2) were applied using hand-held pressure (Hozelock) sprayers, until run-off. Bacteria were applied to the plants once, between the third and fourth fungicide application. Prior to inoculation (also by spraying), the onion leaves were mechanically wounded by gentle scraping with a plastic comb to remove the wax cuticle and part of the epidermal layer. Two different doses of bacteria were tested on two otherwise identical trials. Bga bacteria were maintained in LB media at 28 ^{o}C and stored at – 80 ^{o}C in glycerol stocks. Prior to infection, bacteria were grown to early stationary phase in LB media at 18 ^{o}C and immediately before inoculation, diluted to either 10^6 colony forming unit / milliliter (cfu/ml) or 10^4 cfu / ml in sterile distilled water.

Onion bulbs were harvested one week after the final treatment, detached from the leaves using sterile scissors and heat-treated (28 ^{o}C) for three weeks. The bulbs were then stored in paper bags in cold storage (1 – 4 ^{o}C) for at least four weeks.

To assess the extent of disease, Bga bacteria were quantified from representative samples of each bulb. A sterile cork borer was used to take two samples (total ~ 1 g) from the bulb, suspended in 2.5 ml PBS media and vortex'ed for 30 seconds. The suspension was left at room temperature for 2 hours to allow the bacteria to migrate from the onion tissue to the PBS. Serial dilutions of the suspension were made, plated on Bga selective media (Salles*, et al.*, 2006) and incubated at 28 ^{o}C for 48 – 60 hours. White colonies were verified as Bga from PCR amplification of Bga-specific sequences (Whitby*, et al.*, 2000). No other contaminating bacteria were detected on the agar plates.

A Generalised Linear Model (glm) with binomial error and logit link was used to detect whether the probability of onion infection was influenced by treatment. The analysis was based on presence/absence of bacteria in the replicate samples (Genstat software). The average number of bacteria per treatment was used to generate charts (Excel and Minitab software).

Laboratory experiments were set-up to determine which concentration of bacterial inoculum resulted in development of symptomatic disease in onion bulbs. The outer layer of skin was removed from mature red onion bulbs, the bulb surface sterilized with 70 % ethanol, and stab inoculated with approximately 0.25 ml of Bga at 10^8, 10^6 or 10^4 using a 1 ml syringe and sterile needle. The infected bulbs were stored in a sealed box at 28 ^oC and the symptomatic disease assessed after one week by visual examination of the halved bulbs.

Table 1 Treatment Programme

Application (Date)	Treatments				
	1 SFP	**2 Amistar**	**3 Unicur**	**4 Unicur + Cuprokylt**	**5, 6, or 7 Elicitors**
1 (30/05/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur + Cuprokylt	SFP + elicitor AB, AD, CD
2 (08/06/11)	Valbon, Switch	SFP	SFP	SFP + Unicur + Cuprokylt	SFP
3 (17/06/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur + Cuprokylt	SFP + elicitor AB, AD, CD
24/06/11	Apply bacteria	Apply bacteria	Apply bacteria	Apply bacteria	Apply bacteria
4 26/06/11)	Fubol Gold, Switch	SFP	SFP	SFP	SFP
5 (05/07/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur + Cuprokylt	SFP + elicitor AB, AD, CD
6 (14/07/11)	Valbon, Switch	SFP	SFP	SFP + Cuprokylt	SFP
7 23/07/11)	Invader	SFP + Amistar	SFP + Unicur	SFP + Unicur	SFP + elicitor AB, AD, CD
8 (01/08/11)	Invader	SFP	SFP	SFP	SFP

SFP = Standard Fungicide Program.

Table 2 Fungicides and elicitors used in the trial

Fungicide	Concentration	Label	Treatment Number
Amistar	1 L / Ha	azoxy	2
Unicur	1.25 L / Ha	pro+fluox	3, 4
Cuprokylt	2 kg / Ha	cup oxy	4
Invader	2 kg / Ha	n/a	SFP
Valbon	1.6 kg / Ha	n/a	SFP
Switch	1 kg / Ha	n/a	SFP
Fubol Gold	1.9 kg / Ha	n/a	SFP

Elicitor combinations	Concentration	Label	Treatment Number
BABA + Bion	1 mM + 1 mM	AB	5
BABA + Probenazole	1 mM + 0.2 mM	AD	6
cis-jasmone + Probenazole	3.2 mM + 0.2 mM	CD	7
No treatment control	n/a	NTC	8

Results

Glass-house trials were established to test whether elicitors incorporated into a standard fungicide programme affected Bga infection in onions. Onions were grown from seed in compost and 20 replicate plants were tested per treatment, as detailed in Tables 1 & 2. Two sets of trials were established to test different concentrations of bacterial inoculum: 10^6 cfu/ml and 10^4 cfu/ml. Infection with Bga was assessed as (i) extent of infection, comparing the average number of bacteria in infected bulbs for each treatment, and (ii) incidence, on a presence or absence basis of the bacteria in the onion bulbs.

The extent of infection in the bulbs varied between each treatment. The number of bacteria recovered from elicitor-treated bulbs was significantly less than those treated with Amistar, Unicur or Unicur plus Cuprokylt (Fig 1A). The same result occurred with both bacteria inoculums, although at the lower dose of bacteria, the extent of infection was also very low with Cuprokylt (Fig. 1B). There was no significant difference between the elicitor combinations (Figs 1A&B). No infection was detected in the no-treatment control (NTC) bulbs, despite inoculation with bacteria. It is thought that this because they were not treated with any chemicals (just water), which appeared to result in no leaf damage at all and visibly 'greener' foliage before the bacteria were applied (Appendix 1, Fig. 4). It is possible this occurred because the work was conducted under glass in a controlled climate and presumably infection would have resulted if the trial was run under field conditions.

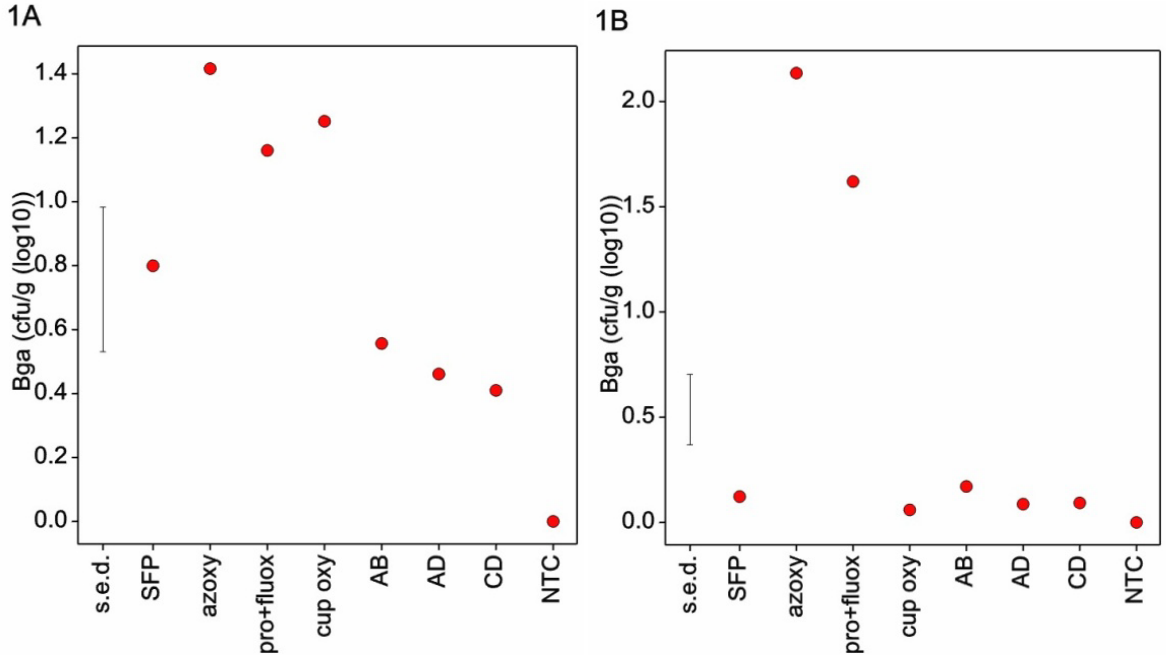

Figure 1 Charts showing the average number of Bga bacteria from 20 replicate bulbs, for each treatment. Two doses of Bga infection were used, 10^6 cfu/ml (A) or 10^4 cfu/ml (B). The standard error of the difference (s.e.d.) is also shown. Refer to Table 2 for treatment descriptions and labels.

Measurement of the extent of infection showed that Bga was not detected in all replicate bulbs in each treatment group. Therefore, the level of disease was also assessed by measuring the incidence of infection, i.e. presence/absence of infection, using a binomial analysis. This approach also revealed statistically significant differences between the treatments for the higher dose of infection, where less than half the onions in the elicitor treatment groups contained bacteria, compared to the treatments with different fungicides (Fig 2). There were no significant differences between the different types of elicitors used. The lower dose of infection resulted in a significant reduction in incidence of Bga for the elicitor group compared to SFP with either Amistar or Unicur.

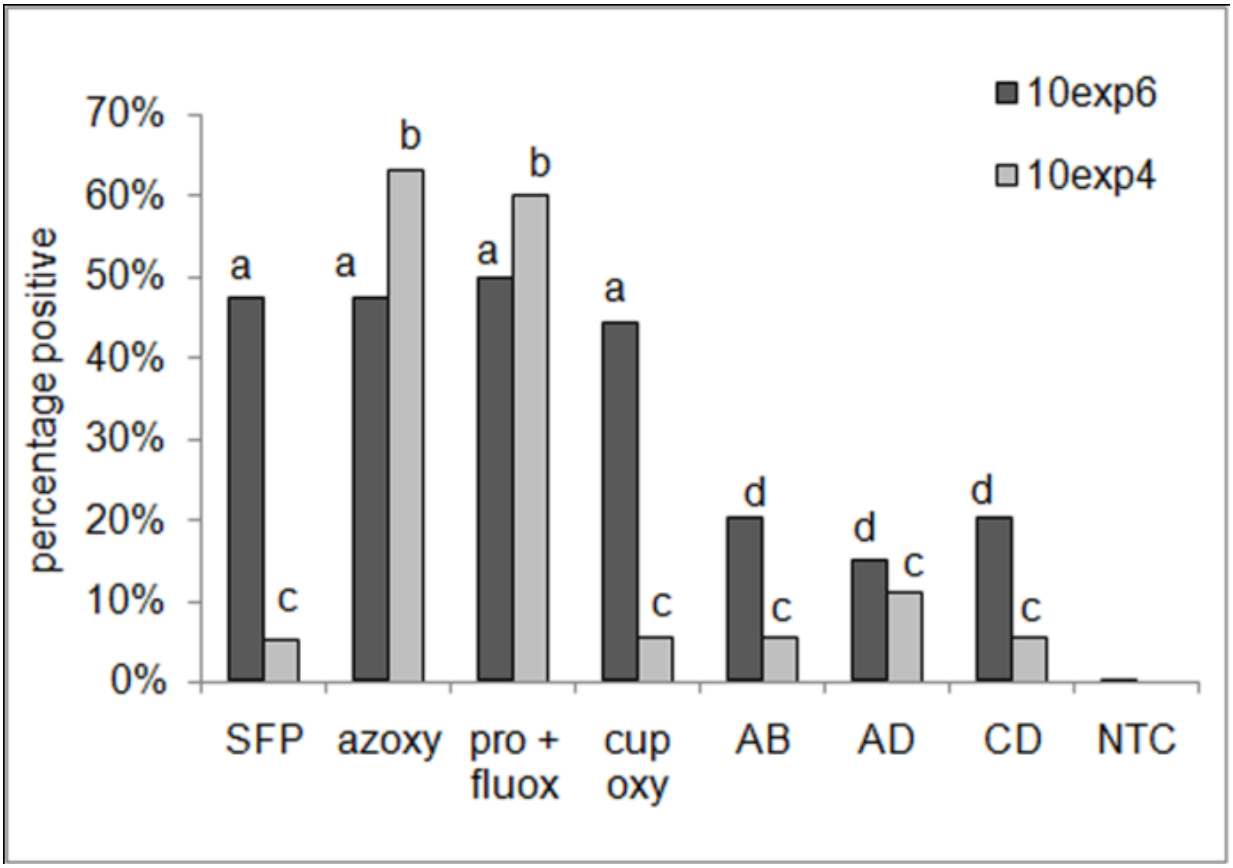

Figure 2 Bar chart showing the number of infected bulbs, for two different infection doses, expressed as a percentage. 10 exp6 (dark grey) refers to 10^6 cfu/ml; 10 exp4 (light grey) refers to 10^4 cfu/ml. The superscript letters refer to differences in statistical significance ($p > 0.05$) between groups. Each group is significantly different from each other: in the 10exp6 dose group 'a' differs from group 'd' and in the 10exp 4 dose group 'b' differs from group 'c' Refer to Table 2 for treatment descriptions and labels.

Laboratory experiments showed that symptomatic disease only occurred in mature red onion bulbs that were stab inoculated with relatively high doses of Bga, at 10^8 or 10^6 cfu/ml (Fig. 3). Sections were taken from each stab-inoculated area and Bga was found to be present in every infected sample, as indicated by specific PCR (not shown). Therefore, symptomatic disease with Bga, i.e. brown rot, only occurred at relatively high levels of inoculum.

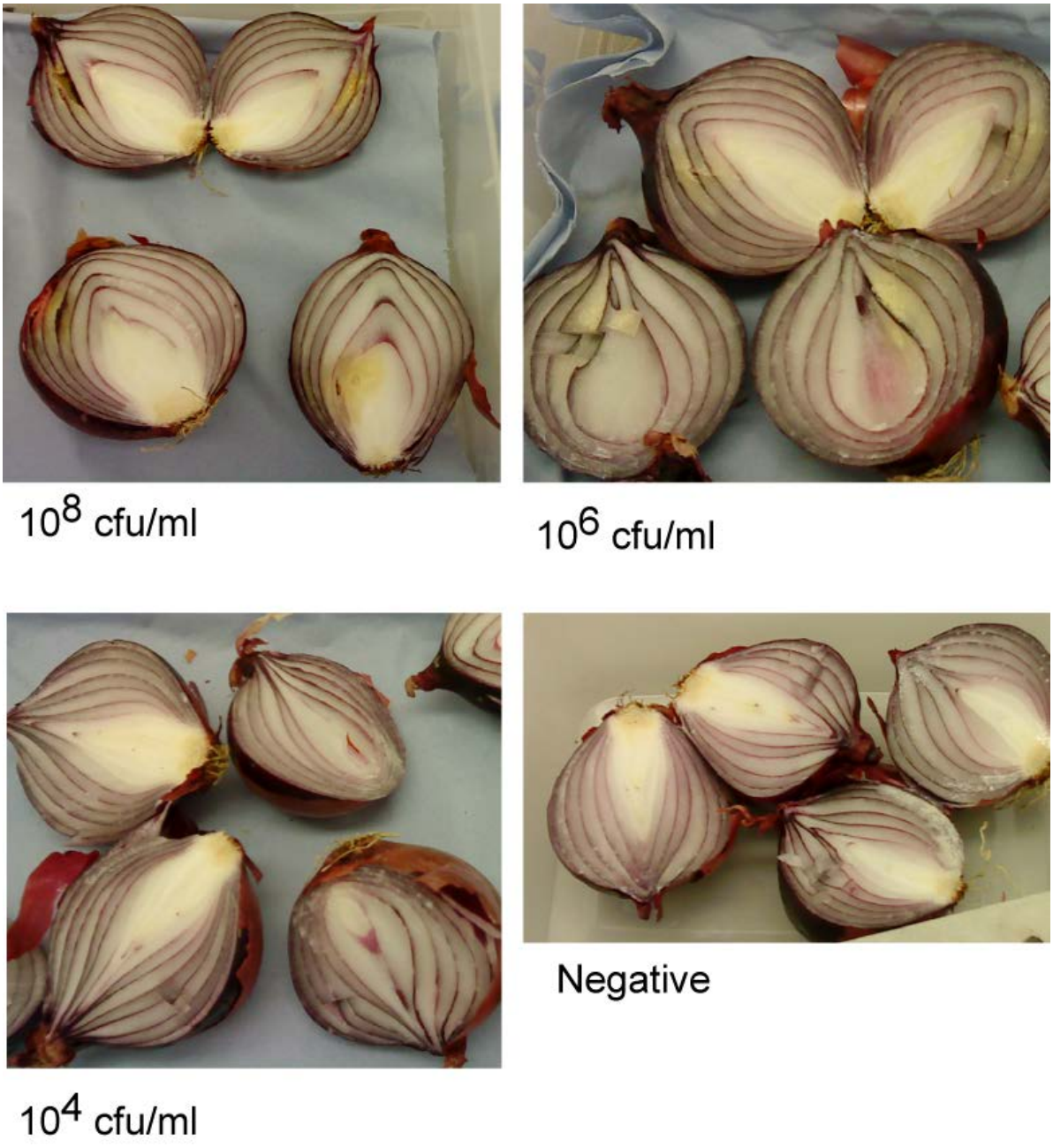

Figure 3 Photographs of artificially infected mature red onion bulbs, at different concentrations of inoculum. The values refer to the inoculum concentration and the uninfected control (Negative).

Discussion

In glasshouse trials with red onion seeds, we were able to show that plant defence elicitors can significantly reduce bacterial infection in onions caused by Bga (*Burkholderia gladioli*

pv. *alliicola*), compared to standard fungicide treatments. Completion of three specific project objectives resulted in:

i. Establishment of glasshouse trials using commercial samples of red onions, grown from seed.
ii. Application of different treatment strategies to determine whether addition of compounds eliciting plant defence responses is as effective as copper oxychloride and standard fungicides alone.
iii. Determination of the extent and incidence of bacterial infection in bulb onions following a period of heat-treatment.

Two different infectious doses of Bga were tested and both trials showed the lowest level of infection in the elicitor-treated onions. However, there were differences in the treatments with added fungicides, in particular with Cuprokylt and the SFP-only treatments. The differences are likely to have arisen because the infectious dose of 10^4 cfu/ml was very low and resulted in inconsistent infection rates in the onion bulbs. **At the higher level of inoculum, treatment with the elicitors resulted in significantly less infection than treatment with Cuprokylt.**

The trial was established to test elicitors on onions grown from seed, because it is thought that onion sets provide the infective inoculum. When they are planted, Bga bacteria in the immature bulb scales can be transferred to the surrounding soil and maturing bulb as the plants develop. This effectively provides an inoculum for the maturing bulb as well as re-infecting the surrounding soil. This trial was not designed to test whether elicitors would continue to provide protection against Bga infection for mature bulbs developed from infected sets, but this study should now be conducted. In addition, further work on the minimum level of inoculum required to cause disease would be beneficial for growers in determination of the effectiveness of alternative treatments.

Elicitors were incorporated into a standard fungicide programme and applied four times in 18-days intervals. Three different combinations, each with two elicitors were selected on the basis of their reported activity elsewhere. A total of four elicitors were used in the Bga-onion trial: Probenazole is used as a standard treatment against rice blast and rice blight in Asia (Sakamoto*, et al.*, 1999), Bion is also used in Asia to treat cucumbers (Deepak*, et al.*, 2006), and both Bion and BABA have been used in broccoli trials (Pajot & Silue, 2005). *cis*-jasmone is an analogue of a defensive plant hormone (jasmonate) and has been shown to provide protection against herbivores and also other pathogens (Smith*, et al.*, 2009). We have used these elicitors previously in field trials of broccoli, in an attempt to control head-

rot bacteria (FV 378). It is common practice to use elicitors in combination, either with each other or with licensed fungicides, to trigger multiple defensive pathways in the plant host and so confer protection against a broad range of pathogens. It is interesting that all three combinations of elicitors had a beneficial effect on Bga infection of onions. This would suggest that Bga is sensitive to a basal level of plant defense resistance that is common to the four different elicitors used in this trial. Further work is required to determine if any of the elicitors had a dominant effect on Bga infection of onions. 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

Plant defence elicitors were tested against Bga (*Burkholderia gladioli* pv. *alliicola*) infection of red onions (var. Red Baron). The onions were grown from seed in a glasshouse trial and harvested at maturity. The bulbs were dried at 28 ^{o}C for three weeks and stored at 1 – 4 ^{o}C for four weeks before the level of Bga was measured. Therefore, the trial assessed whether plant defence elicitors provided any protection against Bga infection in onion bulbs compared to commercial treatments such as Cuprokylt.

Addition of plant defensive elicitors into a standard fungicide programme resulted in a significant and large reduction in the incidence (and extent) of infection of red onions by Bga that was greater than standard treatments, opening the way to possible use of these elicitors in commercial onion production. Future work is now required to understand the underlying mechanisms of protection, which in turn, will provide information for a targeted use of the elicitors.

Knowledge and Technology Transfer

Scientific meetings:

Work on plant defense elicitors in relation to this and another HDC-funded trial (FV 378) 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 2012 (Dundee, UK) as a poster and as a paper in the conference proceedings.

Growers meetings:

Work on this project was presented by Andy Richardson at the UK Onion and Carrot Conference in Peterborough on 16th November 2011.

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APPENDICES

Appendix 1 Supplementary figures.

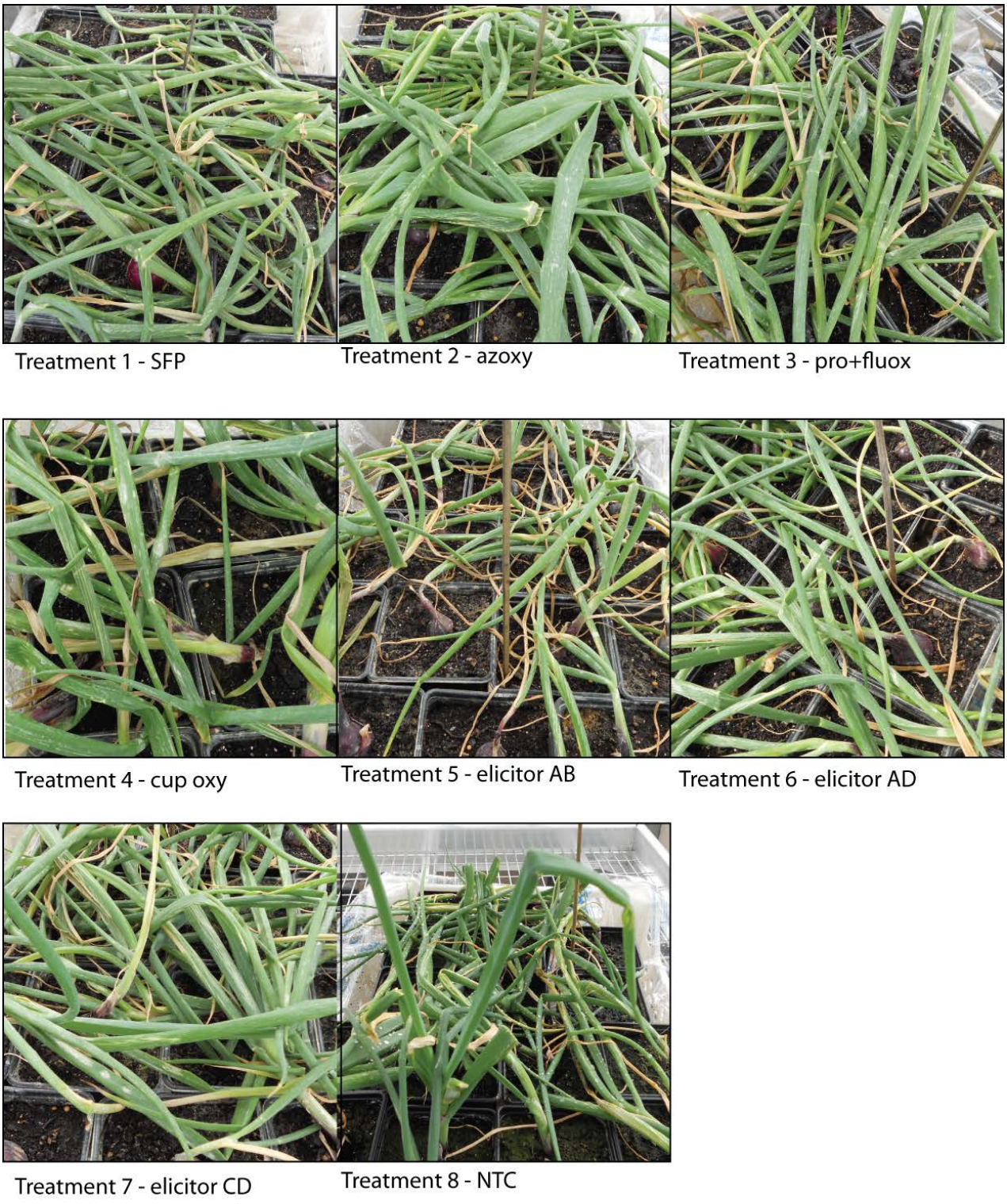

Figure 4 Photographs showing onion plants immediately prior to harvest. The treatments are described in Table 2.

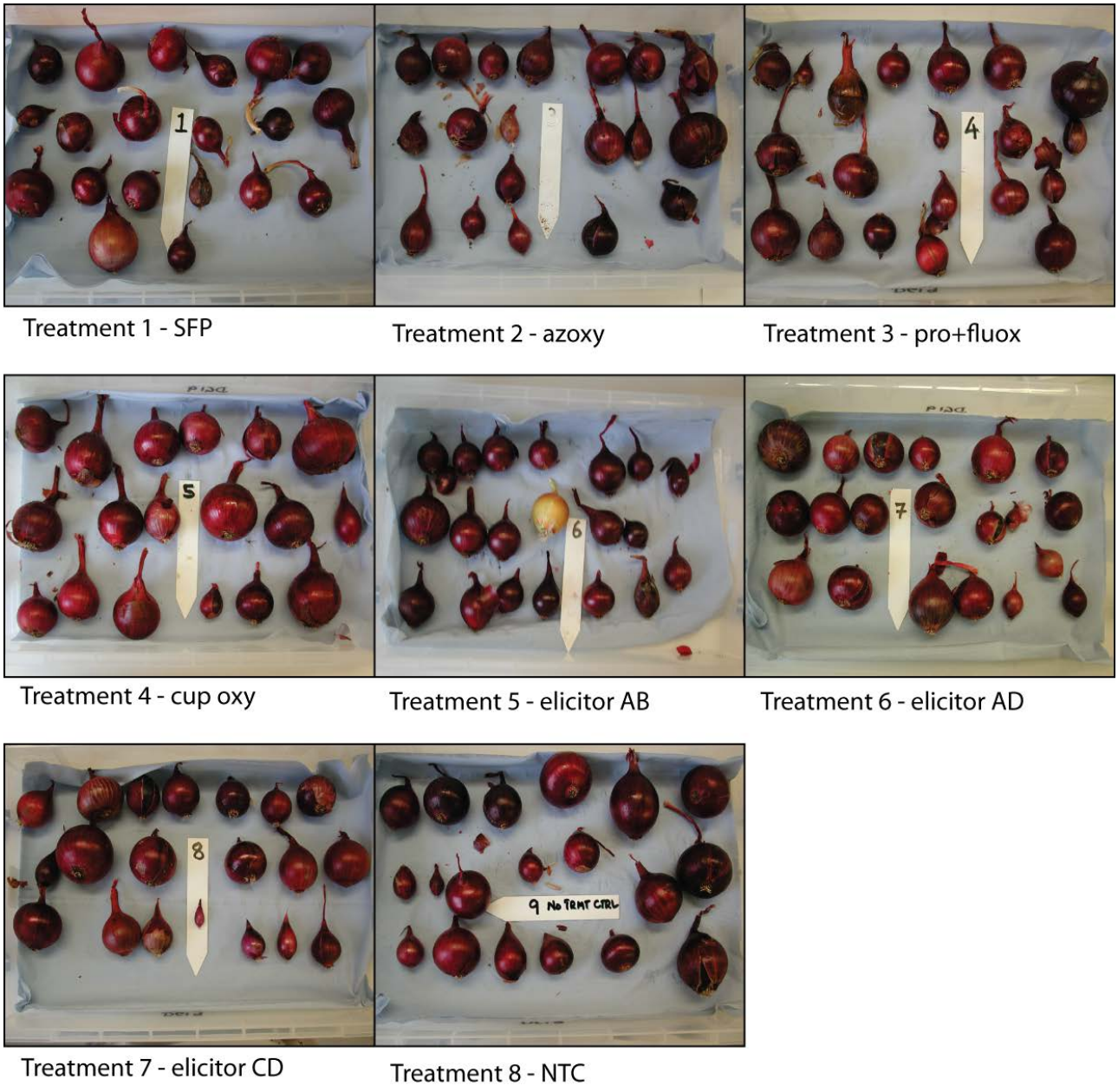

Figure 5 Photographs showing onion bulbs post drying and immediately prior to sampling. The treatments are described in Table 2. (N.B. plant labels in photos correspond to the plant tray number used in the glasshouse, not the Treatment number).

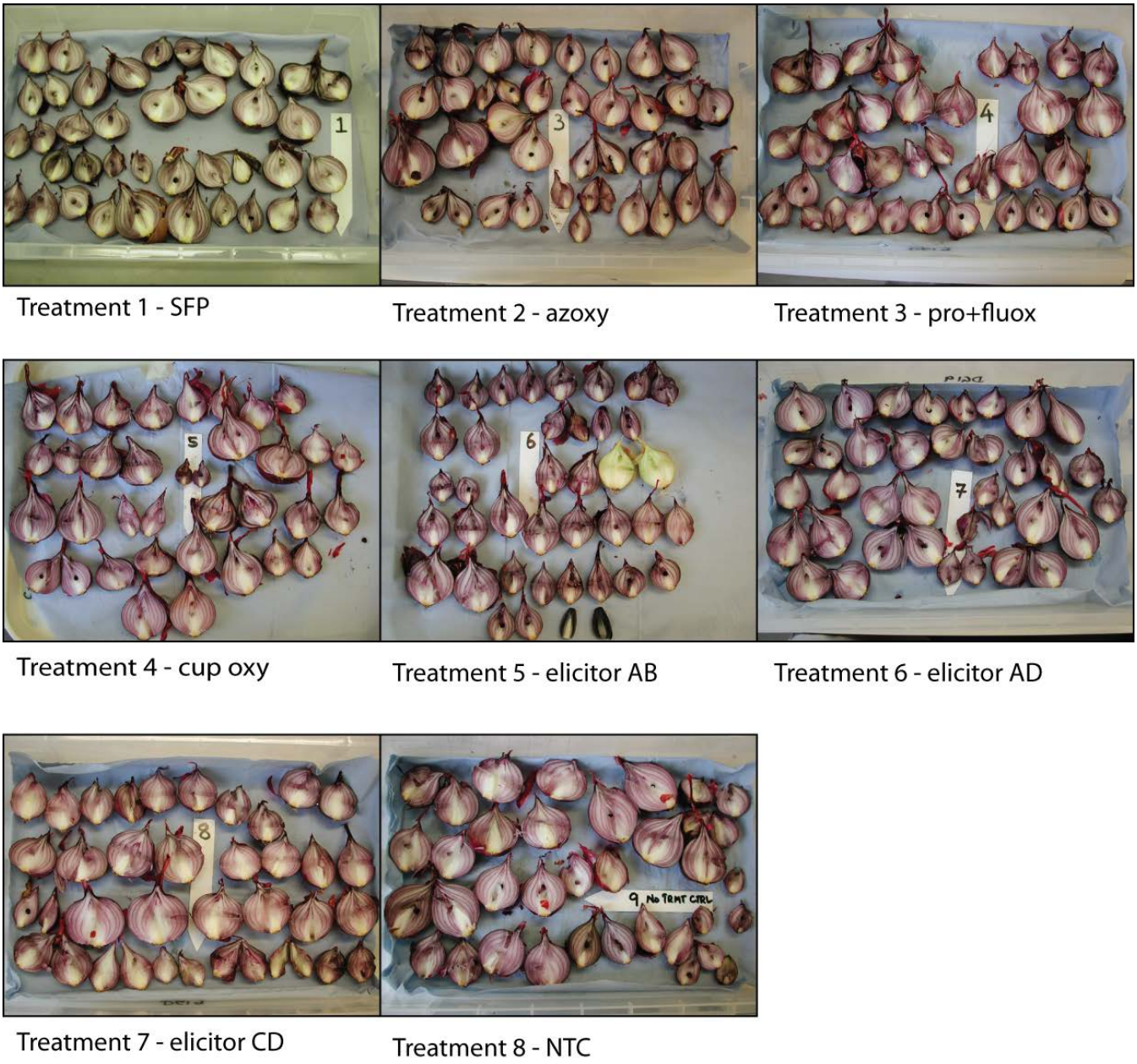

Figure 6. Photograph showing onion bulbs post sampling (coring). The treatments are described in Table 2. (N.B. plant labels in photos correspond to the plant tray number used in the glasshouse, not the Treatment number).

