Project title:	Determining the effectiveness of seed treatments on the occurrence of neck rot disease in onions caused by *Botrytis* spp.
Project number:	FV 423
Project leader:	Dr Charles Lane, FERA
Report:	Final report, December 2013
Previous report:	
Key staff:	Victoria Barton- seed health analyst Dr Stéphane Pietravalle – statistical consultant
Location of project:	FERA, Sand Hutton
Industry Representative:	Alastair Findlay, The Lakes Farm
Date project commenced:	01/05/2013
Date project completed (or expected completion date):	20/12/2013

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.

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GROWER SUMMARY

Headline

Nine seed treatments were found to be effective at controlling Botrytis neck rot.

Background

Neck rot of onion is a serious and damaging pathogen which may cause significant losses in stored onions. In 2011, it was estimated that 12% of the UK crop was either dumped or downgraded due to the neck rot with a total loss of £9 million at farmgate.

There are up to 8 different species of Botrytis associated with onions causing a variety of diseases including neck rot, bulb rot, scape (inflorescence stalk) and umbel (inflorescence) blight. *Botrytis aclada*, *B. allii* and *B. byssoidea* are commonly found to be the cause of the rot but other species such as *B. squamosa, B. tulipae*. *B. elliptica, B. porri* and *B. cinerea* have also been recorded but are not considered typically to be the primary cause of neck rot.

Seed treatments are an essential part of the management strategy for the control of this disease. Therefore, there is a need to evaluate a range of treatments in order to ensure effective control of these pathogens for the future using a range of potential treatments.

Summary

A single lot of known infected seed (variety Solution) was treated with 11 different products including eight chemical treatments, one biological control agent and a combined physical and chemical seed treatment. The range of products included a number of experimental products as well as current seed treatments commonly used in both the UK (Hy-Tl) and the Netherlands (Topsin M). Infection levels were assessed by using a growing-on test (blotter test) with infection assessed by visual examination after three weeks. Although not quantified, there was no visual difference in germination rates between the various treatments. The infection levels within the untreated control were quite variable (3.5-8.5%) necessitating assessment of five replicates each comprised of 400 seeds. Due to the significant increase in assessments, it was not possible to determine the species diversity of Botrytis for each treatment. However, 13 isolates from the untreated control revealed the presence of predominantly *Botrytis aclada* (12/13) but also *B. allii* (1/13). No other species of Botrytis was observed in the untreated control.

Financial Benefits

The work has evaluated the effectiveness of different seed treatments for the control of onion neck rot, demonstrating that the majority, but not all, products can reduce infection levels to a level (less than 1%). It is anticipated that this should reduce post-harvest storage levels to a commercially acceptable level.

Action Points

- Use this report to review the effectiveness of seed treatments against Botrytis neck rot.
- It is important for scientists to monitor the effectiveness of current and future products to ensure effective control of Botrytis on onion seed.

SCIENCE SECTION

Introduction

Neck rot of onion is a serious and damaging pathogen which may cause significant losses in stored onions. The disease is most common on bulbs after harvest only developing symptoms once in store thus preventing grading out prior to storage. Affected scales initially become sunken and soft, but with time, tissue may become grey in colour and eventually fungal growth appears between the scales. On some occasions, the fungus may produce hard, black bodies (sclerotia) that form around the neck. Secondary infection by other organisms may further exacerbate decay in store.

Extensive sampling of around 40,000 tonnes of crop (10% of total UK bulb onion production) by the Allium & Brassica Centre in 2011 showed that around 2% of bulbs sampled had neck rot symptoms. However infection levels in individual stores were as high as 48% in badly affected crops. Commercially infection levels in excess of 8% in stored samples will lead to the crop either being dumped or downgraded to the lower value processing market. In 2011 it is estimated that 12% of the UK crop was either dumped or downgraded due to neck rot with a total loss of £9 million at farm gate.

There are up to 8 different species of Botrytis associated with onions causing a variety of diseases including neck rot, bulb rot, scape (inflorescence stalk) and umbel (inflorescence) blight. *Botrytis aclada, B. allii* and *B. byssoidea* are commonly cited as the cause of the rot but other species such as *B. squamosa, B. elliptica, B. tulipae, B. porri* and *B. cinerea* have also been recorded but are not considered typically to be the primary cause of neck rot (Chilvers et al., 2006).

Identification to species of Botrytis is a very complex matter and although cultural and morphological parameters can be used to separate many of the species, unfortunately, two of the most important species *B. aclada* and *B. allii* cannot be reliably separated using morphometric characters. This has led to some debate and confusion as to whether this is a species complex or two discrete species. At one stage, two subgroups of *B. aclada* (AI and AII) were proposed but further analysis showed these were two discrete species *B. aclada* (AI, small spored isolates) and *B. allii* (AII, larger spored isolates). Molecular techniques have confirmed that these two taxa are separate species and correctly should be referred to as *B. aclada* and *B. allii* (Yohalem et al., 2003; Chilvers, et al., 2007). This difficulty in reliably identifying these two species has resulted in little clarity about the

epidemiology and geographical distribution of these organisms at the species level.

Seed treatments are essential part of the management strategy for the control of this disease. Maude & Presly (1977, a, b) demonstrated that a major source of the pathogen was infected seed. In 1972 and 1973, 39.5 and 71.4% respectively of commercial onion seed samples were infected. The pathogen was internal in seed and persisted for 3 ½ years in infected seeds kept in a seed store at 10°C and 50% relative humidity. Seedlings raised from diseased seed became infected by mycelial invasion of the senescing cotyledons. They also demonstrated that the amount of neck rot in onion stores was directly related to the percentage infection of onion seeds. Although the rate of infection of bulbs was subject to modification by weather conditions during the growth of the onion crop the ratio of seed to bulb infection for different percentages of seed infection remained constant. They demonstrated that 10% infected seeds gave neck rot losses of commercial significance (greater than 10%) in both wet and dry years; whilst 1% seed infection caused significant losses only in wet summers. Therefore, they proposed that levels of less than 1% seed infection would be necessary to ensure acceptably low levels of the rot in stored bulbs in most years.

Project aim:

To identify if multiple species of *Botrytis* are infecting onion seeds and leading to failure of seed treatments for neck rot disease in emerging seedlings.

Project objectives:

1. Conduct controlled growing-on tests of onion seeds treated with up to 11 industry standard products and physical procedures.
2. Identify and quantify the *Botrytis* species found to be infecting growing seedlings.
3. Analyse the species occurrences between and within the treatments.
4. Inform grower community of the likelihood that seed treatment failures are either due to the presence of *Botrytis* species which are not controlled or resistance to active ingredients within the established population of the pathogen.

Materials and methods

A single seed lot (var. Solution), known to be infected with Botrytis (circa 11 kg, reported as having an internal Botrytis infection of 8% but of unknown species), was provided to Elsoms for sub-division and application of treatments using standard industry protocols and according to the European seed treatment assurance scheme (ESTA). QA statements for seed loading were provided by Elsoms.

Treatments:

1	Untreated
2	Thiram
3	Topsin M
4	HyTL
5	Fludioxonil
6	HDC F137 experimental chemical control product
7	Thiram + Fludioxonil
8	Thiram + HDC F137
9	HDC F138 experimental biological control product
10	Fludioxonil+ HDC F137
11	Physical /Chemical (hot water thiram soak)

Initial evaluation:

At the onset of the project, a representative homogeneous sample was drawn from the untreated control to determine the level of infection using a blotter method. Following this initial evaluation results were discussed with FERA's statistical consultant to develop a robust design for the evaluation experiments in order to demonstrate that treatments could reduce infection to less than 1%.

Treatment evaluation:

Blotter technique

The level of Botrytis for each treatment was determined using a blotter technique (figures 1 to 3) (Lane *et al*., 2012). A representative sample was drawn from each treatment and 400 seeds counted out (referred to as a 'lot'). This was further divided into four subdivisions, each of 100 seeds, which were placed aseptically onto each blotter tray (filter paper kept constantly damp due to contact with a reservoir of water beneath the tray) and seeds evenly distributed. The seed was not surface sterilised prior to assessment. The tray was placed in a seed germinator and covered with a lid to ensure high humidity at all times. Incubation chambers were placed in a large walk-in incubator with a constant temperature of 21°C and 12 hours dark/12 hours UV light. Incubation chambers were checked on a regular basis to ensure moisture levels were maintained and that fast-growing saprophytic fungi did not contaminate the experiment. Blotter trays were examined under a dissecting microscope on a weekly basis with final assessment recorded after 21 days incubation. Potential colonies of Botrytis were examined under high magnification with a dissecting microscope or if required, a small amount of fungal material was removed aseptically and microscope preparations made and examined under a high power compound microscope. The number of seeds infected with Botrytis for each treatment was determined.

Isolate purification

Isolates of Botrytis were obtained from the untreated control by removing a small portion of the fungus and transferring it aseptically to agar. Colonies were incubated as described above to permit purification and identification using morphological techniques.

Morphological assessment

Isolates were grown on a standard medium (potato dextrose agar) and incubated under the standard conditions as described above. After 10 days incubation, the length and breadth of conidia (asexual spores) were measured. Other morphological features (e.g. such as the presence of sclerotia) was monitored for over an extended period (4 to 8 weeks).

Molecular assessment

Representative cultured isolates were characterized by PCR amplification of the ribosomal IGS region (Khan et al., 2013) and sequencing of the resulting product. The consensus sequences from each of the cultures were aligned with published representative sequences of *Botrytis* and *Sclerotinia* using the ClustalW algorithm to permit species discrimination.

Results

Initial evaluation

The results showed that infection levels varied from 3.5 to 8.5% (Table 1). Following these results statistical analysis demonstrated that in order to detect less than 1% level of infection with a 95% confidence interval five lots (each of 400 seeds, total 2000 seeds) should be tested.

Table 1. Total infection for the untreated control of each lot of 400 seeds tested after 20 days.

	Total seed out of 400 infected with *Botrytis* spp.	Infection (%)
Lot1	14	3.5
Lot2	33	8.3
Lot3	22	5.5
Lot4	34	8.5

Species identification

Morphological identification indicated the presence of isolates belonging to either *Botrytis aclada* or *B. allii* with molecular sequencing confirming the dominance of *B. aclada* (12/13) compared to *B. allii* (1/13) (Appendix 1).

Treatment evaluation

Results for the number of seeds infected with Botrytis for each lot (400 seeds) with five replicates are presented in Table 2, in addition to estimates for 95% confidence intervals for the mean prevalence in each bag.

No Botrytis was detected in treatment number 11 (chemical and physical seed treatment) with levels of Botrytis in the untreated control (treatment number one) varying between 0 to 14 infected seeds per 400 (0-3.5%).

For all the other remaining treatments apart from treatment three and nine no Botrytis was detected.

For treatment three (Topsin M) Botrytis was detected varying from 13 to 37 infected seeds per 400 (3.25-9.25%).

For treatment nine (experimental biological control agent) although very low levels of Botrytis were detected in each 400 seed lots (0 to 2) and prevalence the in bag was estimated at between 0.005 to 0.4% this is still well below 1%. It should not be inferred from this result that it is any less effective than the other treatments in which no Botrytis was observed and estimated prevalence was less than 0.22%. It is also worth noting, that seed tests do not give a good indication of the efficacy of biological control agents and transmission studies are preferable (Roberts, 2013).

Table 2. Observations and estimates of disease prevalence in bags.

Treatment number	Lot	Number of seeds infected with *Botrytis* spp. out of 400	Estimated prevalence in bag (%) (95% C.I.)
1 Untreated	1	14	0.58 to 4.3
	2	6	
	3	11	
	4	1	
	5	0	
2 Thiram	1	0	<0.22
	2	0	
	3	0	
	4	0	
	5	0	
3 Topsin M	1	13	3.3 to 8.2
	2	32	
	3	12	
	4	11	
	5	37	
4 HyTl	1	0	<0.22
	2	0	
	3	0	
	4	0	
	5	0	
5 Fludioxonil	1	0	<0.22
	2	0	
	3	0	
	4	0	
	5	0	
6 HDC F137	1	0	<0.22
	2	0	
	3	0	
	4	0	
	5	0	
7 Thiram & Fludioxonil	1	0	<0.22
	2	0	
	3	0	
	4	0	
	5	0	
8 Thiram & HDC F137	1	0	<0.22
	2	0	
	3	0	
	4	0	
	5	0	
9 HDC F138	1	2	0.05 to 0.4
	2	1	
	3	0	
	4	0	
	5	0	
10 Fludioxonil	1	0	<0.22
	2	0	
	3	0	
	4	0	

& HDC	5	0

Treatment number	Lot	Number of seeds infected with *Botrytis* spp. out of 400	Estimated prevalence in bag (%) (95% C.I.)
11 Physical seed treatment to disinfect	1	0	<0.22
	2	0	
	3	0	
	4	0	
	5	0	

Statistical analysis

Prevalence of infection in the untreated lot showed evidence of variation indicating infected seeds were not evenly distributed within the initial starting material making a heterogeneous starting sample. It is believed that the composite 11 kg sample had been obtained from four different sources before consolidation and therefore may not have been fully mixed. Hence, estimates of prevalence in bags which returned non-zero results were made by fitting a beta binomial model to account for within bag variation. Estimates of the upper limit for prevalence in each bag that returned all zero results (the upper limit for prevalence is equal to the limit of detection of examination) was made assuming that potential within bag variation was equal to the within bag variation estimated for the untreated bag.

Phytotoxicity

Although no quantitative assessments of seedling germination were carried out during this test, no obvious differences between the numbers of seedlings were observed.

Figure 1. Blotter trays in large incubation room.

Figure 2. Visual examination of blotter trays under a dissecting microscope.

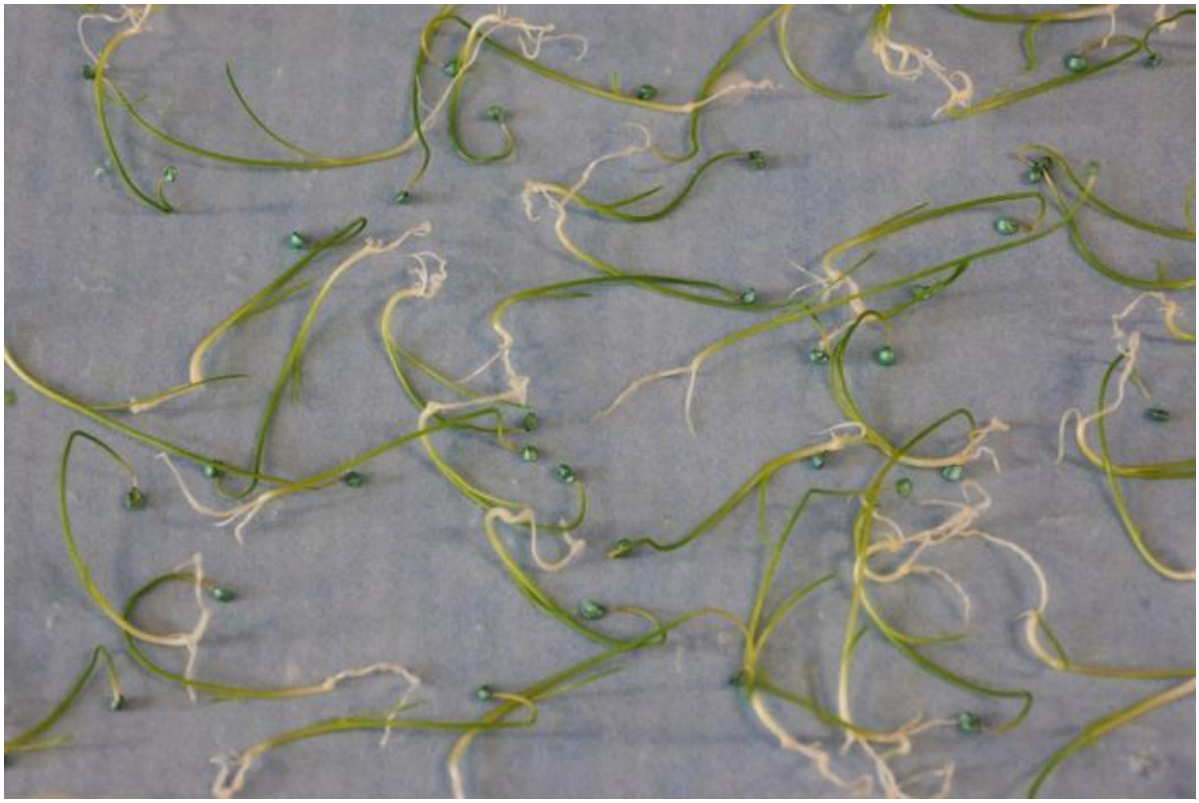

Figure 3. Onion seedlings for assessment.

Discussion

The prevalence in the untreated bag was estimated to be somewhere between 0.58 and 4.3% seeds. For all other treatments, with the exception of treatment three (Topsin M), a mean prevalence significantly below 1% was recorded. For treatment three, the prevalence was estimated to be somewhere between 3.3 and 8.2%. Hence the results provide evidence that, with the exception of treatment three, all treatments have the potential to reduce prevalence to below 1%.

The extent to which any treatment will reduce prevalence to this level in practice will depend on the uniformity of infection in the bulk to the treatment is applied and the uniformity with which treatment can be applied throughout the bulk.

It is important to remember that the assessment of the efficacy of treatments was only possible on one known infected seed lot and caution should be drawn before extending this result to encompass all seed lots.

Assessment of the species diversity revealed the dominance of *Botrytis aclada* in comparison to *B. allii* but with no other species found. Traditionally in the UK, onion neck rot has been attributed to *Botrytis allii*, however, due to the difficulty in separating species this is probably an historical assumption. Du Toit (*pers. comm.*) stated that in her experience in Washington State that neck rot was caused equally by *B. allii* and *B. aclada* and they would not routinely speciate the causal agent of neck to rot. However, now identification of Botrytis species is easier due to molecular techniques it would be interesting to re-evaluate the current species diversity within UK onion crops.

Due to the variable infection level in the untreated seed lot (3.5-8.5%) it was necessary to assess five times the number of replicates than originally planned thus preventing assessment of species diversity for individual treatments. Therefore, it was not possible to assess species diversity within treated lots (objective 3).

Conclusions

The current UK standard treatment Hy-Tl was found to be effective at reducing infection levels to less than 1%. However, Topsin M was found not to be effective at reducing infection levels to less than 1%. Fludioxonil and two new experimental products (HDC F 137 and 138) were also effective at reducing incidence to less than 1%.

Morphological identification indicated the presence of isolates belonging to either *Botrytis aclada* or *B. allii* with molecular sequencing confirming the dominance of *B. aclada* compared to *B. allii*.

Further work is required to establish if this seed lot is representative of seeds from other provenances.

Knowledge and Technology Transfer

Interim report concerning treatment efficacy (September 2013).

The Eighth UK Onion and Carrot Conference and Exhibition, Peterborough, November 20, 2013, Platform presentation by Charles Lane.

Vegetable Consultancy Association Annual Meeting, Stilton, November 2013, presentation by Charles Lane.

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Appendix 1

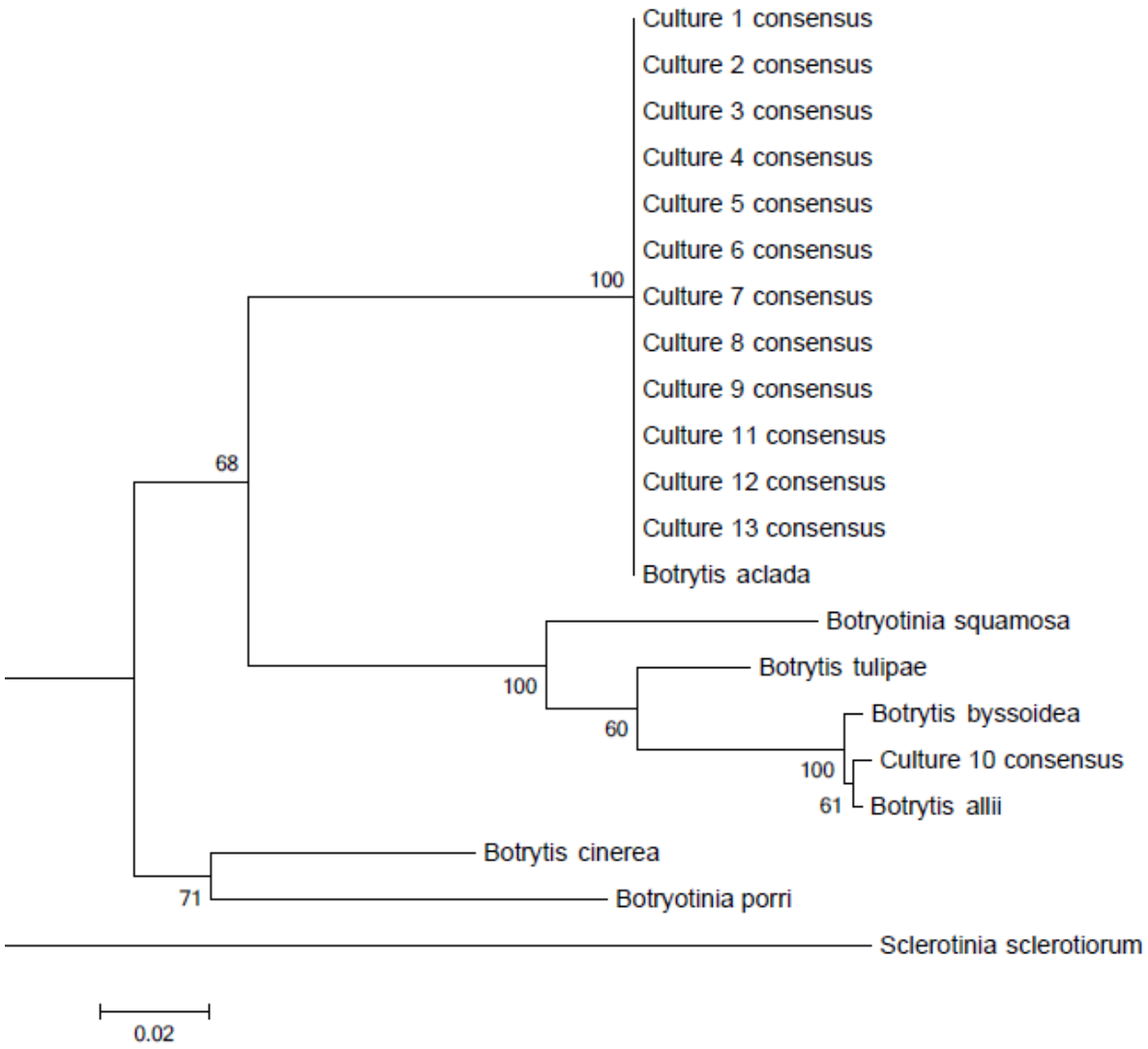

Figure 3. Phylogenetic tree of *Botrytis* 13 isolates from the untreated control in comparison to reference isolate of appropriate species.

Reference isolates:

B. aclada sequence:

JX399167 submitted by Khan,I., Marroni,V., Keenan,S., Viljanen-Rollinson,S., Scott,I. and Bulman,S.

(100% identical over this region to *B. aclada* sequence from Chilvers,M.I., du Toit,L.J. and Peever,T.L

B. allii sequence:

DQ462236 submitted by Chilvers,M.I., du Toit,L.J. and Peever,T.L.