Project title:	**Onion neck rot: seed infection, pathogens and treatments**
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Key staff:	Dr S J Roberts
Location of project:	PHS Laboratory, Ryton Gardens
Industry Representative:	Mr Sam Rix
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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.

Dr S J Roberts

Plant Pathologist

Plant Health Solutions Ltd

Signature ..... Date .....

Report authorised by:

Dr S J Roberts

Director

Plant Health Solutions

Signature ..... Date .....

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GROWER SUMMARY

Headline

Around 26% of 2015 commercial bulb onion seed lots were found to be infested with either *B. allii, B. aclada* or both. Both species seem to be equally prevalent.

Background

Neck rot can be a major cause of losses in stored onions in the UK. The extent of losses is variable. Losses of over 50% were reported in the late 60s and early 70s, and more recently losses of up to 40% been reported in individual crops.

The disease can be caused by three different species of *Botrytis*: *B. aclada, B. allii,* and *B. byssoidea. B. byssoidea* is thought to be less important; *B. allii* and *aclada* were previously lumped together as one species, (usually called *B. allii*), hence the vast majority of the literature and reports of the disease during the 20th century refer to neck rot as caused by *B. allii;* we should now interpret these reports as referring to either *B. allii* or *B. aclada* or both. In this project we will refer to *Ba* to represent both/either of the two main neck rot pathogens, *B. allii* and *B. aclada.*

There is no historical information on the relative distribution or significance of these two species, and it is also not known if there are any differences in their biology and epidemiology or sensitivity to fungicides.

The disease is seed-borne but symptoms are not apparent in the field and only develop in store. It is likely that most seed is tested by seed companies, and most seed is treated with fungicides. Nevertheless major losses still occur in some years. These losses could be a result of failure to control seed-borne infection or alternative sources of inoculum.

Until recently, the industry standard seed treatment for neck rot has been HyTL (thiabendazole + thiram) under a Specific Off-Label Approval (SOLA), and emergency approvals, but the registration has now expired. Recent work has shown that Thiram and Maxim and potentially some new products may be effective, but recent studies have so far been limited to direct effects on low levels of apparent seed infection, there have been some contradictory results, and there is no recent information on resistance or on differences between the neck rot pathogens.

It is also possible that some fungicides applied to the growing crop may have an impact by reducing the rate of spread in the field (and so contribute to control of disease in store) but there is little information on this aspect.

The absence of field symptoms means the link between seed-borne infection and storage losses is obscure; seed-to-seedling transmission depends on pathogen loading, and disease in store is further affected by the weather conditions in the growing season and at harvest, therefore there remains some controversy about the importance of seed infection.

There is no formal standardisation of the seed test method used for *Ba* or of the health standard that needs to be achieved. There is also no assessment of inoculum load. Therefore although seed may have been tested/treated what is considered as 'clean' or 'healthy' may differ depending on the source of the seed and the test laboratory, and the methods and standards applied.

Much work on neck rot in the UK was done during the 1970s at Wellesbourne by Robert Maude and colleagues, some MAFF funded work was done at Wellesbourne by the author as part of a project on organic seed production in the early 2000s. More recently there have been some limited HDC studies on seed treatments and a 3 year TSB-funded project on biological seed treatments (Roberts 2013, 2014).

This main aim of the project was to provide an independent assessment of the current prevalence and incidence of the disease in commercially available onion seed in the UK. Specific objectives were to:

1. Determine the prevalence and incidence of *Botrytis* neck rot pathogens on commercially available onion seed.
2. Obtain isolates of *Botrytis* neck rot pathogens from stored bulbs.
3. Identify isolated neck rot Botrytis spp. to species level.
4. Determine the fungicide sensitivity of selected isolates *in vitro.*
5. Determine the efficacy of fungicide seed treatments on apparent seed infection and seed transmission.
6. Determine if there is a difference in seed-to-seedling transmission between the two *Botrytis allii/aclada* species.

Summary

Seed testing

Thirty bulb onion seed lots from six seed companies, and representing all of the most popular varieties were tested for the presence of the neck rot pathogens *Botrytis allii/aclada* (*Ba*) by direct-plating on a selective medium. We detected *Ba* in eight of the thirty seed lots (i.e. 27%). Two of the positive seed lots were untreated. Six of the positive seed lots had

been treated with fungicides, and gave negative results when tested 'as received', but were then positive when re-tested after a short (10 min) wash. The percentage infestation levels ranged from 0.5% to 59% (2 and 59% in the untreated lots; 0.5 to 33% in the treated/washed).

In addition we also received and tested ten seed lots pre- and post- physical (steam) treatment. All of the pre-treatment tests were positive and all of the post-treatment tests were negative. As these were specially selected as 'known to be infected' by the seed company concerned they are not included in the above statistics.

Stored bulbs

Three bulb samples with typical neck rot symptoms were received. *Ba* was successfully isolated in each case.

Identification of species from seed and bulbs

Over eighty isolates were sub-cultured from seed-test plates or bulbs for further characterisation. Based on colony characteristics, sporophore and spore morphology, etc. these were reduced to about forty for testing by PCR. Isolates were initially tested using neck rot specific primers that give a positive result with *B. allii, B. aclada* and *B. byssoidea*. The initial PCR results generally confirmed the expectations based on colony morphology.

A further PCR with *Botrytis* specific primers followed by digestion (cutting the DNA at a specific place) was used to separate the *Ba* isolates into *B. allii* and *B. aclada*. The results indicated that both *B. allii* and *B. aclada* are present in UK commercial onion seed. Some seed lots contained only one or the other species, but some seed lots contained both species. Both species were also found in stored bulbs with neck rot; in two samples all isolates were *B. allii*, in one all isolates were *B. aclada*.

DNA extracts were also sent to Wellesbourne for sequencing of an IGS region, the sequencing data confirmed the identification based on PCR/digestion, but has also provided tentative indications of two distinct types within the *B. allii* isolates.

Note that the occurrence of *B. aclada* is not new. Before 2002, both *B. allii* and *B. aclada* were lumped together as one species: *B. allii*, and many strains reported as *B. allii* in the pre-2003 literature have since been re-identified as *B. aclada*. *B. allii* is probably a hybrid of *B. aclada* and *B. byssoidea.*

Fungicide sensitivity

A selection of eleven isolates (six *B. allii* and five *B. aclada*), from different seed lots and from bulbs, were tested for sensitivity to selected seed treatment fungicides. The fungicides

were incorporated into the agar medium at the same rate as used for seed treatment. The fungal isolates were inoculated onto the plates as 5 mm diameter agar plugs, and then growth was assessed by measuring the diameter of the resulting growth, if any.

Apron XL (metalaxyl-M) had little effect on any of the isolates. This was expected as metalaxyl-M is not considered to have activity against *Botrytis* spp.

Maxim 480FS (fludioxonil) gave variable results: although the growth and sporulation of all isolates was inhibited compared to the controls, some isolates were completely inhibited, but other isolates eventually grew to the edge of the plates. All of the most fludioxonil-resistant isolates were *B. aclada*.

Thiram gave similar results with all isolates: very limited growth, but not completely inhibited.

Table 1. Effect of chemical fungicides incorporated into the agar medium on the growth rate of neck rot isolates.

Isolate	Year	Source	Lot	Species	Radial growth rate (mm/day)			
					Control	Apron	Maxim	Thiram
8336	2003	seed	n/a	*B. aclada*	8.0	5.0	3.1	0.6
9736	2015	seed	2054W	*B. aclada*	8.0	5.0	0.4	0.5
9738	2015	seed	2056W	*B. aclada*	8.0	5.4	2.4	0.4
9752	2015	bulb	2105	*B. aclada*	8.5	5.0	1.0	0.5
9744a	2015	seed	2058W	*B. aclada*	8.5	4.6	1.1	0.4
9737	2015	seed	2054W	*B. allii*	8.0	5.0	0.5	0.5
9745	2015	seed	2058W	*B. allii*	7.0	5.0	0.4	0.5
9749	2015	seed	2099	*B. allii*	7.5	5.0	0.4	0.5
9754	2015	bulb	2016	*B. allii*	8.0	5.0	0.4	0.5
9757	<1972	bulb	n/a	*B. allii*	7.8	5.3	0.4	0.6
9722B	2015	bulb	2037	*B. allii*	8.0	5.0	0.6	0.5

Seed treatments

Four isolates, two *B. allii* and two *B. aclada* were used to inoculate untreated bulb onion seed. Seed was then treated with either fungicides or biological treatments at recommended rates, or hot water. The hot-water and fungicide treated seed was then tested by direct-plating on selective medium. [Note there is no value in direct-plating of seed treated with biologicals as the applied microbes are either inhibited by the medium (bacteria) or overgrow the plates (fungi)]. The inoculated-treated seed was also sown in modules in the glasshouse to examine seed-to-seedling transmission. Seedlings were then harvested 3-4 weeks after sowing to check for transmission.

The results indicated:

Apron XL is ineffective against *B. allii/aclada* (as expected).

Maxim 480 FS is inhibitory but not eradicating and gave significant reductions with all four isolates, but notably was less effective with one of the *B. allii* isolates (9737).

Thiram is inhibitory but not eradicating, and results were less consistent than Maxim. It was less effective than Maxim in direct plating tests and similar for three out of the four isolates in transmission tests. One of the *B. allii* isolates (9737) was not controlled.

Hot water was the most effective treatment for three of the four isolates in the direct plating assays and although it appeared to fail for one isolate, inoculum loading was visually reduced. Hot water gave significant reduction for all isolates in the transmission tests.

The two bacterial BCAs (HDC195, HDC196) were overall less effective and varied depending on the *Ba* isolate. The fungal BCA (HDC194) was almost as effective as the chemical fungicides.

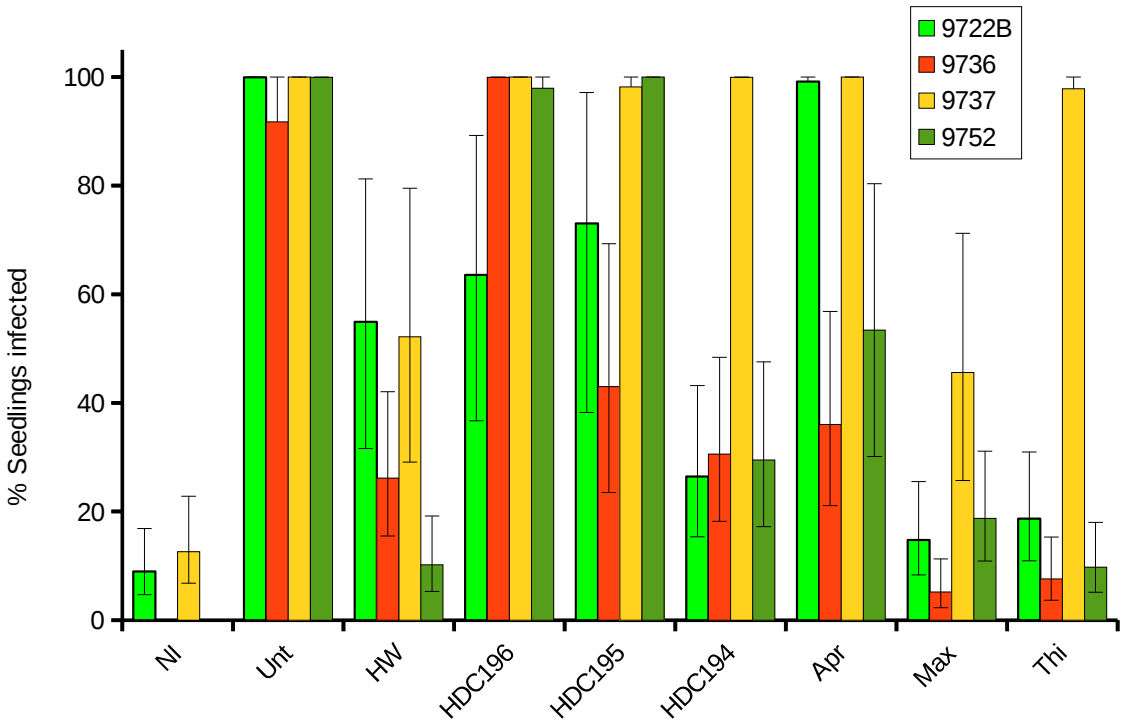

Figure 1. Effect of seed treatments on the % onion seedlings infected with four different neck rot isolates: *B. allii* (9722B, 9737); *B. aclada* (9737, 9752). Error bars represent the 95% confidence limits.

Dose-response assays

Unfortunately the dose response assays to compare transmission between the two species provided little information, as the range of doses used was insufficient. However, at the time

of sampling (3-4 weeks after sowing) there was evidence that secondary spread had already occurred with both *B. allii* isolates but not with the *B. aclada* isolates.

Financial Benefits

There are no clear direct financial benefits from this project as it sought to provide background data to explain variability in control and disease outbreaks. Based on average loses of around 10% p.a. direct financial losses from neck rot are estimated at around £7.5 million p.a.

Action Points

- Do not assume that fungicide-treated onion seed is free from neck rot pathogens.
- Do not assume that chemical fungicide treatments will be fully effective against neck rot.
- Request information from seed suppliers on their policies and steps taken to minimise the risk from neck rot: e.g. do they test all seed lots; do they reject seed lots with high levels; do they apply a physical treatment; etc.
- Further work is needed to understand possible differences in the epidemiology of the two pathogens and the impact on control options.
- Further work is needed to understand field spread and the impact, timing and efficacy of field-applied fungicides.
- The manufacturer/supplier of the experimental BCA HDC 194 should be encouraged to bring the product to market as a seed treatment.

SCIENCE SECTION

Introduction

Neck rot can be a major cause of losses in stored onions in the UK. The extent of losses is variable. Losses of over 50% were reported in the late 60s and early 70s, and more recently losses of up to 40% been reported in individual crops.

The disease can be caused by three different species of *Botrytis*: *B. aclada, B. allii,* and *B. byssoidea. B. byssoidea* is thought to be less important; *B. allii* and *aclada* were previously lumped together as one species, (usually called *B. allii*), hence the vast majority of the literature and reports of the disease during the 20th century refer to neck rot as caused by *B. allii;* we should now interpret these reports as referring to either *B. allii* or *B. aclada* or both. In this project we will refer to *Ba* to represent both/either of the two main neck rot pathogens, *B. allii* and *B. aclada.*

There is no historical information on the relative distribution or significance of these two species, and it is also not known if there are any differences in their biology and epidemiology or sensitivity to fungicides.

The disease is seed-borne but symptoms are not apparent in the field and only develop in store. It is likely that most seed is tested by seed companies, and most seed is treated with fungicides. Nevertheless major losses still occur in some years. These losses could be a result of failure to control seed-borne infection or alternative sources of inoculum.

Until recently, the industry standard seed treatment for neck rot has been HyTL (thiabendazole + thiram) under a Specific Off-Label Approval (SOLA), and emergency approvals, but the registration has now expired. Recent work has shown that Thiram and Maxim and potentially some new products may be effective, but recent studies have so far been limited to direct effects on low levels of apparent seed infection (this can result in products appearing more effective than they really are). There have also been some contradictory results, and there is no recent information on resistance or on differences between the neck rot pathogens.

It is also possible that some fungicides applied to the growing crop may have an impact by reducing the rate of spread in the field (and so contribute to control of disease in store) but there is little information on this aspect.

The absence of field symptoms means the link between seed-borne infection and storage losses is obscure; seed-to-seedling transmission depends on pathogen loading, and

disease in store is further affected by the weather conditions in the growing season and at harvest, therefore there remains some controversy about the importance of seed infection.

Infestation levels in untreated seed lots can be relatively high (>30%). Seeds may be infested with the pathogen(s) both externally and internally. Presumably internal inoculum is less susceptible to treatments.

There is no formal standardisation of the seed test method used for *Ba* or of the health standard that needs to be achieved. There is also no assessment of inoculum load. Therefore although seed may have been tested/treated what is considered as 'clean' or 'healthy' may differ depending on the source of the seed and the test laboratory, and the methods and standards applied.

Much work on neck rot in the UK was done during the 1970s at Wellesbourne by Robert Maude and colleagues, some MAFF funded work was done at Wellesbourne by the proposer as part of a project on organic seed production in the early 2000s. More recently there have been some limited HDC studies on seed treatments and a 3 year TSB-funded project on biological seed treatments (Roberts 2013, 2014).

This project aims to provide an independent assessment of the current prevalence and incidence of the disease in commercially available onion seed in the UK. We will also examine the sensitivity of the pathogen(s) to fungicides and the ability of seed treatment fungicides to control the disease (reduce seed-to-seedling transmission). We will also begin studies to determine if it matters which of the neck rot pathogen(s) are present on seed.

Materials and methods

Seed sources

In collaboration with the grower coordinator (S. Rix), and consultants (A. Richardson of the Allium and Brassica Centre; and T. Will of Vegetable Consultancy Services) a list of the most widely grown bulb onion varieties from each of the major onion seed suppliers in the UK was drawn up, with the number of varieties from each company approximately in proportion to market share. Treated and untreated seed of each of the target varieties was then requested from each of the seed companies, 'as supplied to growers'. Suppliers were aware that the seed would be tested for the presence of *Ba*. We also obtained 'left-over' residual seed after drilling from some growers and from trials work.

Seed testing

Seed was tested by direct plating on semi-selective Kritzman's agar medium (Kritzman & Netzer 1978). Twenty-five seeds were spaced evenly on each 9.0 cm plate. Plates were

then incubated for up to 14 d in the dark at 20°C. Individual seeds on each plate were then examined for the presence of typical *Ba* (based on sporophores and conidia), using a stereo microscope, after 5-7 days and then again at up 14 days depending on earlier results. Where appropriate, a selection of suspect *Ba* was sub-cultured from individual seeds to plates of PDA (Potato Dextrose Agar) and incubated at 20°C in the dark for further identification.

Isolation from bulbs

Bulbs were cut in half, and tissue pieces from the leading edges of lesions were excised with a sterile scalpel and plated on PDA. Where typical sporulating *Ba* was present, conidia were directly sub-cultured onto plates of PDA. Where typical sclerotia were present, these were excised with a sterile scalpel, briefly surface sterilised in 0.3% chlorine, rinsed in SDW and then plated directly on PDA. PDA plates were incubated at 20°C in the dark.

Identification of isolates

A selection of isolates from each positive seed test and from each of the bulb samples were subject to further tests to identify them to the level of species. The macro appearance of growth and sporulation, and production of sclerotia was observed on plates of PDA. Preparations of conidia from PDA plates was also observed by light microscopy, micrographs obtained, and spore size measurements made using the measurement tool in Adobe Photoshop (calibrated to the appropriate magnification of the image).

To extract DNA for PCR and sequencing, isolates were sub-cultured to potato dextrose broth (PDB) in 1.5 ml microtubes and incubated for 5-7 d at 20°C. Tubes were then centrifuged and the culture supernatant removed. DNA extraction was then done using either Sigma extract-n-amp or Qiagen DNeasy kits, following the manufacturers' instructions.

Isolates were tested by conventional PCR using 'neck-rot' specific primers (Chilvers *et al.* 2007). These primers give a single 114 bp product with *B. aclada*, *B. allii*, and *B. byssoidea,* whereas no product is obtained with other *Botrytis* species that may be present on onion seed.

In addition, to discriminate between the three 'neck-rot' species, a selection of isolates was tested using PCR followed by RFLP analysis (enzymatic digestion of the products to produce patterns which are specific to each of the three species) (Nielsen, Yohalem & Jensen 2002). A 413 bp product is produced by all three species and this is then digested by XapI: this results in a single 413 bp band for *B. aclada*, a 413 bp and 298 bp product for *B. allii*, and a 298 bp produce for *B. byssoidea.*

Finally to confirm the above results a selection of DNA extracts were sequenced by Dr Andrew Taylor at Warwick Crop Centre.

Fungicide sensitivity

A selection of eleven isolates (six *B. allii* and five *B. aclada*) from different seed lots and from bulbs, were tested for their ability to grow on agar plates containing the fungicide at the standard recommended concentration for seed treatment. The appropriate volume of fungicide was added to molten PDA after autoclaving and cooling to 50°C and then poured into 9 cm Petri dishes, approx 20 mL per plate. Isolates for testing were sub-cultured to plates of PDA and incubated until growth reached the edge of the plates. The fungicide-containing plates plus control plates containing no fungicide were then inoculated in the centre with a 5 mm diameter agar plug of growth from PDA. Plates were then incubated at 20°C in the dark and the diameter of growth recorded at 5 and 11 days after inoculation, when growth in key treatments had reached the edges of the plates.

Seed treatment

A sample of non-treated seed was kindly provided by Bejo Seeds. Four isolates (two *aclada* and two *allii*) were each used to inoculate sufficient seed for each treatment. Seed was inoculated using a proprietary method developed during an earlier Technology Strategy Board project. The inoculation method results in seeds that mimic a heavily naturally infested seed lot that is both externally and internally infested, and where the infestation cannot be removed by surface disinfection or washing.

For the dose response assays, aliquots (3, 3, 3, 24 g) of seed were exposed to inoculum for different periods of time (1.6, 16, 30 and 48 hours).

Nine days after inoculation, 3 g aliquots of seed were then treated with hot water, biologicals, or fungicides (see Table 2) and then tested by direct plating as above (hot water and fungicides only) and in transmission tests.

Table 2. List of seed treatments examined

Product	AI	Approved	Supplier	Rate
Untreated				
Apron XL	metalaxyl-M (339.2 g/L)	y	Syngenta	0.5 mL/kg
Maxim 480FS	fludioxonil (480 g/L)	y	Syngenta	1 mL/kg
Thiram	Thiram	y	Agrichem	5 mL/kg
HDC196	Bacillus sp.	n	Confidential	100 g/kg
HDC195	Bacillus sp.	n	Confidential	100 ml/kg
HDC194	Fungal BCA	n	Confidential	133 g/kg
Hot water		y		50°C 30 min

Transmission testing

'P60' module trays were filled with Fertile Fibre Seed growing medium, levelled off, then lightly compressed. Approx 7 seeds were sown in each cell, with 30 cells (half a tray) per isolate x treatment combination. Trays were then placed in glasshouse and given an initial watering in by hand. Subsequently all further watering was via an overhead sprinkler system controlled by an irrigation timer set to water daily at 08:30 and 17:30, with the duration adjusted according to need.

Statistical analysis

The effect of treatments on the proportion of infested seed estimated by direct plating was analysed by fitting a series of generalised linear models with binomial error distribution and a logit link-function. Means and standard errors were obtained as predictions from the model, after fitting the appropriate model terms.

The effect of treatments on apparent transmission was analysed by fitting a series of generalised linear models with binomial error distributions and complementary log-log link function. Treatment means were obtained as predictions from the relevant model.

All analyses were performed using Genstat (Payne *et al.* 2005).

Results

Seed testing

Thirty bulb onion seed lots from six seed companies, and representing all of the most popular varieties were tested for the presence of the neck rot pathogens *Botrytis allii/aclada* (*Ba*) by direct-plating on a selective medium. *Ba* was detected in eight of the thirty seed lots (i.e. 27%). Two of the positive seed lots were untreated. Six of the positive seed lots had been treated with fungicides, and gave negative results when tested 'as received', but were then positive when re-tested after a short (10 min) wash. The percentage infestation levels ranged from 0.5% to 59% (2 and 59% in the untreated lots; 0.5 to 33% in the treated/washed). (See Table 3).

Table 3. Results of direct plating seed tests for *Botrytis allii/aclada*

Lab No	Variety[1]	Type	Treatment[2]	% Ba[3]		Species[4]
				As rec'd	Washed	
S2038	A1	Natural	Th, Flu, Met	<2	<2	
S2039	B1	Natural	Th,Thio	<2	<2	
S2054	C1	Natural	Flu, Met	<2	33	Bac+Bal
S2055	D1	Natural	Flu, Met	<2	<2	B. cinerea present
S2056	E1	Natural	Flu, Met	<2	9	Bac
S2057	F1	Natural	Flu, Met	<2	12	Bac
S2058	G1	Natural	Flu, Met	<2	2	Bac+Bal
S2059	H1	Pelleted	Flu, Met	<2	<2	
S2060	J1	Natural	None	2	<2	Bal
S2061	J1	Pelleted	Th, Flu, Met	<2	<2	
S2062	K1	Natural	None	<2	<2	B. cinerea present
S2063	K1	Natural	Th, Flu, Met	<2	<2	
S2064	L1	Pelleted	None	<2	3	Bac+Bal
S2065	M1	Natural	None	59	nt	Bac+Bal
S2066	M1	Natural	Th, Thio	<2	<2	
S2067	N1	Natural	Th	<2	<2	
S2087	O1	Natural	Unknown	nt	<1.5	
S2088	P1	Natural	Unknown	nt	<1.5	
S2089	Q1	Natural	Unknown	nt	<1.5	
S2090	R1	Natural	Met, Flu	nt	<1.5	
S2091	S1	Natural	Th, Flu, Met	nt	<1.5	
S2092	T1	Natural	Th, Flu, Met	nt	<1.5	
S2093	A1	Natural	Th, Flu, Met	nt	<1.5	
S2094	U1	Natural	Th, Flu, Met	nt	<1.5	
S2095	V1	Natural	Th, Flu, Met	nt	<1.5	
S2096	W1	Natural	Th, Flu, Met	nt	<1.5	
S2097	X1	Natural	Th, Flu, Met	nt	<1.5	
S2098	Y1	Natural	Th	nt	<1.5	
S2099	Z1	Natural	Th	nt	0.5	Bal
S2100	A2	Natural	Th	nt	<1.5	
S2101	A3	Natural	Th	nt	<1.5	

Notes:
[1] Variety code, samples with the same code represent the same variety.
[2] Fungicide treatment abbreviations: Th – thiram, Flu – fludioxonil, Met = metalaxyl, Thio = thiophanate methyl.
[3] Percentage seeds on which either *B. allii* (Bal) or *B. aclada* (Bac) detected
[4] Which of the two Botrytis species were present.

In addition we also received and tested ten seed lots pre- and post- physical treatment (see Table 4). Although only a limited number of seeds was tested (100), in all cases steam treatment reduced the infestation levels to below the detection limit. As these were specially selected as 'known to be infected' by the seed company concerned they are not included in the above statistics, as they do not represent 'as supplied to growers' seed.

Table 4. Results of seed tests for *Botrytis allii/aclada* on known infected seed pre- and post-steam treatment by the seed company.

Lab No	Type	Treatment (%Ba)	
		None	Steam
S2121	Natural	4	<3
S2122	Natural	12	<3
S2123	Natural	5	<3
S2124	Natural	3	<3
S2125	Natural	3	<3
S2126	Natural	94	<3
S2127	Natural	29	<3
S2128	Natural	10	<3
S2129	Natural	7	<3
S2130	Natural	24	<3

Isolation from bulbs

Three bulb samples with typical neck rot symptoms were received. *Ba* was successfully isolated in each case.

Identification of species from seed and bulbs

Over eighty isolates were sub-cultured from seed-test plates or bulbs for further characterisation. Based on colony characteristics, sporophore and spore morphology, etc. these were reduced to about forty for testing by PCR.

All isolates later confirmed as *Ba* by PCR tended to sporulate profusely on short (<1mm) sporophores in the dark on PDA.

Isolates that sporulated sparsely if at all, producing more rounded conidia on long sporophores and readily produced sclerotia on PDA were tentatively identified as *B. cinerea* and gave a negative result with the neck rot specific primers.

A further PCR with *Botrytis* specific primers followed by digestion (cutting the DNA at a specific place) was used to separate the *Ba* isolates into *B. allii* and *B. aclada*. Isolates producing a single 413 bp product after digestion were identified as *B. aclada*; isolates producing a second 281 bp band in addition to the 413 bp band were identified as *B. allii*. Both *B. allii* and *B. aclada* were detected in seed and in bulbs. Some seed lots contained only one or the other species, but some seed lots contained both species (see Table 3). Both species were also found in stored bulbs with neck rot; in two samples all isolates were *B. allii*, in one all isolates were *B. aclada*.

DNA extracts were also sent to Wellesbourne for sequencing of an IGS region, the sequencing data confirmed the identification based on PCR/digestion, but has also provided

tentative indications of two distinct sub-types within the *B. allii* isolates, the importance and relevance of these two sub-types may warrant further investigation.

Fungicide sensitivity

Results are shown in Table 5.

Apron XL (metalaxyl-M) had little effect on any of the isolates; although there was some slight reduction in radial growth rate compared to the controls (5 vs 8 mm/d), all isolates had grown to the edge of the plate by 11 d.

Maxim FS (fludioxonil) gave variable results depending on the species/isolate. Although the growth and sporulation of all isolates was inhibited compared to the controls, some isolates were completely inhibited (<0.5 mm/d), whereas other isolates eventually grew to the edge of the plates (3.6 mm/d). There was a marked difference between the species: all of the most fludioxonil-resistant isolates were *B. aclada*.

Thiram gave similar results with all isolates: very limited growth, but not completely inhibited.

Table 5. In vitro sensitivity of six isolates of Botrytis allii (Bal) and five isolates of B. aclada (Bac) to chemical fungicides

Days	Isolate	Year	Source	Lot	Species	Colony diameter (cm)			
						Control	Apron	Maxim	Thiram
5	8336	2003	seed	n/a	Bac	8	5	2.5	0.7
5	9736	2015	seed	2054W	Bac	8	5	0.5	0.6
5	9738	2015	seed	2056W	Bac	8	5.4	1.5	0.6
5	9752	2015	bulb	2105	Bac	8.5	5	0.5	0.6
5	9744a	2015	seed	2058W	Bac	8.5	4.6	0.7	0.6
5	9722B	2015	bulb	2037	Bal	8	5	0.5	0.6
5	9737	2015	seed	2054W	Bal	8	5	0.5	0.6
5	9745	2015	seed	2058W	Bal	7	5	0.5	0.6
5	9749	2015	seed	2099	Bal	7.5	5	0.5	0.7
5	9754	2015	bulb	2016	Bal	8	5	0.5	*
5	9757	<1972	bulb	n/a	Bal	7.8	5.3	0.5	0.7
11	8336	2003	seed	n/a	Bac	>8.5	>8.5	8	1
11	9736	2015	seed	2054W	Bac	>8.5	>8.5	0.6	1
11	9738	2015	seed	2056W	Bac	>8.5	>8.5	7.2	0.5
11	9752	2015	bulb	2105	Bac	>8.5	>8.5	3.2	0.8
11	9744a	2015	seed	2058W	Bac	>8.5	>8.5	3.5	0.6
11	9722B	2015	bulb	2037	Bal	>8.5	>8.5	1.5	1
11	9737	2015	seed	2054W	Bal	>8.5	>8.5	1.3	1
11	9745	2015	seed	2058W	Bal	>8.5	>8.5	0.5	0.8
11	9749	2015	seed	2099	Bal	>8.5	>8.5	0.5	0.6
11	9754	2015	bulb	2016	Bal	>8.5	>8.5	0.5	1
11	9757	<1972	bulb	n/a	Bal	>8.5	>8.5	0.5	1

Seed treatments

Results for direct-plating of fungicide treated seed are shown in Figure 2. Statistical analysis indicated that there were significant main effects of isolate, treatment and a significant treatment x isolate interaction. Thus, none of the treatments were consistently effective against all of the isolates. Apron was ineffective against all isolates. Hot water was the most effective for three the four isolates, but was apparently ineffective for one (9736), but even in this case visibly reduced inoculum loading per seed. Maxim reduced apparent infestation with all isolates but efficacy varied with isolate. Thiram reduced apparent infestation with all isolates, but again efficacy varied with isolate.

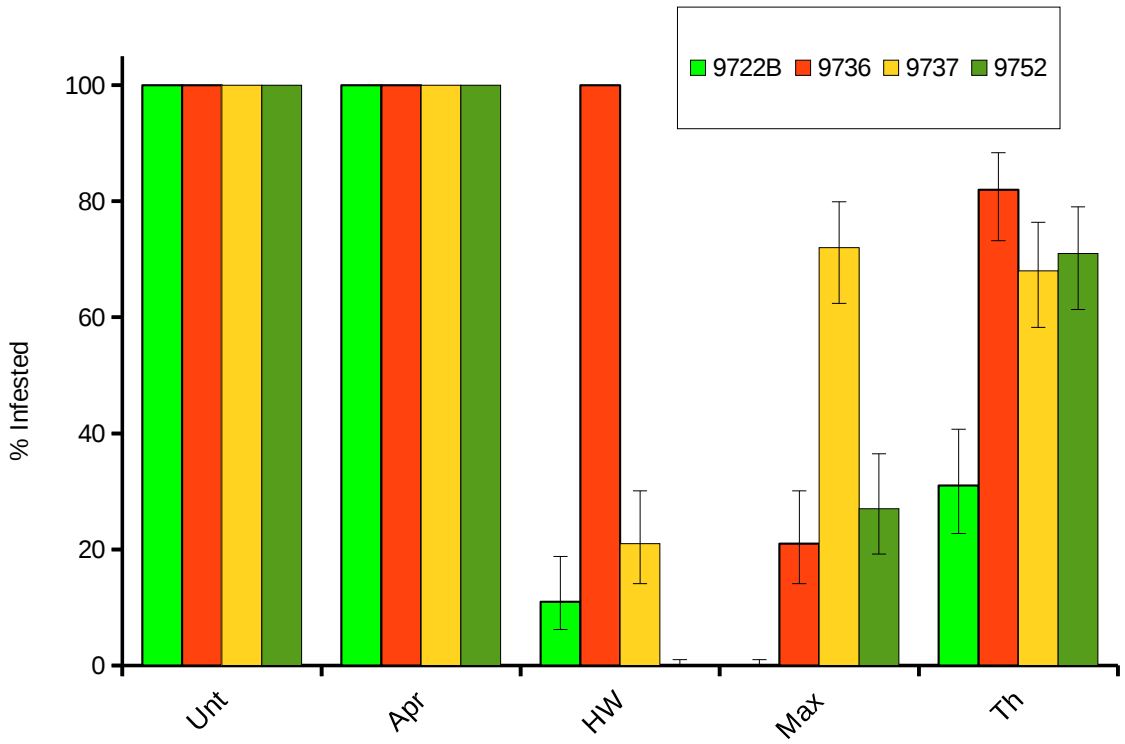

Figure 2. The effect of fungicide seed treatments on seed infestation levels determined by direct plating in onion seed inoculated with four different neck rot isolates: *B. allii* (9722B, 9737); *B. aclada* (9737, 9752). Error bars represent the 95% confidence limits.

Transmission studies

Effect of treatments on emergence.

Statistical analysis indicated significant effects of inoculation x species and treatment on emergence. Emergence was reduced in seed inoculated with *B. allii* (isolates 9722B and 9737) but not *B. aclada*, and overall emergence was improved in inoculated seed by treatment with Maxim and Thiram (Figure 3).

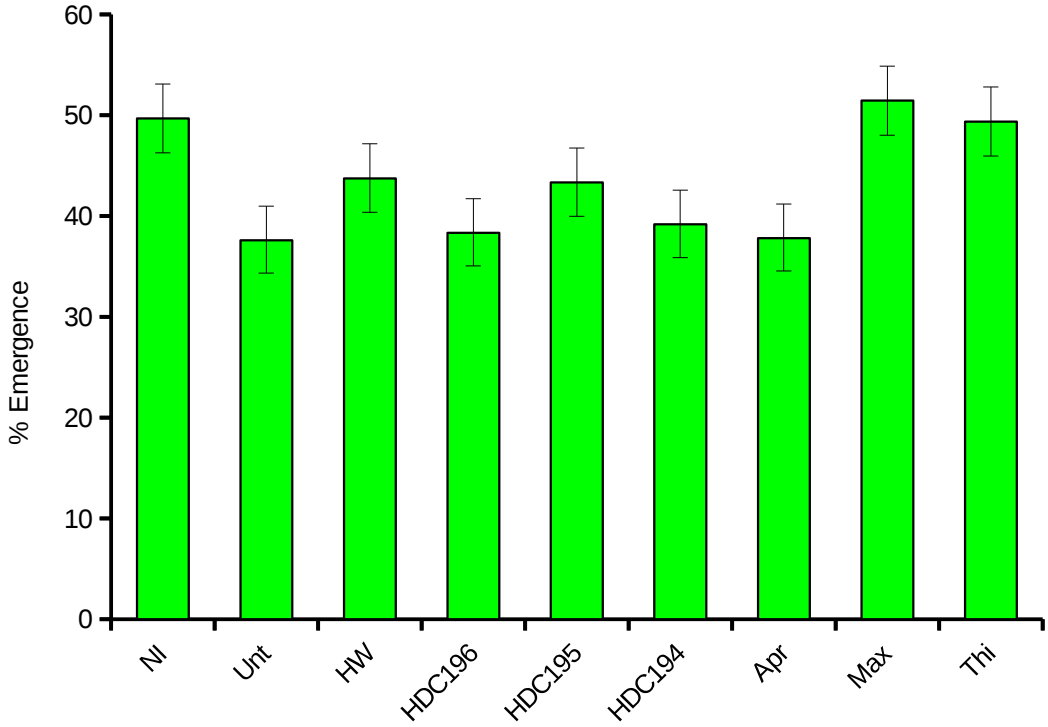

Figure 3. Effect of seed treatments on emergence for seed inoculated with four different neck rot isolates. Values represent the predicted means of the four isolated. Error bars represent the 95% confidence limits.

Effect of seed treatments on transmission

Statistical analysis indicated a major effect of species on apparent transmission, a smaller effect of isolate within species, and an effect of seed treatment. Results are summarized in Figure 4. Hot water reduced transmission for all isolates but appeared to have a bigger effect on *B. aclada* (~75% reduction) than on *B. allii* (~50% reduction). The experimental BCA HDC194, Maxim, and Thiram reduced transmission by 70 to 80% for three of the four isolates, but appeared to be much less effective for one of the *B. allii* isolates (9737). The results for Apron indicated significant reduction in transmission for the two *B. allii* isolates but no effect for the two *B. aclada* isolates. The results for the other two biological treatments (HDC195, HDC196) were less clear with indications of reductions for one (HDC 196) and two (HDC 195) isolates respectively.

A low level of *Ba* was detected in both of the non-inoculated, untreated seed seed lots in the blocks of seed inoculated with *B. allii*, but not in the blocks of seed inoculated with *B. aclada*.

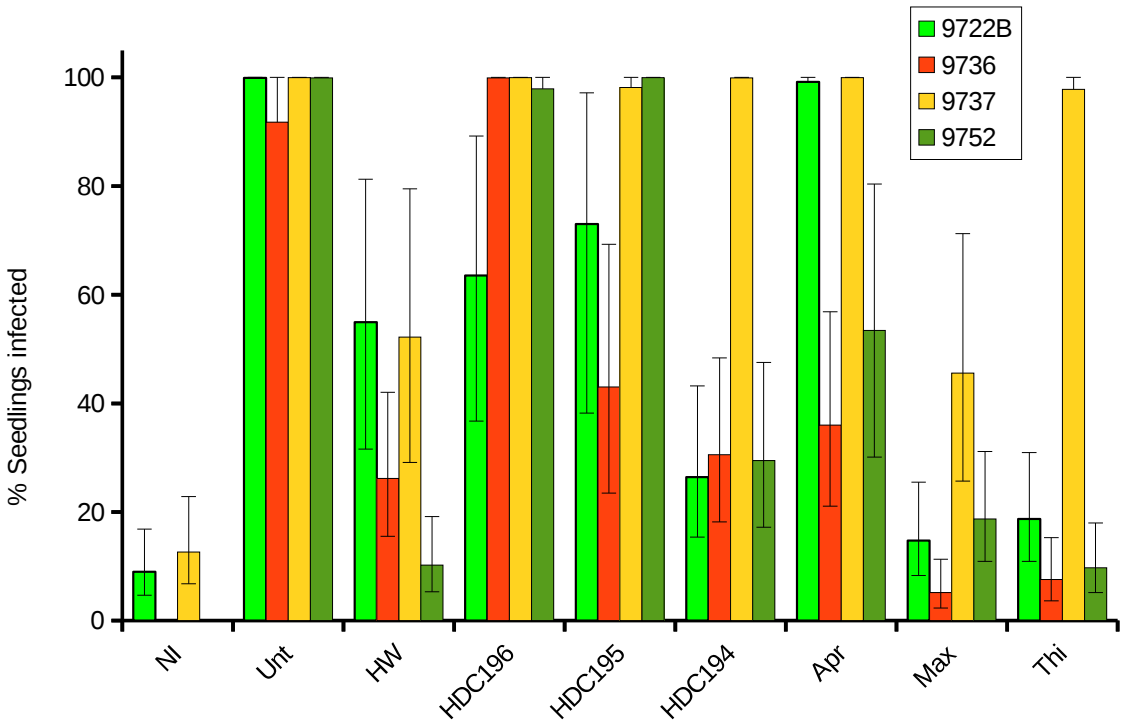

Figure 4. Effect of seed treatments on the % onion seedlings infected with four different neck rot isolates: *B. allii* (9722B, 9737); *B. aclada* (9737, 9752). Error bars represent the 95% confidence limits.

Effect of doses on transmission

Inoculum doses ranged from 5 x 10^3 to 1 x 10^5 CFU/seed. Apparent transmission was 100% or close to 100% for all inoculum doses for all isolates. Inevitably, therefore, model fitting was problematical and statistical analysis did not reveal any significant differences between isolates.

Discussion

Both *B. aclada* and *B. allii* were detected in commercial UK onion seed marketed for the 2015 season, including from fungicide-treated seed, and in stored bulbs with neck rot symptoms (2014 seed). Prior to 2002, both *B. allii* and *B. aclada* were lumped together as one species. Thus any reports of the pathogen/disease referring to *B. allii* prior to 2003 should be considered as referring to either or both species. Many strains originally reported as *B. allii* in the pre-2003 literature have been re-identified as *B. aclada*. For example, one strain used in this study (8336) which was isolated from UK onion seed in 2003 and originally identified as *B. allii* has been shown to be *B. aclada*; other isolates from the UK

isolated in 1988 and originally recorded as *Ba* (Linfield, Kenny & Lyons 1995) have also been subsequently identified as *B. aclada* (Nielsen *et al.* 2002). It is thus clear that both *B. allii* and *B. aclada* are now present and have been present in commercial onion seed for some time. In this study, both species appear to be equally prevalent. It was suggested in the previous HDC project (FV 423) (Lane 2013) that *B. aclada* is the dominant species, however this was based on isolates from only a single seed lot, and is not supported by this study.

The two species are reported as being morphologically similar and can only be reliably differentiated using molecular methods. Nevertheless, we found that spore size was generally a good predictor of species: uniformly small conidia indicated *B. aclada,* whereas more variably sized with occasional much larger conidia indicated *B. allii*. We also found a number of indicators which we found to be useful predictors; e.g. most of the isolates that we initially thought might be mixed (due to uneven 'lumpy' appearance of growth) were later found to be *B. allii* whereas cultures with a smooth even surface were usually *B. aclada*. *B. allii* cultures were often a darker tan when viewed from below, and *B. allii* cultures sometimes produced sclerotia (in contradiction to some previous reports), but this was not observed in *B. aclada*.

The *in vitro* fungicide sensitivity tests indicated that Apron XL (metalaxyl-M) has little effect on *Ba*, and this was consistent with expectations. The results for Maxim 480 FS (fludioxonil) indicated that there is a major difference in sensitivity between *B. aclada* and *B. allii*, with four out of the five isolates of *B. aclada* relatively insensitive. All isolates, irrespective of species, appeared to be equally sensitive to Thiram.

To examine the efficacy of seed treatments, seed was inoculated with four isolates, two *B. allii* and two *B. aclada.* Using inoculated seed ensured that seeds had a high and uniform level of infestation, thereby obviating some of the issues with variability encountered in FV 423, and enabling more reliable cost-effective comparisons of the relative efficacy of treatments. The hot-water and fungicide treated seed was then tested by direct-plating on selective medium and in seed-to-seedling transmission studies. Seed treated with biologicals was only tested in transmission studies as previous work has shown that direct plating is unreliable as a means of assessing BCAs: the applied microbes are either inhibited by the medium (bacteria) or overgrow the plates (fungi).

Hot water treatment appeared to give variable results in the direct plating tests, but was more consistent when assessed in the transmission studies. No doubt this is because although in all cases hot water reduced the inoculum load per seed, this is not quantifiable in direct-plating seed tests. There were indications that *B. allii* may be more sensitive than

B. aclada, but further work would be needed to confirm this. It should be noted that in this study a single hot water treatment regime was applied (50°C for 30 min), this temperature/time regime was derived from some earlier work on onion seed treatment (Wood R. *et al.* 2003) and appears to be relatively safe for onion seed. However, work done as part of the EC STOVE project (Schmitt *et al.* 2006) indicated that seeds vary in their sensitivity to physical treatments from lot to lot (depending on physiological maturity), so it is important to optimise on a per seed lot basis.

In agreement with the *in vitro* sensitivity tests, Apron XL did not have any effect on the apparent seed infestation level by direct plating. However it did appear to reduce apparent transmission in both of the *B. allii* isolates.

Maxim 480 FS gave similar results in both direct plating and in transmission tests, giving significant reductions with all four isolates, but notably was less effective with one of the *B. allii* isolates (9737).

The results for the transmission tests with Thiram seem to be inconsistent with those for the *in vitro* testing, with one of the *B. allii* isolates (9737) appearing to be little affected.

In addition to the seed treatments applied during the study we also received ten 'known to be infected' seed lots from one company before and after steam treatment. These were not included in the main prevalence testing results as they would have biased the results. In all cases the steam treatment reduced infestation to undetectable levels.

Note that Topsin (thiophanate-methyl) which was included in the previous work (FV 423) was not examined in this study as it was (a) clearly ineffective in FV 423 and (b) is no longer approved.

Taken together, these results indicate that the current fungicide treatments should not be relied on to control *Ba*, as efficacy seems to vary according to the particular species and strains present on a seed lots, combined with inoculum load and location. Indications are that *B. aclada* is more likely to be less sensitive. This also explains some of the variable reports about the efficacy of chemical fungicides. The steam treatment used by one seed company seems to be particularly promising, and hot water treatment seems worthy of further refinement/optimisation. Thus simply applying a fungicide to *Ba* infested seed lot provides no guarantee that that seed is clean and safe to use. We would recommend that seed lots with high levels of infestation should be discarded, and that seed lots with lower levels should be treated with a combination of a physical treatment followed by re-testing and treatment with a fungicide or biological.

The low but detectable level of *Ba* in seedlings grown from non-inoculated seed in the *B. allii* treatment blocks and not in the *B. aclada* treatment blocks suggests that some secondary spread and infection had already occurred at the time of sampling in *B. allii* but not in *B. aclada*. This was consistent with observations of visible sporulation on occasional dying cotyledons a few days prior to sample collection, at 21 d after sowing. It is possible that the two species have different latent periods (sporulation was observed later in the *B. aclada* inoculated seedlings) or that sporulation is triggered by different conditions, that may lead to the dominance of one or other species in different environments, or in different growing seasons. Further work is needed to investigate this.

The dose-response assay was not successful in determining whether there is any difference in transmission between the two species/strains. This was likely due to too narrow a range of inoculum doses, despite using an approach that had previously been successful, plus the confounding effect of early secondary spread in *B. allii*. Thus whilst at the lowest doses there were slightly fewer infected seedlings from *B. aclada* inoculated seed, the higher levels from *B. allii* inoculated seed could have been due to secondary spread. Potentially, this could be resolved by producing seed with a wider range of doses combined with earlier sampling (i.e. prior to sporulation and secondary spread).

In addition to seed health, treatment, and transmission, there are two key issues that are likely to be important determinants on the final level of disease in store: the rate of spread in the field and the impact of foliar applied fungicides in the field (e.g. depending on timing and the rate of field spread, foliar applied fungicides may have more or less impact on final disease levels and depending on the sensitivity of the pathogen).

Most routine seed testing for *Ba* does not differentiate between the two species. One of the questions we set out to answer was *Does this matter?* The answer is probably yes, as there are differences in fungicide sensitivity, and indications of differences in the epidemiology. However, whether there is much value in routinely discriminating in seed tests would also depend on what approaches will be subsequently taken to control the seed infestation.

Conclusions/Recommendations

- Both *B. allii* and *B. aclada* can be present in commercial UK onion seed and neck rot affected bulbs.
- The apparent efficacy of fungicide seed treatments depends on the particular species and strain present on the seed, and the inoculum loading.
- There are no completely reliable seed treatments for *Ba* on onion seeds.

- All bulb onion seed should be tested for *Ba* and seed with high levels should be discarded.
- For seed with lower levels of infestation, a combination of a physical treatment (e.g. steam or optimised hot water) plus a subsequent chemical fungicide or biological is likely to prove most effective, but seed should be re-tested, post treatment to ensure efficacy.
- There are indications that the two *Botrytis* spp may have subtle differences in their epidemiology.
- There are indications that *B. allii* may be a more aggressive pathogen.
- Secondary spread of neck rot may occur within two weeks of emergence.
- There are still major gaps in our knowledge about the field spread of neck rot and the impact of field-applied fungicides; further work is needed to understand disease spread, its timing, and identify addition control options.
- Growers should continue to practice high temperature curing to dry bulb necks limit the impact of the disease during storage.

Knowledge and Technology Transfer

Summary of results provided for BOPA Technical committee, September 2015.

Poster at Onion and Carrot Growers conference, November 2015.

Article in HDC News (scheduled for March 2016).

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