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Biological control of *Sclerotinia pseudotuberosa* and other fungi during moist storage of *Quercus robur* seeds

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Abstract

The fungal pathogen *Sclerotinia pseudotuberosa* is a major cause of deterioration during storage of *Quercus robur* seeds (acorns) and along with other, mainly saprotrophic fungi, contributes to the decline of viability and vigour in the acorn population. Hot-water thermotherapy (HWT; 41 °C for 2.5 h) killed the fungal pathogen *S. pseudotuberosa* and prolonged the storage life of acorns. The addition of the systemic fungicide benomyl to the HWT and/or the broad-spectrum fungicide thiram as a seed dressing further enhanced the storage life of acorns. Three fungal antagonists, *Coniothyrium minitans*, *Trichoderma* sp. (KW3) and *Trichoderma virens* (G20), were also applied as a film-coating to acorns using a polyvinylacetate sticker achieving ca. 10^7–10^8 viable conidia per acorn. The biological treatments provided protection against infection and the spread of infection of *S. pseudotuberosa* and other fungi on the acorns during storage over several months. All treated and stored acorns grew normally following sowing in seedbeds. The *Trichoderma* species were more effective than *C. minitans*, with *T. virens* being the most effective. *T. virens* reduced pathogen spread from acorns infected with *S. pseudotuberosa* to 'clean' acorns when *T. virens* coated infected and 'clean' acorns were mixed together. However, *T. virens* was less effective than HWT at preventing the proliferation of this pathogen within individual acorns that were infected before coating. A combination of HWT and subsequent coating with *T. virens* provided a more effective control against both *S. pseudotuberosa* and saprotrophic fungi than when either treatment was applied alone.

Introduction

Quercus robur, the pedunculate or English oak is a tree commonly found in Europe forests and woodlands that has great ecological importance as well as commercial timber value. The fruit (acorn) comprises a seed, commonly 3–6 g in weight surrounded by a thin hard pericarp that is retained throughout storage and seeding. The number of acorns produced varies greatly between years with mast (high production) years in many cases separated by three to eight years of poor harvests (Suszka and Tylkowski, 1980). Storage life of the acorns is limited because they are desiccation sensitive and must be stored moist. This sensitivity has been recognised for many years (Zederbauer, 1910), but has been characterised in greater detail more recently (e.g. Gosling, 1989; Poulsen and Eriksen, 1992; Finch-Savage, 1992; Grange and Finch-Savage, 1992). These so called recalcitrant seeds (Roberts, 1973) have gained greater significance as an increasing number of species are found to have desiccation sensitive seeds, in particular those from tropical moist environments (Pammenter and Berjak, 1999). The limited storage potential causes problems for genetic conservation and continuity of supply in commerce and reforestation programmes.

Viability declines progressively throughout the acorn population within a few months in moist storage at ambient temperatures. This problem has generated much interest over the years and a wide range of storage methods have been developed and used in practice (see Suszka and Tylkowski, 1980; Gosling, 1989;

Kehr and Schroeder, 1996; Suszka et al., 1996). Moist conditions encourage germination making the acorns more susceptible to damage. A wide range of fungi can also proliferate on the acorns in store and in particular the primary pathogen, *Sclerotinia pseudotuberosa*. This pathogen can be largely eliminated by hot-water thermotherapy (HWT) of the acorns (Delatour, 1978; Delatour et al., 1980). While effective against *S. pseudotuberosa*, thermotherapy does not eliminate all seed-borne fungi and these can become a significant problem as viability begins to decline in the acorn population (Kehr and Schroeder, 1996). There is also an undocumented view that HWT can reduce seed vigour. Saprotrophic fungal growth can be minimised by storage in open containers, but excessive drying which reduces viability can occur. Moisture content should be maintained in the region of 40–48% during storage (Gosling, 1989; Suszka et al., 1996). At this moisture content fungal proliferation takes place even in cold storage, especially as the high metabolic rates of the acorns (Szczotka, 1978; Tylkowski, 1977) increases the temperature in bulk stores. Chemical seed dressings can be used to control saprotrophic fungal growth (Suszka et al., 1996), but these are not without drawbacks (Kehr and Schroeder, 1996).

High acorn viability can be maintained for over two years when fungal control treatments are combined with more recent developments in storage technology involving limited drying and storage at sub-zero temperatures (Suszka and Tylkowski, 1980; Suska et al., 1996). These treatments were found to have no permanent negative effects on subsequent seedling growth (Tylkowski, 1982). Kehr and Schroeder (1996) speculate that by using best harvest practice and artificial cold-hardening of the acorns, maintenance of high viability over five years storage at $-8\,^{\circ}C$ will be possible. However, accurate temperature control is required for HWT to avoid physical damage to the acorns and extensive cold stores are required for these bulky fruits. For many nurseries that operate independently, this level of investment is not possible and their principal aim is to maintain viability from harvest in autumn to the following spring in years when there are sufficient acorns. Consequently, there is a need for simpler solutions to reduce plant pathogens on acorns and provide alternatives to the use of chemicals to control saprotrophic growth in acorn storage. This need may become greater as chemicals are removed from sale and the general acceptability of their use declines. One alternative technique is to use biological control to reduce fungal proliferation during acorn storage.

There has been a considerable amount of work on the use of fungal biological control agents applied as seed coatings, most often to control soil borne diseases (reviewed by McQuilken et al., 1997b). *Coniothyrium minitans* is a mycoparasite that can infect a range of ascomycetous sclerotia under laboratory conditions (Whipps and Gerlagh, 1992) and control *S. sclerotiorum* in both field and glasshouse trials (e.g. McQuilken and Whipps, 1995; McQuilken et al., 1995; Jones and Whipps, 2002). Furthermore, conidia of *C. minitans* have been successfully applied to seeds using a fluidised-bed film-coating process (McQuilken et al., 1997a). *Trichoderma* sp. including *Trichoderma* (*Gliocladium*) *virens* can provide effective broad-spectrum biocontrol against a wide range of fungal species and have several modes of action (Hjeljord and Tronsmo, 1998; Harman and Björkman, 1998). These *Trichoderma* antagonists can also be applied as a seed treatment to protect seeds and to colonise and protect roots (reviewed by Hjeljord and Tronsmo, 1998; Harman and Björkman, 1998).

In the present work, isolates of three fungal antagonists were selected to investigate their potential to control the pathogen *S. pseudotuberosa* and saprotrophic fungal growth during storage of *Q. robur* acorns. The first, *C. minitans* (Conio) has been shown to control *S. sclerotiorum* in lettuce (Jones and Whipps, 2002). The second, *T. virens* (G20, synonym GL21) is a known antagonist of pathogens such as *Sclerotium rolfsii*, *Pythium ultimum* and *Rhizoctonia solani* and has been developed into a commercial product in the USA (Ristaino et al., 1994; Wilhite et al., 1994; Lumsden et al., 1996). The third, *Trichoderma* sp. (KW3) has shown activity against the onion white rot pathogen *Sclerotium cepivorum* (Clarkson et al., 2002).

Materials and methods

HWT, measurement of moisture content, germination and infection with S. pseudotuberosa

HWT was applied to acorns in a 30 l plastic container. The water was circulated using an in-flow water heater (Grant Instruments (Cambridge) Ltd, Cambridge, UK). Acorns were placed into the water at $41 \pm 0.5\,^{\circ}C$ where they remained for 2.5 h after the temperature had returned to $41\,^{\circ}C$ (ca. 10 min). Acorns were drained, spread out on a laboratory bench and left to dry to their original weight in the flow of air from a fan.

At regular intervals during drying the acorns were stirred to facilitate even loss of water. HWT did not reduce percentage viability in germination tests carried out immediately after treatment. Acorn moisture content was measured after HWT to ensure comparability with the acorns of other treatments. Moisture content was determined on a minimum of two replicates of five seeds, by oven drying for 17 h at 103 °C (ISTA, 1999). Before drying, all samples were cut into pieces smaller than cubes with 4-mm sides. All moisture contents were expressed on a fresh weight basis.

Acorns were prepared for germination, after an initial 24-h immersion in water, by removing the pericarp and cutting off the basal third of the cotyledons. The cut surface of the acorn was observed for *S. pseudotuberosa* infection and was then pushed into moist heat-sterilised sand, leaving the top third visible for assessment. There were 25 acorns in each replicate plastic tray covered with a transparent polystyrene propagation lid. There were four such replicates for each treatment held at 20 ± 1 °C in the dark. The acorns were exposed only briefly to light when germination (10 mm of radicle growth) was recorded. The acorns were then either removed or exposed to light ($60\,\mu mol\,m^{-2}\,s^{-1}$) after germination and seedlings were evaluated for normality at 28 days (Bekendam and Grob, 1979). On some occasions, germination was not recorded, but the acorn was cut into slices (cut test) to observe the extent of fungal infection. An acorn was recorded as infected with *S. pseudotuberosa* when it developed a characteristic black colouration of the cotyledons.

Mycoparasites and their application to acorns

T. virens (G20), *Trichoderma* sp. (KW3) and *C. minitans* (Conio) were maintained on potato dextrose agar (PDA, Oxoid, UK) at 20 °C. Three-week-old plate cultures were flooded with sterile tap water and the colony surface was gently scraped with a spatula to produce small volumes of conidial suspensions. Conidial concentrations were assessed using a haemacytometer and adjusted to ca. 10^6 conidia ml^{-1}. The conidial suspensions (200 ml) containing each antagonist were then used to inoculate separate mushroom spawn bags (Van Leer Ltd, Poole, UK) that contained autoclaved 2-l quantities of a 15% v/v mixture of maize (Midland Shire Farms Ltd, Worcester, UK) and horticultural grade perlite (Silvaperl Products Ltd, Harrogate, UK) and 400 ml tap water. After incubation for 30 days, at 20 °C, conidia were harvested by flooding the spawn bags with water and passing through a 300-μm sieve to remove the maize and perlite. The suspensions were adjusted to ca. 1.5×10^8 conidia ml^{-1} before application to acorns.

In a series of preliminary experiments, a number of alternative methods of applying conidial suspensions of these antagonists to acorns were examined, including slurry treatment and film-coating using different polymer stickers. Film-coating allowed a greater number of conidia to be applied to the acorns with ca. 10^7–10^8 per acorn compared to ca. 10^3 by slurry application. Conidia applied by film-coating remained attached to acorns and retained high levels of viability during moist storage for up to 9 months. Conidia viability was assessed 36 h after washing them from acorns and placing them on PDA in 9-cm diameter Petri dishes incubated at 18 °C in the dark (McQuilken et al., 1997a). The following protocol for applying conidial suspensions was developed and used in experiments. Polyvinylacetate (PVA, Vinamul, Vinamul Ltd, Carshalton, UK) was added to the suspension to give a 1% (w/w) solution. A coating was built up on the acorns by repeating a cycle of wetting with the PVA conidia suspension using a pressurised hand sprayer during shaking and then drying in a flow of air at 20 °C.

HWT and chemical treatment: Experiment 1

Acorns were harvested from the ground at intervals over 30 days from a single tree at Wellesbourne, UK in 1989, and placed in nylon mesh sacks at 1 ± 1 °C. Immediately after the final harvest, undamaged acorns were selected and subjected to four treatments. The treatments were: (1) untreated control; (2) HWT; (3) HWT in a 0.1% a.i. w/v solution of benomyl (as Benlate Fungicide; 50% a.i. WP; DuPont); (4) HWT, surfaced dried and dressed with thiram (as Tripomol; 80% a.i.; Bos Chemicals Ltd) at 1 g a.i. kg^{-1}. Following treatment the acorns were placed at 1 ± 1 °C in mesh-lined plastic potato chitting trays contained within loosely folded black polyethylene sacks. This arrangement allowed aeration without significant loss of moisture from the acorns. There were four replicate trays, each divided by solid plastic boards into four sections assigned at random to acorns from one of the four treatments. At two-week intervals the acorns were stirred and on some occasions a sample was removed to record their moisture content, the extent of fungal infection and their viability in germination tests.

Efficacy of three fungal antagonists in the control of S. pseudotuberosa*: Experiment 2*

Acorns collected in the New Forest, UK that had a mean moisture content of 42% and thought to be extensively infected with fungi were acquired from a commercial source on 17 November 1999. The extent of fungal infection and viability of untreated acorns during over-winter storage at 3 ± 1 °C and 10 ± 1 °C was compared to acorns given four different film-coating treatments. The film-coating treatments were: (1) PVA only; (2) PVA containing *T. virens*; (3) PVA containing *Trichoderma* sp.; (4) PVA containing *C. minitans*. There were four replicates each containing 1 kg of acorns (ca. 200) per treatment. Each replicate was placed in an open topped container having a 1-cm layer of nylon mesh in the perforated base. The container was placed within a self-sealing polyethylene bag with each corner (1-cm triangle) removed for aeration. At two-weekly intervals the acorns were mixed. After 97 days in store, samples were removed to record their moisture content and the extent of fungal infection in cut tests. After 130 days of over-winter storage, acorns were sown in uncovered soil seedbeds on 3 April 2000. There were 25 acorns per replicate sown in rows 40 cm apart. The number of seedlings emerging was recorded at intervals following sowing. Seedlings were left growing until mid-August when their growth was observed for normality.

Can fungal antagonists prevent the spread of infection?: Experiment 3

Acorns were collected in 1999 from nets laid on cleared ground under a single tree at Wellesbourne, UK to prevent contact between the acorns and the ground to minimise fungal infection. Their mean moisture content at harvest was 43%. Following harvest, the acorns were placed in nylon mesh sacks at 1 ± 1 °C. After ca. 30 days the acorns were given HWT and then subjected, at the same time as the New Forest acorns in Experiment 2 to the same film-coating treatments. All Wellesbourne acorns were marked with red nail varnish. Acorns from both Wellesbourne and the New Forest were stored at 10 ± 1 °C in the same type of containers as those used in Experiment 2. The following treatments were established to evaluate the efficacy of antagonists to suppress the development of *S. pseudotuberosa* in infected acorns and the spread from infected to uninfected acorns. Untreated New Forest and HWT Wellesbourne acorns were stored both separately and mixed together. In addition, acorns from both provenances, film coated with each antagonist separately, were mixed together. There were four replicate containers of the four mixed-acorn lots (control (no antagonist added); + *C. minitans*; + *T. virens*; + *Trichoderma* sp.) and two unmixed controls. The extent of infection with *S. pseudotuberosa* was evaluated in cut tests at 54 and 87 days and recorded separately for the two acorn provenances.

HWT combined with biological control: Experiment 4

Acorns of UK origin harvested in 2000 were acquired from a commercial source that had a mean moisture content of 46% on arrival. The acorns were subjected to four treatments: (1) no treatment; (2) HWT; (3) film-coating with *T. virens*; (4) HWT and then film-coating with *T. virens*. A sample of acorns (2 kg) from each treatment was placed to store at 3±1 °C in the same type of containers to those used in Experiment 2. There were three replicate containers of each treatment. In addition, 3 kg of acorns from each treatment were mixed together to determine whether the different treatments interacted. Before mixing, acorns were individually marked with a different colour nail varnish for each treatment. The mixed bulk was divided into three equal replicates and stored in potato chitting trays as for Experiment 1. Samples were removed at intervals up to 420 days. The number of acorns were recorded in each of four categories: (1) those with limited discolouration of the cotyledons; (2) those showing the full symptoms of *S. pseudotuberosa* infection; (3) those germinated in store; and (4) those which remained unblemished. Viability was also assessed in germination tests at the final harvest.

Statistical analysis

All experiments, germination tests and sowings were laid out as randomised blocks. Data were expressed as percentages and the angular transformations were subjected to analyses of variance.

Results

HWT and chemical treatment: Experiment 1

Moisture content was maintained at 44 ± 3% and acorns did not germinate during more than 840 days

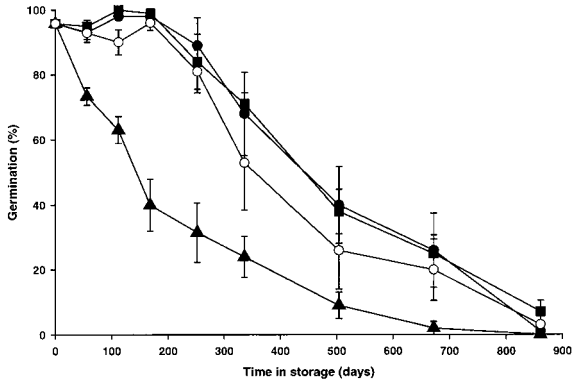

Figure 1. Effect of storage period at 1 °C on percentage germination of acorns. ▲, untreated control; ○, HWT; ●, HWT in a 0.1% a.i. w/v solution of benomyl (as Benlate Fungicide; 50% a.i. WP; DuPont) ■, HWT, surfaced dried and dressed with thiram (as Tripomol; 80% a.i.; Bos Chemicals Ltd) at 1 g a.i. kg^{-1}. Values are ±standard error of the mean.

of storage. There were significant ($P < 0.001$) effects of both treatments and time in storage. Percentage germination of untreated acorns in germination tests (viability) declined throughout storage, falling to 40% over 168 days (Figure 1). In contrast, all HWT treatments retained full viability over the first 168 of storage. Viability then declined in all treatments to leave less than 10% germination in any treatment by 840 days. Up to 672 days, HWT treated acorns retained higher ($P < 0.001$) viability than untreated acorns. From day 336, maintenance of viability was further improved ($P < 0.001$) by the addition of benomyl to HWT or by the addition of thiram as a seed dressing after treatment.

Efficacy of three fungal antagonists in the control of S. pseudotuberosa: *Experiment 2*

At the start of storage, film-coating applied ca. 1×10^8, 2.3×10^7 and 1.8×10^8 conidia per acorn and their viability was 76%, 88% and 80% for *C. minitans*, *T. virens* and *Trichoderma* sp., respectively. During storage, percentage acorn germination was less than 15% and did not differ significantly between treatments. Damaged and infected acorns had been removed before acorns were placed into treatments and so there were no visible external signs of infection at the start of the experiment. However, by 97 days of storage, there was a significant ($P < 0.001$) effect of both treatment and temperature on the percentage of acorns infected with *S. pseudotuberosa* (Figure 2). There was no significant interaction between acorn treatments and storage

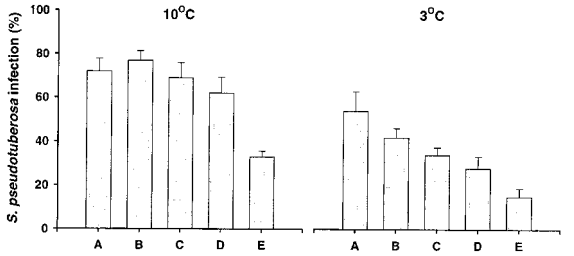

Figure 2. Effect of biocontrol agents on percentage acorns infected with *S. pseudotuberosa* after storage at 10 and 3 °C for 97 days. There were five treatments: A, untreated control; B, PVA only; C, PVA containing *C. minitans*; D, PVA containing *Trichoderma* sp.; E, PVA containing *T. virens*. Standard errors of means are shown as vertical bars.

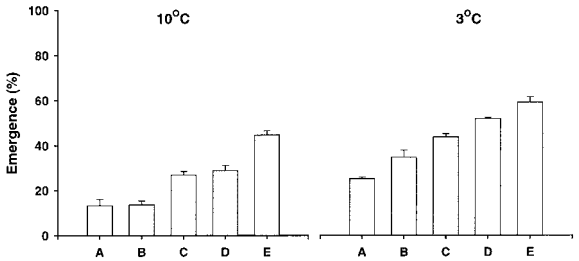

Figure 3. Effect of biocontrol agents on percentage seedling emergence following spring sowing of acorns stored at 10 and 3 °C for 97 days. There were five treatments: A, untreated control; B, PVA only; C, PVA containing *C. minitans*; D, PVA containing *Trichoderma* sp.; E, PVA containing *T. virens*. Standard errors of means are shown as vertical bars.

temperature. At both temperatures, percentage infection was high so that in untreated acorns there was more than 70% and 50% of the acorns stored at 10 and 3 °C, respectively that had completely blackened cotyledons and were not viable. At both temperatures the antagonists significantly reduced the percentage of acorns recorded as infected. *T. virens* provided significantly ($P < 0.001$) greater control and reduced percentage infection compared to the other two antagonists at both temperatures. The PVA sticker applied in the absence of an antagonist provided no protection at 10 °C. At 3 °C, percentage infection on acorns was lower when the sticker was applied, but was not significantly different from that on acorns without the sticker.

The amount of infection in storage was reflected in the numbers of acorns that produced normal plants following sowing in the spring (Figure 3). There was a significant effect ($P < 0.001$) of temperature and seed treatment on seedling emergence. There was no significant interaction and the treatment effects were similar

following storage at both temperatures. More seedlings emerged from acorns that had been film-coated with antagonists than from the untreated acorns and the greatest number of seedlings emerged from those coated with *T. virens*. Seedlings left to grow until mid-August all produced normal plants.

Can fungal antagonists prevent the spread of infection?: Experiment 3

At the start of storage, film-coating was applied ca. 2.4×10^8, 2.8×10^8 and 4.7×10^8 conidia of *C. minitans*, *T. virens* and *Trichoderma* sp., respectively to Wellesbourne acorns. Conidia viability was 81%, 97%, and 88%, respectively. Application of conidia to New Forest acorns and conidia viability after film-coating were as reported for Experiment 2. During storage, Wellesbourne acorns did not germinate and germination of New Forest acorns remained less than 15%.

New Forest acorns had a higher ($P < 0.001$) level of infection with *S. pseudotuberosa* than Wellesbourne acorns so that more than 80% of the unmixed commercial acorns were infected after 54 days in store, whereas, no unmixed HWT Wellesboune acorns were infected (Figure 4). However, more than 60% of untreated Wellesbourne acorns that were mixed with New Forest acorns were showing signs of infection after 54 days of storage. This suggests that fungi on the New Forest acorns had infected the acorns from Wellesbourne. There was a significant increase ($P < 0.001$) in percentage infection between 54 and 87 days

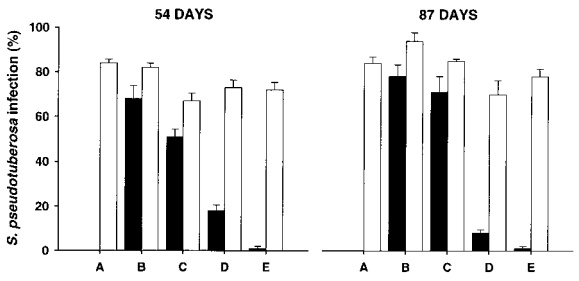

Figure 4. Percentage of acorns infected with *S. pseudotuberosa* after storage at 10 °C for 54 and 87 days. There were five treatments. Untreated New Forest (open columns) and Wellesbourne (solid columns) acorns were stored separately (A) or mixed together (B). Acorns from both provenances were also mixed after the following film-coating treatments: PVA containing *C. minitans* (C); PVA containing *Trichoderma* sp. (D); PVA containing *T. virens* (E). Standard errors of means are shown as vertical bars.

storage and a significant ($P < 0.001$) treatment effect. There was also a significant ($P < 0.001$) interaction between treatment with antagonists and acorn provenance such that infection was reduced by treatment of Wellesbourne acorns, but not those from the New Forest. This result implies that the treatments were less able to control the development of infection in New Forest acorns, but were able to prevent the spread of infection to the Wellesbourne acorns. Although the percentage infection of Wellesbourne acorns was significantly reduced by all three treatments, film-coating with *T. virens*, provided almost complete protection from the spread of infection up to the second harvest at 87 days.

The experiment ended at 87 days because of the high level of infection in the controls and acorns film-coated with *C. minitans*. However, acorns film-coated with *Trichoderma* sp. and *T. virens* were stored for a further 150 days and the percentage infection in Wellesbourne acorns did not increase above those recorded at 87 days (data not shown). Percentage infection on New Forest acorns approached 100% at 237 days (data not shown).

HWT combined with biological control: Experiment 4

At the start of storage there were no visible external signs of infection and 1.2×10^8 conidia of *T. virens* were applied to acorns by film-coating. The effects of treatments in acorns that were stored separate and where the treatments were mixed together were similar and so for brevity only those stored separately will be reported. There were significant effects ($P < 0.001$) of treatment on both the percentage *S. pseudotuberosa* infection and the percentage of acorns that germinated in store (Figure 5). HWT both with and without the addition of *T. virens* prevented the development of infection with *S. pseudotuberosa* throughout storage for more than 400 days, whereas the percentage infection increased in the absence of HWT. However, application of *T. virens* alone significantly reduced infection compared to the untreated control. The percentage of normal seedlings in the germination test made at the final harvest were significantly ($P < 0.001$) different with 21%, 67%, 56% and 66% in the untreated, HWT, *T. virens* only and HWT plus *T. virens* treatments, respectively.

Over the first 120 days of storage, sufficient to over-winter acorns, germination was greatest in untreated control acorns. As storage time increased above 120 days there was an increasing percentage

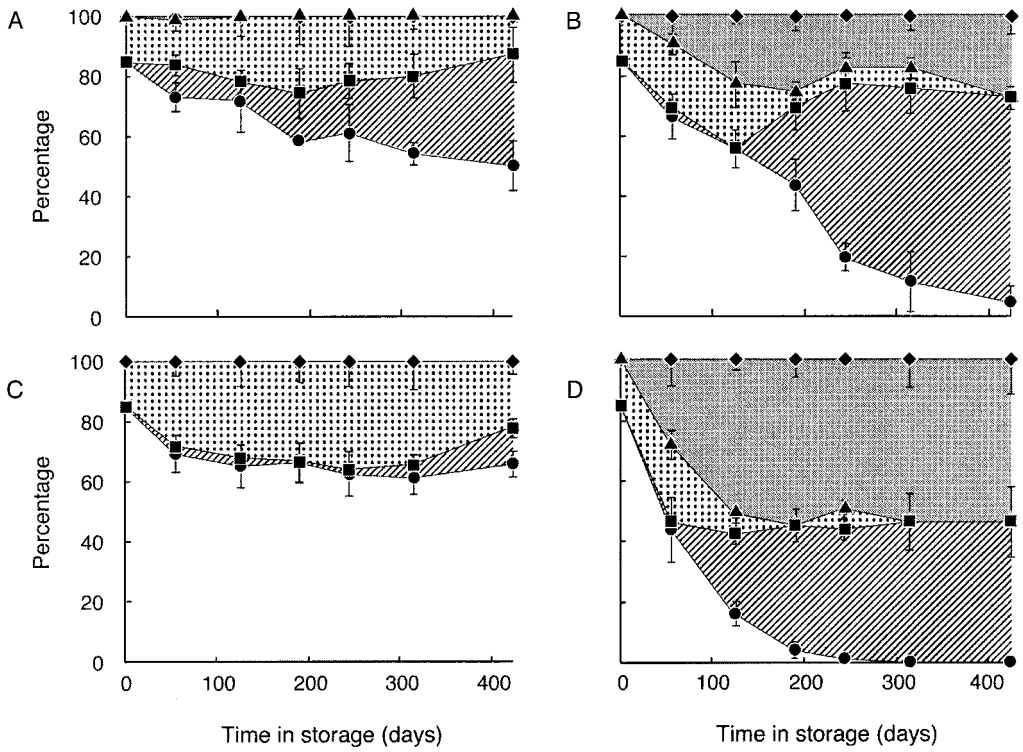

Figure 5. Percentage of acorns that remained unblemished (open), germinated (hatched) during storage at 1 °C, showed some discolouration of the cotyledons (dotted), or became visibly infected with *S. pseudotuberosa* (shaded). There were four treatments: A, HWT; B, film-coated with *T. virens*; C, HWT plus film-coated with *T. virens*; D, untreated control. Values are ±standard error of the mean for each category.

of the acorns that germinated in all treatments. The increase was largest in acorns that were treated with *T. virens* only. The smallest amount of germination was recorded in HWT acorns that were also coated with *T. virens*. During storage, visual estimates were made of the amount of saprotrophic growth that accumulated between mixing occasions (data not shown). The amount of germination was clearly linked to the extent of saprotrophic growth and was least on HWT acorns that were coated with *T. virens*. HWT or *T. virens* alone did not prevent saprotrophic growth. There were insufficient acorns to measure moisture content throughout the experiment, but the treatments with increased saprotrophic growth appeared visibly wetter on the surface of the acorns during mixing.

Discussion

HWT killed *S. pseudotuberosa* and prolonged the storage life of acorns. Experiment 1 shows that the addition of both a broad-spectrum (thiram) and systemic (benomyl) fungicide enhanced the storage life of acorns. This general pattern of results was repeated using acorns harvested in 1991 and 1992, and following storage, acorns were shown to grow normally in uncovered seedbeds (data not shown). These findings are in general agreement with other published work (e.g. Delatour, 1978; Suszka and Tylkowski, 1980; Kehr and Schroeder, 1996; Suszka et al., 1996). The present work also shows that fungal antagonists can be applied to acorns in sufficient numbers using a PVA sticker to provide protection against infection (and the spread of infection) of *S. pseudotuberosa* and other fungi on the acorns. Of the three antagonists compared, the broad-spectrum *Trichoderma* sp. and *T. virens* were more effective than *C. minitans*, which is primarily a mycoparasite of sclerotia (Whipps and Gerlagh, 1992). The most effective antagonist was *T. virens*. In general, the antagonists used have limited activity at lower temperatures (Whipps and Gerlagh, 1992; Hjeljord and Tronsmo, 1998), but *S. pseudotuberosa*

conidia will germinate and grow at temperatures below zero (B. Suszka, pers. comm.). In the present work, *T. virens* (G20) reduced *S. pseudotuberosa* infection at 3 °C (Experiments 2 and 4), but further work is required to investigate how effective this antagonist would be at lower temperatures. The greater effect achieved with *T. virens* compared with that of the other two antagonists may therefore be due to greater activity at low temperatures.

In mixed acorn lots containing both infected New Forest and 'clean' HWT Wellesbourne acorns (Experiment 3) the symptoms of *S. pseudotuberosa* infection developed in acorns from both sources. However, when the antagonists, in particular *T. virens*, were applied to acorns before mixing, infection continued to develop in the New Forest, but not in the Wellesbourne acorns. It is therefore likely that biological control was achieved by preventing the spread of infection. The antagonists appear less effective at penetrating the pericarp to control *S. pseudotuberosa* in acorns that have been in contact with the soil surface. This result is similar to that achieved with fungicides. A range of chemical treatments can be applied to control the spread of fungi in acorns, but when there is a heavy infection of *S. pseudotuberosa* they are less effective than HWT as they do not penetrate below the pericarp (Suszka et al., 1996). HWT is most effective when carried out immediately after harvest (Suszka et al., 1996; Kehr and Schroeder, 1996) and the same is likely to be true for biological control. However, it is clear from the results of Experiment 3 that storage, both with and without the antagonist or HWT, is greatly improved by the collection of acorns that have not made contact with the soil surface where infection by *S. pseudotuberosa* occurs (Delatour, 1978). The collection of acorns from nets and application of antagonists can greatly improve survival in storage (Experiment 3). However, *Q. robur* acorns can be extensively colonised by a variety of fungi during fruit development on the tree (Kehr and Schroeder, 1996).

Kehr and Schroeder (1996) have shown that HWT does not kill the full spectrum of fungi present on the seeds and those remaining can produce copious amounts of growth on the acorns and significantly reduce storage potential. Several seed-borne fungi have been shown to be resistant to temperatures as high as 41 °C (e.g. *Penicillium* spp., Kehr and Schroeder, 1996; *Fusarium moniliforme*). The more heat-resistant fungi may then proliferate in the absence of their normal competitors removed by HWT. Even if they do not cause damage directly these fungi can reduce aeration and generate free moisture that initiates germination. Fungal proliferation may exacerbate problems caused by the high respiration of acorns (Szczotka, 1978; Tylkowski, 1977) which can cause overheating in bulk, although high metabolism *per se* is not the cause of viability loss in acorns (Greggains et al., 2000).

In the present work, *T. virens* and to a lesser extent the other antagonists significantly reduced visible saprotrophic fungal growth on the surface of acorns during storage. In Experiment 4, *T. virens* delayed the proliferation of saprotrophic fungal growth compared to that on untreated acorns by 120 days, which is sufficient to store seeds over winter for spring sowing. In that experiment, commercial seeds were purchased and so the acorns were not treated until delivery some 6 weeks after harvest. The acorns were heavily infected with a range of fungi, nevertheless a combination of HWT and film-coating with *T. virens* effectively controlled the proliferation of both *S. pseudotuberosa* and saprotrophic fungal growth. In Experiment 3, clean acorns were collected locally and could therefore be treated immediately. In this case more effective control of saprotrophic growth was possible using *T. virens* in the absence of HWT. There may also be further benefits from the antagonists if rhizosphere competent strains are used. For example, *T. virens* applied as a seed coating can also provide long-term protection against root pathogens during subsequent seedling growth in the seedbed (reviewed by Harman and Björkman, 1998). If this were the case then biological control agents may offer distinct advantages over the use of fungicides to control fungi on recalcitrant seeds in moist store. Further work is required to test this potential benefit of biological control agents used during recalcitrant seed storage.

Acorn seedlots are often mixtures from a number of trees and even different areas. The extent of infection will vary between these seedlots and it is thus important to prevent the spread of infection from badly infected sources within the bulk. The results of mixing infected and uninfected seeds following film-coating *T. virens* in Experiment 3 illustrates that biological control may be effective under these conditions. A further problem is that the harvest moisture content of seeds differs between trees and years. This may be related to the maturity of the seed at shedding (Finch-Savage and Blake, 1994). Less mature seeds have higher moisture content and are more likely to germinate in store. This is not just because of their moisture content, but at any given moisture content a greater proportion of the water is unbound and available for

germination (Grange and Finch-Savage, 1992; Finch-Savage, 1992). Experience over several years suggests that these immature acorns cannot be prevented from germinating without drying, whereas 'fully mature' acorns with lower moisture content and a greater proportion of bound water at shedding will not germinate unless supplied with water (Finch-Savage and Clay, 1994). The presence of immature acorns during storage also tends to increase problems with saprotrophic fungal growth in line with the greater availability of moisture from these acorns.

This study has provided evidence for the value of applying *T. virens* onto *Q. robur* acorns, as a film-coating using PVA as a sticker, to reduce fungal proliferation in storage. The control of fungi during storage is a widespread problem for all recalcitrant seeds because they must be stored moist (Mycock and Berjak, 1990; Berjak, 1996; Calistru et al., 2000). The present work suggests that use of a broad-spectrum biological control agent such as *T. virens* may be helpful, under these conditions, to control fungi during storage and therefore aid seed supply in forest plantings.

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