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RESEARCH ARTICLE

# Long-term preservation of a collection of *Rhizoctonia solani* using cryogenic storage

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## Abstract

*Rhizoctonia solani* is an important plant pathogen for a number of crops and maintaining an extensive collection of reference isolates is important in understanding relationships of this pathogen with multiple hosts. Current long-term storage methods typically call for frequent transfer increasing the risk of changes in morphological, physiological or virulence characteristics. Cryopreservation using storage in liquid nitrogen (LN) was evaluated to examine the potential for storage of a *R. solani* culture collection containing 106 isolates (primarily from sugar beet). Cultures were stored on autoclaved barley grains in the vapour phase of LN. After 60 days, 5 years and 10 years in storage, all isolates were tested for viability by calculating the percentage of barley grains from which *R. solani* mycelia grew. Five years after initial storage, all isolates except one had no change in viability. After 10 years in storage, 67 of 106 isolates had no significant decrease in viability, 39 of 106 isolates had a significant decrease in viability but only 9 isolates had less than 10% growth, with 4 having no growth. A subset of isolates stored for 10 years were tested for pathogenicity on a susceptible (FC901) and resistant (FC703) sugar beet germplasm. All isolates tested maintained approximately the same level of virulence that they had prior to storage on both germplasms. This indicates that cryogenic methods are suitable for the preservation or storage of *R. solani* culture collections, although efficacy may vary with individual isolates.

## Introduction

Pathogen culture collections provide a vast amount of genotypic and phenotypic information from previously studied isolates and are invaluable resources for future scientists to advance research (Kang *et al*., 2006). Pathogen collections allow researchers to compare past and present population diversity, predict future changes and facilitate the identification of emerging pathogens (Kang *et al*., 2006). Maintenance of culture collections can be difficult depending on the pathogen in question (Ryan *et al*., 2000; Elliott, 2005) and often is a 'side effect' and not the primary concern during the initial storage of the material. A primary cause of culture collection loss has been the inability to store and maintain individual isolates in the state in which they were originally collected (Day & Stacey, 2008). An ideal storage method maintains viability of the fungus, causes no loss of virulence in pathogenic isolates, causes no alterations to physiological or morphological characteristics, and allows for simple preparation and removal of material (Day & Stacey, 2008; World Federation for Culture Collections, 2010). Ryan *et al*. (2000) published a decision key for fungal pathogens that aid in selecting the best storage method; however, no methodology can be applied to all fungi. Many methods require frequent fungal transfer (Sinclair, 1970; Butler, 1980); however, repeated subculture can alter fungi (Butler, 1980; Bacon, 1988; Hajek *et al*., 1990;

Sneh & Adams, 1996) requiring periodic passage through the host to retain virulence (Butler, 1980; Sanchez-Pena & Thorvilson, 1995). In addition, frequent handling can increase the risk of contamination (Smith & Onions, 1983). Many fungi can be stored in a freeze-dried state, reducing cellular activity. This is achieved by freeze-drying spores in a milk/inositol suspension (Tan, 1997; Smith & Ryan, 2004). But those fungi that do not produce spores, such as *Rhizoctonia*, must be stored on media that support their growth.

*Rhizoctonia solani* Kühn [teleomorph, *Thanatephorus cucumeris* (Frank) Donk] is an important pathogen of many crops (Sneh *et al*., 1996), including sugar beet (*Beta vulgaris* L.; Windels *et al*., 2009). *Rhizoctonia* can be stored at room temperature (22–25°C), 4°C or −20°C on media or barley (*Hordeum vlugare* L.) grains (Sinclair, 1970; Butler, 1980). *Rhizoctonia* grows well on potato dextrose agar (PDA) but as a result of loss of water from the agar, cultures must be transferred at least once a year (Butler, 1980; Sneh & Adams, 1996). This constant transferring may cause *R. solani* to lose viability, vigour, virulence and also increase the chance of contamination over time (Smith & Onions, 1983). Alternatively, some *R. solani* cultures have been stored on agar slants under mineral oil for many years; however, this method often retards growth of the retrieved culture and is reported to provide poor genetic stability (Smith & Onions, 1983). A soil-wheat bran storage method has been published for *R. solani*, with isolates reported viable for at least 4 years; however, some problems with bacterial contamination were reported and different soil types and nutrient sources affected storage efficacy (Butler, 1980). Storage of *R. solani* on barley has been used successfully for a number of isolates, with viability and virulence remaining high after 10 years for five of seven anastomosis groups (AGs) tested (Naito *et al*., 1993; Sneh & Adams, 1996).

Maintaining a culture collection is time consuming and labour intensive; therefore, finding a long-term storage (greater than 5 years) method that maintains viability and genetic integrity of *Rhizoctonia* isolates is important. A potential method is cryopreservation in liquid nitrogen (LN) at a temperature of −196°C or in the vapour above the LN (−150°C and below; Sneh & Adams, 1996). Cryopreservation allows isolates to be preserved in a metabolically inactive state in temperatures that are less than −139°C, allowing little re-crystallisation of damaging ice to occur (Sneh & Adams, 1996). Additionally, space and ease of access to cultures are important for a usable storage method. A cryogenic storage method for *Rhizoctonia* has been developed (Smith & Onions, 1983, 1994); however, this method required that the entire sample be thawed when used and was for relatively small aliquots (Sneh & Adams, 1996).

A method for storing larger amounts of material long term, allowing for extraction of small amounts from the stored material, was of interest to our program for maintenance and long-term storage of a working *Rhizoctonia* reference culture collection. For our purposes, we adapted a method for storage of material on autoclaved barley grains at −80°C (Sneh & Adams, 1996) to cryogenically store isolates of *R. solani*. First, we determined the most appropriate method(s) to cryopreserve *R. solani*. Once the best method was identified, the entire *R. solani* culture collection from the USDA-ARS Sugar Beet Research Unit (SBRU) was preserved in the LN vapour phase and assayed for viability after 60 days, 5 years and 10 years in storage. Selected isolates were evaluated to determine if they maintained their virulence on sugar beet.

## Materials and methods

### Colonisation of barley grains with *R. solani*

All isolates used for this study had been originally stored under standard conditions at −20°C on autoclaved colonised barley grains. Isolates are re-cultured at least every 5 years. To prepare colonised barley, hulled barley grains were hydrated with distilled water over night then autoclaved for 1 h at 121°C. The autoclaved barley grains were allowed to cool for 24 h, then autoclaved again for 1 h at 121°C. Autoclaved barley grain was cooled and placed onto PDA plates containing 1- or 2-day-old *R. solani* cultures and incubated at 24–27°C for 3 weeks to allow colonisation of barley grains. To culture from storage, isolates were grown on PDA by placing three colonised barley grains from storage onto the centre of a fresh plate of medium.

### Response of isolates to cooling and storage methodology

The potential impacts of cooling rate and storage methodology were tested on three *R. solani* isolates (R-9; AG2-2, Shiba2; AG1 and R101; AG4) known to be pathogens of sugar beet. Five cryovials were prepared for each of the three isolates, with each cryovial containing 50 colonised barley grains. Cryovials were stored in the vapour phase of LN using a combination of one of three cooling treatments and one of two warming treatments. Cooling treatments were (a) fast cool: samples were directly placed into the vapour phase of LN cooling at a rate of 25–50°C $min^{-1}$ (Vertucci, 1989), (b) slow cool: samples were placed in a LN controllable freezer (Thermo-Forma CryoMed Freezer model # 7951, Thermo Fisher Scientific Inc., Waltham, MA, USA) programmed to hold samples at −5°C for 1 h, after which samples


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were cooled slowly at a constant rate ($1^{\circ}C\ h^{-1}$) to $-30^{\circ}C$ and held for 24 h, before being plunged into the vapour phase of LN (Christina Walters, personal communication) and (c) refrigerator/freezer: samples were first cooled at $4^{\circ}C$ then at $-20^{\circ}C$, each for 12 h, before placing in the LN vapour. Warming treatments included (a) fast warm: samples were warmed in a water bath at $70^{\circ}C$ for a few minutes, this was to allow the samples to quickly warm to room temperature and prevent re-freezing as they thaw (Christina Walters, personal communication) or (b) slow warm: samples were warmed at room temperature for 24 h. These treatments were compared to storage of the same isolates either in a freezer ($-20^{\circ}C$) or in a refrigerator ($4^{\circ}C$). After 30 days in storage, 15 colonised barley grains per treatment were plated onto PDA and the number of grains out of which there was visible growth of *R. solani* was counted at 2 and 5 days after plating. Methods were compared using analyses of variance (ANOVA) on the number of grains with fungal growth at each time using the PROC MIXED for repeated measures procedure in SAS statistical software (SAS Institute, Cary, NC, USA).

### Effectiveness of long-term storage of *R. solani* culture collection using LN

Based on results of the above trial, each of 106 isolates from the SBRU *R. solani* culture collection, representing nine AGs, as well as a bridging isolate was prepared for long-term storage in LN using the fast cool/slow warm procedure. Six cryotubes per isolate were prepared and each contained approximately 25 colonised barley grains; two vials were intended for testing viability for each time period. The isolates were stored by directly placing the samples into the vapour phase of LN. Two vials were removed at each of the time periods tested (60 days, 5 years and 10 years) and allowed to warm to room temperature for 24 h. Barley grains were removed from cryovials, placed on PDA plates and incubated at $24^{\circ}C$. Plates were examined at 2 and 5 days after plating for fungal growth. As a measure of viability, the number of barley grains out of which *R. solani* grew from the total number of grains plated was recorded as percentage fungal growth. At 10 years, a subset of isolates that had been stored cryogenically (R-9, RZC98, RZC68, RZC65, RZC54; all AG2-2) were evaluated for pathogenicity on a susceptible (FC901; Gaskill *et al*., 1967) and a resistant (FC703; Hecker & Ruppel, 1977) sugar beet germplasm. For each isolate tested and a negative control (sterile ground barley), five pots containing pasteurised potting soil were planted with two seeds of each sugar beet germplasm and placed in a greenhouse at $22 \pm 5^{\circ}C$, with a 16-h photoperiod. For inoculum preparation, 200 g of barley grain was soaked in beakers overnight with distilled water (water was poured off to within approximately 1 cm below the grain surface after soaking) and autoclaved as described above. Agar plugs (7-mm diameter) from PDA cultures were placed approximately 1 cm deep in the autoclaved barley, with one plug per 200 g of barley, and incubated for 21 days at $24–27^{\circ}C$. Infested barley was removed from beakers and dried for 5–7 days, then ground in a Waring blender (Waring Laboratory, Torrington, CT, USA) for 5 min, sterilising the blades with 70% ethanol between isolates. Ten weeks after sowing, approximately 0.5 g of ground inoculum (or sterile ground barley as the negative control) was placed in the soil next to the tap root (about 2-cm deep) of each sugar beet plant. Plants were maintained in the greenhouse in a completely randomised design at approximately $27^{\circ}C$. Four weeks after inoculation, roots were harvested, washed free of soil and examined for root rot symptoms. Roots were rated for disease severity on a scale of 0 (*no visible symptoms*) to 7 (*plant dead and root completely rotted through*; Ruppel & Hecker, 1982; Panella, 1998). The experiment was repeated twice. To compare cryogenic storage methods to standard storage conditions, isolates stored for the same period (10 years) in a freezer ($-20^{\circ}C$) were compared with the same isolates stored cryogenically. Isolates that had been stored at $-20^{\circ}C$ had been transferred at least once during the 10-year period. Five isolates (R-9, RZC98, RZC65, RZC68 and RZC54) were again inoculated to FC901 plants grown as described above. Inoculum was prepared and applied to each experimental unit as previously described with the experiment being replicated twice. Results were compared with ANOVA on mean disease severity ratings using a PROC MIXED procedure and a Dunnett's *t*-test was used to compare each isolate to the negative (autoclaved barley) and positive (R-9 stored with traditional methods) controls. For comparison of isolates stored at $-20^{\circ}C$ versus cryogenic storage, ANOVA on mean disease severity were analysed using a PROC MIXED and LSMeans used to compare all isolates.

## Results

### Effectiveness of long-term storage of *R. solani* culture collection using LN

Significant differences in the percentage of barley grains with fungal growth among the individual isolates were found using the different cooling and warming treatments at 2 days after plating ($P$ value $<0.0001$); however, most of these differences were not significant when evaluated at 5 days after plating ($P$ value $>0.05$; data not shown). At the final evaluation day (5 days after plating), only a single treatment had significantly lower fungal viability

(as measured by percentage growth; $P < 0.001$). As a result of the low viability of R101, storage in a refrigerator (CR) was the only treatment that was significantly less effective on the final evaluation day (5 days after plating) with a mean fungal viability of the three isolates of 98.6% (all $P$ values <0.0001; data not shown).

Because initial testing indicated that the method of sample preparation had no impact on effectiveness of LN storage, all isolates representing the SBRU *R. solani* culture collection (106 isolates) were placed into individual cryotubes, plunged directly into LN (fast cool), and stored at −160°C in the vapour phase of LN. At 60 days after storage, all isolates except one (1556; 50% growth) had 100% growth (Fig. 1). At 5 years, this same isolate (1556) was the only isolate that had less than 100% growth (with only 41% growth). The rest of the isolates had comparable viability at 5 years as they did at 60 days after initial storage (100% growth). After 10 years, there were 29 of 106 isolates that had 100% growth, which remained unchanged during the entire time of the study (approximately 27% of the isolates tested). Another 38 of 106 (approximately 36%) isolates had no significant changes in viability during the study, although this amount was less than 100% growth (at 10 years; Fig. 1). For the remaining isolates, there was significant variation in the ability of cryopreservation to maintain viability during the 10-year period. In total, 39 of 106 isolates did have significant decreases in viability (as measured by percentage growth) at 10 years (approximately 37% of the isolates tested; all $P$ values < 0.05). In total, 9 of these 39 isolates had less than 10% growth when tested at 10 years, with 4 isolates having no growth at all in both replicates tested. Three of the four isolates with no growth previously had 100% growth at 60 days and at 5 years, with 1556 the only isolate to have had reduced growth at each time period tested (Fig. 1).

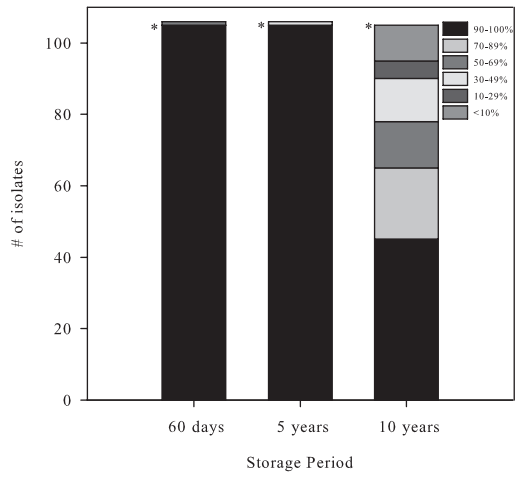


**Figure 1** Effectiveness of long-term storage of *R. solani* isolates over liquid nitrogen (LN) at 60 days, 5 years and 10 years. A comparison of viability (# of barley seeds with fungal growth/# of barley seeds stored) for the number of isolates (total of 106) stored over LN. *Indicates percentage growth of isolate 1556 which is only isolate to have reduced viability in each time period tested.

**Table 1** Virulence of isolates prior to and after cryopreservation

| | Prior to cryopreservation | | After cryopreservation | |
|---|---|---|---|---|
| Isolate | FC901 (S) | FC703 (R) | FC901 (S) | FC703 (R) |
| Autoclaved barley (negative control) | | | 1.2a | 1.4A |
| R-9 (control) | | | 6.2b | 4.3B |
| R-9 | 6.14 | 3.97 | 5.7b | 3.5B |
| RZC98 | 5.97 | 3.97 | 4.2b | 2.3A* |
| RZC68 | 2.14 | 1.81 | 2.1a | 1.8A |
| RZC65 | 3.97 | 2.47 | 2.5a | 1.4A |
| RZC54 | 4.64 | 1.81 | 3.8b | 2.4B |

Lowercase letters indicate significant differences as compared with the autoclaved barley on a susceptible (S) host by Dunnett's *t*-test ($P \leq 0.05$). Capital letters indicate significant differences as compared with autoclaved barley on resistant (R) host by Dunnett's *t*-test ($P \leq 0.05$). *$P = 0.0581$.

To determine if isolates could still be considered pathogenic after being stored in LN for 10 years, a random subset of isolates was inoculated to a susceptible and a resistant sugar beet. In general, all isolates had slightly lower virulence to the susceptible germplasm (FC901) after 10 years in cryopreservation as compared with itself just prior to storage; however, all isolates that were pathogenic prior to storage remained so after cryopreservation (Table 1). One isolate (RZC68) had a virulence of 2.1 to the susceptible variety (FC901) prior to storage in LN. When tested after being stored over LN for 10 years, its virulence to FC901 remained the same (2.1), which was not statistically different from the negative control used (autoclaved barley; Table 1). Additionally, this isolate had a disease severity rating of only 1.8 on the resistant variety (FC703) both prior to storage and after cryopreservation. Based on this information, this isolate should be considered an avirulent *R. solani* isolate on sugar beet; however, storage in LN did not change its virulence. Another isolate (RZC65) was not significantly more virulent to the susceptible germplasm than the negative control (Table 1). When compared with its virulence prior to storage (4.0), we expected this isolate to be moderately virulent to FC901; therefore, this isolate did lose any virulence during storage. When comparing the virulent isolate (R-9) that had been stored in LN for 10 years to an R-9 isolate that had been stored for the same period at −20°C, there was no change in virulence to either the susceptible variety ($P = 0.4829$) or the resistant variety ($P = 0.1618$; Table 1). Therefore,



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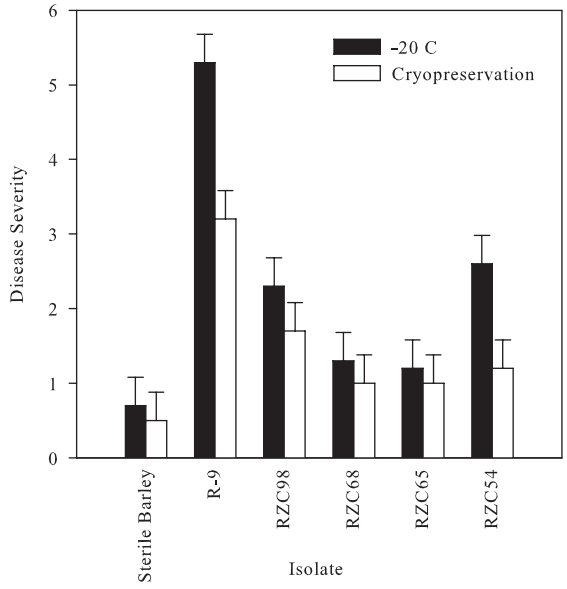


**Figure 2** Comparison of virulence to sugar beet of isolates stored using the freezer method (−20°C) to isolates that were stored over liquid nitrogen (LN) for 10 years. Error bars indicate standard errors of the mean within an isolate treatment.

three of four isolates that were pathogenic to sugar beet prior to storage in LN had no significant changes in virulence after 10 years in storage.

The above study did not compare isolates that had been stored in LN to the same isolate that had been stored over the same period at −20°C. To determine pathogenicity of the isolates stored for 10 years by cryopreservation compared with a standard storage method (−20°C), we again inoculated the same subset of isolates to a susceptible sugar beet variety. In general, isolates stored by cryopreservation were less virulent compared with the same isolates that had been stored by the standard method ($P = 0.0036$; Fig. 2). For example, isolate R-9 that was stored at −20°C had a mean disease severity rating of 5.25, while it had a decreased virulence of 3.15 when the isolate had been stored over the same timeframe cryogenically (Fig. 2; $P = 0.0026$). Again, not all isolates tested would be considered pathogenic to sugar beet as there were significant differences in virulence of the isolates tested ($P < 0.0001$). For example, RZC65 which was non-pathogenic (disease severity rating = 1.2) from the −20°C storage had a disease severity rating of 0.95 after storage in LN (not statistically different at $P = 0.6547$; Fig. 2).

## Discussion

To ensure that pathogen culture collections are not lost due to lack of adequate and reliable storage methodologies, it is important that protocols used to develop and maintain cultures in the collection are appropriate for each pathogen (Smith & Ryan, 2004). Despite the unstable nature of some fungi, the ultimate aim of any preservation methodology should be to ensure the long-term survival of an organism with a minimal amount of change to its physiological and genomic integrity (Smith & Ryan, 2004), while minimising resources needed to maintain large collections. Our results indicate that cryopreservation of *R. solani* on sterile barley stored over LN is a viable method for long-term maintenance of many isolates causing little loss to viability or significant changes to pathogenicity, requiring little investment of resources to routinely maintain isolates over a 10-year period. While storage at −20°C did maintain virulence more effectively, isolates being stored in this manner have to be more routinely re-cultured (transferred at least every 4–5 years). Also, most isolates maintained in a collection are used infrequently, therefore, having a storage method that can maintain them with infrequent handling is beneficial.

Cryopreservation at temperatures below −196°C is considered the best preservation method for many tissues including fungal pathogens (Smith & Allsopp, 1993); however, changes can occur to morphological and pathogenic characteristics during the freezing and thawing process. Furthermore, only a small percentage of an estimated 74 000–120 000 described fungi have had cryopreservation protocols established for their long-term storage and preservation (Ryan *et al*., 2000; Hawksworth, 2001). A methodology that allows for removal of material over time (such as cryopreservation allows), while maintaining isolates in the main collection is highly desirable (Sneh & Adams, 1996; Ryan *et al*., 2000).

It has been previously shown that storage of *R. solani* on infested barley can be successful after 10 years of storage at −20°C, where a number of isolates from five of seven AG groups tested had high viability and virulence after that time (Naito *et al*., 1993). Combining such a method with cryogenic preservation could potentially extend the amount of time during which this material can be stored with little change in the stored material while allowing single colonised grains to be removed to start working cultures of specimens. Another long-time culture storage method using a soil-wheat bran method has been published for *R. solani* (Butler, 1980). However, this method has problems with bacterial contamination, because of the use of different soil types and nutrient sources (Butler, 1980). Use of autoclaved barley in our proposed methodology removes problems associated with potential effects from different soil types and decreases the risk of contamination.

In our study, the *R. solani* isolates tested maintained their overall pathogenicity to sugar beet after 10 years in storage; however, there was loss of virulence (and viability) for some of the isolates tested when compared with storage over the same time period at −20°C. It is important to note that the isolates being stored at −20°C had been actively maintained on a routine schedule every 5 years, which also increased the risk of contamination or loss of characteristics every time they were re-cultured and re-stored. These tradeoffs must be considered whenever a preservation method is considered. Use of cryoprotectant additives could potentially improve the described cryopreservation method for *R. solani*. Some of these cryoprotectants could include glycerol, skim milk, trehalose, dimethyl sulfoxide (DMSO) and others (Bhandal *et al*., 1985; Hubálek, 2003). Additional studies would be needed to modify the described protocol to introduce such a cryoprotectant to the barley grain medium and to compare each additive(s) impact in potentially improving the longevity of storage *R. solani* before modifications could be suggested.

While it appears that storage of *R. solani* over LN may allow for extended viability of cultures in a collection with minimal maintenance requirements, it remains to be known how this method compares to traditional storage methods for isolates that are continually being utilised in a collection and how a 'freeze/thaw' cycle may further impact viability and virulence of particular isolates. A benefit of cryopreserving *R. solani* is that it does allow for an aliquot of colonised grains to be removed for rapidly starting fresh viable cultures without having to thoroughly thaw/warm the entire sample leaving the rest of the sample to be maintained for the long term, thus lowering the risk of changing morphology and/or pathogenic characteristics for individual isolates. To prevent any damage during the freeze/thaw process, drying down the colonised grains prior to storage in LN is recommended (Christina Walters, personnel communication).

Different storage methods may work better for particular fungi (Ryan *et al*., 2000) or, within *R. solani*, for particular AG groups (Naito *et al*., 1993). Our collection primarily contained isolates representing one AG (AG2-2). It is possible that this preservation method does not work for some AG groups; further investigation would be needed to address this. Another optimisation factor that needs further investigation is the impact of initial colonised seed moisture content, which may contribute to isolate degradation in storage. Moisture content has been studied in the culture storage of other fungal pathogens utilising different storage methodologies. Derakhshan *et al*. (2008) reported that higher moisture levels decreased viability over time when storing *Verticillium lecanii* when using a media-based storage method. Other studies have reported that conidia moisture content or relative humidity levels can affect storage effectiveness of fungal pathogens (Daoust & Roberts, 1983; Moore *et al*., 1996). When storing colonised barley, it is difficult to determine the moisture content of the fungus itself compared with the barley seed, acting as a growth medium. It is also possible that the chaff on the outside of the colonised barley could hold additional moisture; we propose that the use of de-hulled barley may improve the methodology which will be investigated in the future.

During long-term preservation, it is important to maintain genomic integrity of species (Smith & Ryan, 2004). Genotypic mutations during storage may change an individual isolate(s) identity with some mutations potentially causing the loss of virulence (Arabi *et al*., 2007). The anastomosis grouping of some *R. solani* can be associated with host specificity as well to virulence (Sneh *et al*., 1991, 1996; Keijer *et al*., 1997); therefore, the impact of cryopreservation on different AGs, as well as any changes in the genetic integrity of stored isolates, should be investigated. Gene sequences, including using described *R. solani* primers currently available to identify anastomosis grouping (Carling *et al*., 2002), could be aligned and used to determine if any simple mutational changes in AG occurred during cryopreservation. *R. solani* also has been characterised using molecular markers including random amplified polymorphic DNA (RAPDs), inter-simple sequence repeat (ISSRs), and amplified fragment length polymorphism (AFLPs) (Ceresini *et al*., 2002; Sharma *et al*., 2005; Guleria *et al*., 2007; Taheri *et al*., 2007) and these technologies could be used to determine if genotypic mutations occur at a higher frequency in isolates that had been cryogenically stored.

Our work here indicates that for the permanent storage of *R. solani* isolates that are known to be pathogenic to sugar beet, cryogenic methods are suited for the preservation of reference culture collections over a long period of time, although efficacy may vary with individual isolates. While we do concede that some isolates did lose viability and/or virulence, cryopreservation does allow for reduction of maintenance activities and could provide the best balance between effort and cost to maintain a collection and ensuring stability of its contents.

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