

Viability and Pathogenicity of *Rhynchosporium secalis* after Long-term Storage

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Long-term storage of *Rhynchosporium secalis* cultures is a challenge for any lab managing a working collection of isolates. In this work, the viability and pathogenicity of *R. secalis* stock cultures were tested after four years of storage at $-20\text{ }^{\circ}\text{C}$ in different concentrations of glycerol. Germinability were measured after each storage by collecting spores by coverslips and placing them on water agar in closed Petri dishes at $20\text{--}22\text{ }^{\circ}\text{C}$ in the dark and allowed to germinate for 24 h. Additionally, at the end of each storage treatment, conidia were collected by coverslips from sporulated leaf lesions of symptomatic barley leaves and placed under similar conditions as non-stored controls.

Cultures of all stored isolates were viable with a spore germination rate of 72.28% (R_{s22}) after four years of storage at $-20\text{ }^{\circ}\text{C}$ in 60% glycerol. Low viability and contamination were observed when spores were stored in sterile distilled water and in Lima bean agar. All isolates continued to infect barley leaves after 4 years of storage. However, the pathogenicity was significantly ($P < 0.05$) reduced in isolates stored in glycerol as compared with controls.

This work helps to preserve *R. secalis* for a long term period at $-20\text{ }^{\circ}\text{C}$ without any contamination; therefore, due to the low costs our results could be applicable for laboratories that have limited resources.

Keywords: *Rhynchosporium secalis*, barely (*Hordeum vulgare* L.), scald, pathogenicity, storage method.

Leaf scald caused by *Rhynchosporium secalis* (Oud.) Davis, is one of the most economically important barley diseases worldwide, causing yield and grain quality reduction (Kavak, 2004; McLean and Hollaway, 2018). Initial inoculum is thought to consist of conidia dispersed by rain-splash or mycelia in infected seeds and crop debris (Fountaine et al., 2010). Studies on *R. secalis* population genetics and pathogenic variation were important to establish better control strategies (Bouajila et al., 2006; Arabi et al., 2010). However, these studies are based on experiments using different numbers of pathotypes which should be properly conserved.

Moreover, isolation of *R. secalis* is critical for certain kinds of studies such as population genetics, virulence comparisons, understanding disease development, and ecological competition (Zhan et al., 2012). After isolation, the obtained *R. secalis* isolates must be maintained by weekly to monthly transfers to fresh growth media, since cultures of this fungus in our preliminary experiments collapse around 1–2 months of storage at temperatures between 6 and $30\text{ }^{\circ}\text{C}$ (Arabi et al., 2010), therefore, frequent transfers of cultures

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are expensive, time consuming and causing risk for contamination. In addition, repeated passages via artificial media may lead to changes in fungal morphology and losses in virulence and sporulation capacity (Butt et al., 2006).

On the other hand, variability of the *R. secalis* is well documented (Williams et al., 2003; Arabi et al., 2008), therefore, a suitable storage method that preserves the characteristics of the isolates should be used. Long-term preservation of fungal cultures is vital for plant pathology, molecular and morphological identification studies (Ravikumar et al., 2016).

Several methods have been used to preserve fungal isolates. However, the traditional method, frequent subculturing of fungal cultures from staled to fresh media was not a very practical means of storing large numbers of fungal cultures. Since, it is time-consuming, prone to contamination and does not prevent genetic and physiological changes. Through the years, different storage methods have been developed in order to reduce these disadvantages. Among them, lyophilization (Grzegorzczuk et al., 2018), cryopreservation (Ohnishi et al., 2017), mineral oil (Uzunova-Doneva and Donev, 2005), sterile soil (Ruiz-Lozano and Azcon, 1996), and silica gel (Trollope, 1975) however, each method has limitations.

This work was aimed for the first time to preserve the pathogenic characteristics of *R. secalis* isolates stored for four years under different concentrations of glycerol at $-20\text{ }^{\circ}\text{C}$.

Materials and Methods

Fungal isolates

Three *R. secalis* isolates (Rs2, 7 and 22) based on physiological criteria (Arabi et al., 2008) were used in this study (Table 1). The mycelia were transferred from a stock culture into Petri dishes containing lima bean agar (LBA) with 13 mg/l kanamycin sulphate and incubated for 2 weeks at $15 \pm 1\text{ }^{\circ}\text{C}$ in the dark. After the positive isolation of fungus, colonies produced from single spores were maintained as pure isolates for testing methods of storage.

Storage methods

Several concentrations of glycerol (vol/vol) (30, 60 and 90%) were examined for storing *R. secalis* isolates. Conidia of each isolate were taken from LBA cultures with

Table 1

Isolates used in this study

Isolates no.	Origin	Year of collection	Pathotype	Initial lesion size (mm)	Reference
Rs2	Aleppo (north)	2004	pt1	1 × 2	Arabi et al. 2008
Rs7	Daraa (south)	2003	pt9	5 × 3	Arabi et al. 2010
Rs22	Hasaka (north-east)	2001	pt4	5 × 9	Arabi et al. 2008

a sterile needle and placed into sterile 2 ml Eppendorf tubes containing 1 ml of each concentration. Tubes were then stored in a refrigerator at -20°C . In addition, tubes containing sterilized LBA (supplied with 13 mg/L kanamycin sulphate) were also used for storing isolates at -20°C . The LBA (15 ml) in Pyrex glass test tubes (150×20 mm) were inoculated with each isolate, incubated at $20 \pm 1^{\circ}\text{C}$ in the dark for 5 days and stored in a refrigerator at -20°C . Samples of these isolates were also taken and put in several sterile glass flasks containing 4 mL of sterile distilled water, which were identified, and hermetically closed with special rubber tops with aluminum belts and stored at -20°C .

Viability tests

Viability was determined by inoculating each culture onto 9 cm culture plates containing LBA followed by incubation at $20 \pm 1^{\circ}\text{C}$ in the dark for three days. Growth indicated that the culture was alive. Spore germination on agar was used as an indicator for viability. Spore germination was determined with four counts of 100 spores each. Germination tests were performed with six slide-coverslips (replicates) per isolate for each storage method. At the end of each storage treatment, coverslips were collected and placed conidia-side down on 1.5% water agar medium contained in 9-cm plastic Petri dishes, incubated at $20\text{--}22^{\circ}\text{C}$ and allowed to germinate for 24 h. Germinated and ungerminated conidia on each coverslip were counted in random fields at $\times 100$ with a light microscope. A total 100 conidia were examined on each coverslip, with the higher number of conidia counted when germinability was low. A conidium was considered germinated if its wall had ruptured and the first cell had started to emerge. As a pre-storage control, we performed identical germination tests with six slide-coverslips carrying spores obtained before storage. At the end of each storage treatment, conidia were collected from sporulated leaf lesions of symptomatic barley leaves using coverslips, placed immediately on 1.5% water agar Petri dishes, incubated at $20\text{--}22^{\circ}\text{C}$ in the dark for 24 h, and served as non-stored controls.

Pathogenicity test

Pathogenicity tests of all stored isolates and controls (non-stored) were performed on WI 2291, the universal susceptible cultivar from Australia, using the method described by Arabi et al. (2008). Seeds were planted in plastic flats ($60 \times 40 \times 8$ cm) filled with sterilized peatmoss, and arranged in a randomized complete block design with three replicates. Each experimental unit consisted of six rows of 18 seedlings. Flats were placed in a growth chamber at $22 \pm 1^{\circ}\text{C}$ (day) and $17 \pm 1^{\circ}\text{C}$ (night) with a 12 h day length and a relative humidity of 80–90%. Seedlings were irrigated with Knop's nutrient solution (1 g NaNO_3 ; 0.25 g KNO_3 ; 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.25 g KH_2PO_4 ; and 10 mg FeCl_3 per 1000 ml of water). Plants were inoculated at growth stage 13 (Zadoks et al., 1974) by uniformly spraying each flat with 25 ml of conidial suspension with a hand-held spray bottle. Disease severity was assessed on the lamina of the second leaf from the base of each plant 17 days after inoculation, using the rating scale 0-to-100 as described by Salamati and Tronsmo (1997), briefly, where 0 = symptom free, 1 = traces or small necrotic flecks, 2 = some chlorosis or necrosis along margins, 3 = necroses but less than 40% affected

tissue, 4 = necroses on 40 to 80% of the lamina, and 5 = more than 80% and up to a fully wilted leaf.

Statistical analyses

All experiments were repeated at least twice. The second experiment confirmed the results obtained in the first one; thus only results from the first experiment are reported. The data were subjected to analysis of variance as a completely factorial design, with six replicates in each experiment, and 'isolates' and 'storage methods' as main factors. When factors were significant, means were compared by Student–Newman–Keuls range tests ($P < 0.05$).

Results and Discussion

In this work, cultures of all *R. secalis* isolates were viable after four years of storage in different glycerol concentrations at $-20\text{ }^{\circ}\text{C}$. The maximum viability and germinability rates (72.28% for isolate Rs22) for all stored isolates were found in 60% of glycerol. Low viability and contamination were observed when spores were stored in sterile distilled water and lima bean agar (Tables 2 and 3). On the other hand, the data showed that germinability of spores was low in all stored isolates in glycerol in comparison to the control (Table 3).

It is well known the role of glycerol as a cryoprotectant for fungi for maintaining regeneration activity and prevent damage (Paul et al., 2015). Therefore, different concentrations of glycerol were used as cryoprotectant in this work. Our results are in agreement with previous works, Yang and Rossignol (1998) and Lalaymia et al. (2014) found that glycerol was suitable appropriate preservative for the long-term preservation of fungi in liquid nitrogen. Nagpal et al. (2012) reported that glycerol was effective for the preservation of fungi maximum up to 90 days at $-70\text{ }^{\circ}\text{C}$. In addition, Kitamoto et al. (2002) found that glycerol was suitable cryoprotectant for the most of macro-fungi up to 33 months. On the other hand, several authors described problems of contamination in collections of fungi stored in PDA due to mites or other causes (Holden and Smith, 1992). Storage of *R. secalis* on LBA and in sterile distilled water resulted in contamination problems as well (Table 2).

Moreover, the results showed that all isolates continued to infect barley leaves, after 4 years of storage. However, the pathogenicity was significantly ($P < 0.05$) reduced in isolates stored on different methods as compared with controls (Table 4). On the contrary, pathogenicity is a characteristic easily lost by fungal isolates in axenic culture (Holden and Smith, 1992). In addition, Silva et al. (1994) found that long-term storage of fungi under mineral oil overlay led to changes in physiological and morphological characters. On the other hand, Page et al. (1992) suggested that dry-stored spores may suffer from chromosome mutations. Beri and Bir (1993) found that levels of reserve substances declined with storage under dry conditions, though to date no comparable information is available for storage under wet conditions.

Table 2

Viability of *R. secalis* isolates stored at -20 °C for 4 years under different storage conditions

Storage method ^b	Isolates		
	Rs22	Rs7	Rs2
Glycerol 30%	+	-	-
Glycerol 60%	+	+	+
Glycerol 90%	+	+	-
LBA	+	-	C
Sterile distilled water	+	C	-

^b a - : no mycelial growth; + : mycelial growth; C : contaminated culture
LBA: lima bean agar

Table 3

Mean disease rating of stored and non-stored isolates of *R. secalis* on barley cv. WI 2291

Storage method	Non-stored (control)	Storage method				
		Glycerol 30%	Glycerol 60%	Glycerol 90%	Sterile distilled water	LBA
Rs22	A92.56a ^a	D26.81a	B72.28a	C36.12a	E17.15a	E19.06a
Rs7	A83.44b	D17.67a	B47.25b	C28.31a	D13.22a	D19.73a
Rs2	A77.50b	C29.64a	B51.05b	C30.00a	D14.63a	D8.91b

^aMeans preceded by different capital letters (row) and followed by the different lowercase letter (column) are significantly different at ($P < 0.05$) according to the Newman-Keuls test. LBA: lima bean agar

Table 4

Mean disease rating of stored and non-stored isolates of *R. secalis* on barley cv. WI 2291

Isolate	Non-stored (control)	Storage method				
		Glycerol 30%	Glycerol 60%	Glycerol 90%	Sterile distilled water	LBA
Rs22	A5.00 ^a	B4.35a	B4.39a	B4.37a	B4.11a	B4.15a
Rs7	A4.29b	B3.25b	B3.75b	C2.24b	C2.10b	C1.65b
Rs2	A4.05b	B2.99b	B3.17b	C1.89c	C1.56c	C1.26b

^aMeans preceded by different capital letters (row) and followed by the different lowercase letter (column) are significantly different at ($P < .05$) according to the Newman-Keuls test. LBA: lima bean agar

Conclusion

In this study, different concentrations of glycerol were used for the first time as a preservative for *R. secalis* in a long-term storage at -20 °C. Among the concentrations tested, the best results were obtained with storage in 60% glycerol. In addition, viability and pathogenicity of isolates were maintained for 4 years using these methods of storage. Contamination was observed when stored in LBA and in sterile distilled water. However, through the glycerol storage method it was clearly showed that fungi were preserved without any contamination, therefore, it appears suitable for the maintenance of stock cultures and homogeneous infection material for routine testing.

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