Effect of freezing of *Neonectria ditissima* inoculum on its pathogenicity

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Abstract The fungus *Neonectria ditissima* causes European canker of apple. To determine the pathogenicity of different isolates, conidial inoculum of each isolate needs to be prepared. Freezing inoculum ensures that conidia do not germinate before inoculation, and facilitates screening of large numbers of isolates. In this study, conidial suspensions of three different isolates and 'field conidia' collected from apple cankers were used to inoculate dormant potted 1-year-old 'Royal Gala' trees in a glasshouse. Each conidial suspension (2×10^5 conidia/ml) was used either fresh or defrosted after 3 days at -20°C . Five buds per shoot were removed and inoculated, with four replicates of two shoots per treatment. Significant differences in disease incidence and lesion size were observed between the different isolates at each assessment date, 5 to 15 weeks after inoculation ($P < 0.003$), but freezing the inoculum had no effect on disease incidence or lesion size. Frozen conidial suspensions can be used for pathogenicity studies and may also be a long-term storage option for cultures.

Keywords European canker, *Neonectria galligena*, *Nectria galligena*, conidia, inoculation, temperature, freezing, pathogenicity, disease incidence, lesion size.

INTRODUCTION

European canker, caused by the fungus *Neonectria ditissima*, (Tul. & C. Tul.) Samuels & Rossman [synonym *Neonectria galligena* (Bres.); anamorph *Cylindrocarpon heteronema* (Berk. & Broome) Wollenw.], can result in the death of apple buds, shoots, spurs and branches (English et al. 1979; Weber 2014). In extreme cases, the main trunk can become girdled, requiring removal of entire trees.

To investigate the pathogenicity of large numbers of isolates of *N. ditissima*, conidia from cultured isolates are required for inoculation. When conidia are collected from these cultures, they are suspended in water or a Tween®20 solution

(Scheper et al. 2014) and all conidial suspensions are adjusted to the same concentration, which can take between 10 and 30 min per isolate. This means that conidia of some isolates can be in an aqueous suspension for many hours, while other suspensions may be used shortly after they were made. Moreover, when large numbers of isolates need to be tested, it may take several days to make all the conidial suspensions.

Conidia start to germinate as soon as they are in an aqueous suspension, with the germination rate dependent on the temperature. Latorre et al. (2002) showed that approximately 4% of conidia germinated per hour at room temperature, but

at temperatures below 6°C conidia obtained from three cultures did not germinate. However, germination studies of conidia collected from sporulating cankers in Motueka, New Zealand, showed that 39% of conidia germinated after 24 h at 3°C, while 93–95% of conidia germinated after 24 h at 15–21°C (RWA Scheper, Plant & Food Research, unpublished data). When this study was repeated using conidia from different sporulating cankers, the conidial germination rates after 24 h at 1, 2, 3, 4 and 5°C were 1, 3, 13, 16 and 28%, respectively.

When investigating the pathogenicity of different isolates of *N. ditissima*, it is important that all conidial suspensions are prepared the same way. Varying storage times of different suspensions, at room temperature or in the refrigerator, should be avoided, because conidia in the suspensions that were stored the longest are more likely to germinate. Germination tubes may break off when inoculating, potentially reducing the pathogenicity of these suspensions.

Freezing of conidial suspensions as soon as they have the required concentration would prevent germination, ensuring more uniform conidial suspensions of the different isolates. Preliminary tests have shown that when defrosted conidial suspensions, which had been frozen at -20°C for up to 1 week, were used to inoculate 'Royal Gala' trees, canker lesions developed. The aim of this study was to determine whether freezing conidial suspensions would affect the germination rate and pathogenicity of these suspensions compared with those of the original suspensions before they were frozen.

MATERIALS AND METHODS

Production of conidial suspensions

Three single ascospore isolates, RS305p, RS324p and RS401p2, isolated from cankers with mature perithecia in Lower Moutere (Nelson), New Plymouth (Taranaki) and Upper Moutere (Nelson) were grown on Matsushima's medium (MM, Matsushima 1961) as adjusted by Dubin & English (1974) for 3 weeks at 20°C under continuous near ultraviolet light, as described by Scheper et al. (2014). The isolates were selected because of their morphological

differences. Isolate RS305p produced very few multi-celled conidia (1%) on MM, while 63% and 75% of the conidia of isolates RS401p2 and RS324p, respectively, were multi-celled (Scheper et al. 2014).

Conidia were collected by flooding each plate with 2 ml sterile distilled water containing 0.005% Tween20 (Tween solution) and pipetting the liquid five times over the culture. Conidia were enumerated using a haemocytometer (Hawksley counting chamber BN15 8TN, UK) and the solutions adjusted to produce conidial suspensions of 2×10^5 conidia/ml.

A suspension (2×10^5 conidia/ml) of field-produced conidia (field inoculum) was made by transferring sporodochia from cankers collected in Motueka (Nelson) to Tween solution, using a binocular dissecting microscope and a sterile scalpel.

Each conidial suspension was divided between two sterile McCartney bottles, one of which was placed in the freezer at -20°C for 3 days and then defrosted by placing the bottle in a water bath at 20°C for 4 min (frozen inoculum). The other was used within 2 h (fresh inoculum).

Conidial germination rates of fresh and frozen inocula were determined by placing four 60- μ l droplets of each conidial suspension on two glass microscopy slides, and covered with a cover slip. The slides were incubated at 23°C for 41 h at 100% relative humidity, and the numbers of germinated and non-germinated spores were counted using a compound microscope at $\times 100$ magnification.

Inoculation

Potted 1-year-old 'Royal Gala' trees on 'M9' rootstocks, with either one or two dormant shoots, were moved into a glasshouse in August 2014, before inoculation. Five bud scars were created on each shoot by breaking off the buds, 1 to 2 h before inoculation. Bud scars were at least 20 cm apart. Each bud scar was marked below the scar with a white paint pen (Pentel White 100W L). Labelled bud scars were inoculated with 10 μ l conidial suspension using a pipette.

The shoots inoculated with the fresh inocula were inoculated first. The shoots inoculated with

the frozen inocula were inoculated 3 days later. After each inoculation, the relative humidity in the glasshouse was increased to 100% for 3 days. The temperature in the glasshouse during both inoculation times was approximately 15°C. After the two three-day periods of 100% humidity, the humidity was kept at 75%.

Trial design and assessments

There were eight treatments (four different inocula either fresh or frozen) arranged in a randomised block design, with four replicates of 10 bud scars per treatment. The 10 bud scars were usually on two trees (five scars per tree), but occasionally on one tree with two shoots (five scars per shoot). Two trees with bud scars treated with Tween solution were randomly placed between the inoculated trees as negative controls.

The bud scars were assessed visually for the presence or absence of canker symptoms, 5 weeks after inoculation, and weekly thereafter, until 15 weeks after inoculation. The length of the lesions was measured every week.

Statistical analyses

Conidial germination rates of fresh and frozen suspensions were analysed using a binomial generalised linear model and a two-factor binomial model, the factors being “isolate” and “fresh v. frozen”.

For the inoculation trial in the glasshouse, data from each of the 11 individual assessments (5 to 15 weeks after inoculation) were analysed with two-factor Analysis of Variance (ANOVA), the factors being “isolate” and “fresh v. frozen”. Residuals were inspected to check the assumptions of ANOVA.

For the proportion of inoculated bud scars that developed canker symptoms (disease incidence) no transformation was needed, and Fisher's Protected Least Significance Differences of Means (LSD, $P=0.05$) were used to determine statistical differences in disease incidence of bud scars among treatments.

For lesion size, a mean was calculated for each replicate of each treatment, and this was analysed. A log transformation was applied to stabilise the variability, and an unbalanced

ANOVA was used because replicates with no lesions were excluded from the analysis. Fisher's Protected LSD ($P=0.05$) were used to determine statistical differences in mean lesion size between treatments.

The data from all 11 weeks were analysed as repeated measures, using mixed models with covariance between the multiple measurements on each replicate (Littel et al. 1998). Inspection of the residuals suggested the disease incidence data did not need to be transformed, but the lesion size data did. All analyses were carried out using GenStat (version 14, 2011, VSNi Ltd, Hemel Hempstead, UK).

RESULTS

Effect of freezing on conidial germination rates

The germination rate of most conidial suspensions was slightly lower when the suspension had been frozen for 3 days (Table 1). The mean germination rate of frozen conidial suspensions (41%) was significantly lower than that of the fresh suspensions (48%), because the main effects of both “isolate” and “fresh v. frozen” were significant ($P=0.002$ and $P=0.011$, respectively), but the interaction was not ($P=0.156$), meaning the effect of freezing was similar for all the isolates.

Freezing of the conidial suspensions did not affect the morphological appearance of the conidia when examined using a microscope.

Effect of freezing on pathogenicity

Disease incidence increased over time with all treatments, except on trees inoculated with frozen inoculum of isolate RS305p, which remained 0% for the duration of the trial (Figure 1). The negative controls did not develop any canker symptoms.

Five weeks after inoculation, significant differences in disease incidence were observed between the different isolates ($P<0.001$), with the disease incidence in trees inoculated with isolate RS324p significantly higher than in those inoculated with any other isolate. There was no significant difference between isolate RS401p2 and field inoculum. However, freezing the inoculum had no significant effect on disease incidence ($P=0.315$).

Table 1 Mean germination rates (%) of conidial suspensions of different isolates of *Neonectria ditissima* in Tween®20 solution (2×10^5 conidia/ml), immediately after preparation (fresh) and after they had been frozen for 3 days at -20°C . Field inoculum was made using conidia from cankers collected in Motueka. Germination rates were based on two replicates of approximately 200 conidia; standard errors ranged from 1.0 to 5.0 (root mean square of 3.1).

Isolate	Fresh	Frozen
RS 305p	48	31
RS 324p	53	45
RS 401p2	35	35
Field inoculum	56	51
Mean	48	41

The first time that the disease incidences of trees inoculated with field inoculum and with isolate RS324p were no longer significantly different, was 8 weeks after inoculation. For the remainder of the trial, there was no significant difference in disease incidence between field inoculum and RS324p.

At the last assessment, 15 weeks after inoculation, significant differences in disease incidence were observed between the different

isolates ($P < 0.001$), with trees inoculated with RS324p and field inoculum displaying significantly more disease than trees inoculated with the other isolates, and trees inoculated with RS305p displaying significantly less than those inoculated with any other isolate (Table 2). However, freezing the inoculum had no significant effect on disease incidence ($P = 0.443$). It is noteworthy that these trends held true throughout the study. At each assessment date, the main effect of “isolate” was significant ($P < 0.001$), but that of “fresh v. frozen” was not (P values ranged from 0.091 to 0.443), nor was the interaction between “isolate” and “fresh v. frozen” (P varied between 0.147 and 0.715), meaning that freezing inoculum did not affect disease incidence at any time between 5 and 15 weeks after inoculation.

Analysis of all 11 weeks as repeated measures showed that there was a significant interaction between “time” and “isolate” ($P < 0.001$), but no significant main effect of “fresh v. frozen”, or interaction between “fresh v. frozen” and “time” or “isolate” (P values ranged from 0.088 to 0.689), meaning that freezing inoculum did not affect disease incidence.

The mean lesion sizes increased over time for all treatments (Figure 2), except on trees inoculated with frozen inoculum of isolate RS305p, which did not produce any lesions. Five weeks after inoculation, the mean lesion sizes on trees inoculated with isolates RS324p, RS401p2 and field inoculum ranged from 5 mm (frozen

Figure 1 Mean disease incidence (%), 5 to 15 weeks after inoculation of bud scars of potted ‘Royal Gala’ apple trees with conidial suspensions of different isolates of *Neonectria ditissima*, immediately after preparation (fresh) and after they had been frozen for 3 days at -20°C . Field inoculum was made using conidia from cankers collected in Motueka.

field inoculum) to 25 mm (fresh RS324p), while no lesions were visible on any of the trees inoculated with RS305p.

The first lesion on a tree inoculated with fresh inoculum of RS305p appeared 7 weeks after inoculation and was 3 mm. Ten weeks after inoculation, this lesion was 30 mm. The sudden drop in mean lesion size 11 weeks after inoculation occurred when a second lesion became visible (Figure 2).

At the last assessment, 15 weeks after inoculation, significant differences in lesion size were observed between the different isolates ($P < 0.001$), with RS324p and field inoculum causing significantly larger lesions than the other isolates, and RS305p causing significantly smaller lesions than any other isolate (Table 2). However, freezing the inoculum had no significant effect on lesion size ($P = 0.161$ for main effect and 0.342 for interaction).

At each assessment date, the main effect of “isolate” was significant for lesion size ($P < 0.003$), but “fresh v. frozen” was not (P values ranged from 0.161 to 0.630), except at 10 weeks after inoculation, when trees inoculated with

frozen inoculum had significantly larger lesions ($P = 0.030$). There was no significant interaction between “isolate” and “fresh v. frozen” (P values ranged from 0.165 to 0.946), except at 10 weeks after inoculation when this interaction was nearly significant ($P = 0.056$). Ten weeks after inoculation, lesions from frozen field inoculum and RS401p2 were larger than those from fresh inoculum, while lesions from fresh and frozen inoculum of RS324p had similar sizes. For the remainder of the trial, there was no difference in lesion size resulting from fresh or frozen inoculum.

Analysis of all 11 weeks as repeated measures showed that there was a significant interaction between “time” and “isolate” ($P < 0.001$), but no significant main effect of “fresh v. frozen”, or interaction between “fresh v. frozen” and “time” or “isolate” (P values ranged from 0.138 to 0.626), meaning that freezing inoculum did not affect lesion size.

Sporodochia were seen in cankers on trees inoculated with both fresh and frozen inoculum of isolate RS324p and fresh field inoculum, 15 weeks after inoculation.

DISCUSSION

Freezing conidial suspensions for 3 days at -20°C did not affect the pathogenicity. Neither disease incidence nor mean lesion size were affected, and sporodochia formed on cankers in trees inoculated with both fresh and frozen inoculum of the most pathogenic isolates. The morphology of the conidia was also not affected by freezing. Although conidial germination in suspensions that had been frozen was significantly lower than in fresh suspensions (41% versus 48% respectively), in reality, the difference was inconsequential, as approximately 2000 conidia were pipetted onto each bud scar, and Dubin & English (1974) showed that only 50 conidia per leaf scar on ‘Red Delicious’ apple trees were required to cause infection. Although higher conidial concentrations resulted in increased disease incidence (Dubin & English 1974), the difference between 41% and 48% of 2000 conidia would have been unlikely to affect the results.

Large and statistically significant differences were observed between different isolates. Isolate RS324p

Figure 2 Mean lesion size (mm), back-transformed from the log scale, 5 to 15 weeks after inoculation of bud scars of potted ‘Royal Gala’ apple trees with conidial suspensions of different isolates of *Neonectria ditissima*, immediately after preparation (fresh) and after they had been frozen for 3 days at -20°C . Field inoculum was made using conidia from cankers collected in Motueka.

Table 2 Mean disease incidence (%) and mean lesion size (mm), 15 weeks after inoculation of bud scars of potted ‘Royal Gala’ apple trees with conidial suspensions of different isolates of *Neonectria ditissima*, immediately after preparation (fresh) and after they had been frozen for 3 days at -20°C. Field inoculum was made using conidia from cankers collected in Motueka. Treatments with the same letter were not significantly different.

Isolate	Disease incidence			Lesion size		
	Fresh	Frozen	Mean	Fresh	Frozen	Mean
RS 324p	90	100	95 a	95	94	95 a
Field inoculum	93	90	91 a	56	87	68 a
RS 401p2	75	83	79 b	43	49	46 b
RS 305p	5	0	3 c	25	-	25 c
Mean	66	68		58	73	

was similar in pathogenicity to field-collected conidia, and both were significantly more pathogenic (lesion size and disease incidence) than the other two isolates. Isolate RS305p was almost non-pathogenic, and RS401p2 was significantly less pathogenic than RS324p and field inoculum, but significantly more pathogenic than RS305p. In previous pathogenicity trials using single conidium isolates of *N. ditissima* in both glasshouse (Scheper et al. 2010) and field studies (Amponsah et al. 2014), trees inoculated with different inocula also developed lesions that varied in number and lesion size.

Scheper et al. (2014) also showed that the isolates used in this study were very different, with regard to sporodochia production on agar and conidial morphology. The most pathogenic isolate RS324p was the only isolate that produced sporodochia on water agar, while the almost non-pathogenic isolate RS305p produced significantly fewer multi-celled conidia (1.8%) than did either the other two isolates or the field inoculum (56.5–86.0%). It is therefore possible that a high percentage of multi-celled conidia is required for pathogenicity. Storage and age of the culture did not seem have an effect, as both RS305p and RS324p were isolated in 2009.

Xu et al. (1998) and Latorre et al. (2002) identified that inoculum dose, duration of wet period, temperature and wound age may affect the success rate of artificial inoculation with *N. ditissima* (Xu et al. 1998; Latorre et al. 2002). In the present trial, these factors were controlled and all inocula were applied at the same dose, to

wounds of similar age and the trees incubated at a similar temperature. The duration of wet period was longer for trees inoculated with fresh inocula because these trees were exposed to 100% humidity for 3 days after inoculation and then again when the remaining trees were inoculated with frozen inoculum. However, this did not affect lesion size or disease incidence.

Freezing conidial suspensions is often used as a long-term (1–10 years) storage technique for various fungi (Dahmen et al. 1983; Satou & Fukumoto 1993; Collins et al. 2003; Chehri et al. 2010; Homolka 2013), but these techniques involve using suitable cryogenic protectants, such as varying concentrations of glycerol, dimethyl sulfoxide (DMSO) or skim milk, very low temperatures (-80°C or -196°C), and sometimes high conidial concentrations (10^8 conidia/ml). The survival of conidia during cryostorage depends on the prevention of ice crystal formation, which can cause physical damage during storage (Barnhart & Terry 1971a, b; Homolka 2013), but different fungi require different conditions (Dahmen et al. 1983). The speed of freezing and thawing may also affect survival of the conidia, because a slow thaw (0.5°C/min) may favour ice crystal growth, which has been shown to damage conidia of *Neurospora crassa*, resulting in a survival rate of less than 10%, while medium warming rates (2–5°C/min) resulted in a survival rate of 70% (Barnhart & Terry 1971a). Dahmen et al. (1983) found that many fungi recovered equally well after rapid (direct immersion in liquid nitrogen)

or slow (1°C/min) freezing and survived both rapid (50-100°C/min) and slow (7-10°C/min) thawing. In the present study, *N. ditissima* conidial suspensions survived and remained pathogenic after slow freezing to -20°C, being frozen for 3 days, and thawing to room temperature at a warming rate of approximately 10°C/min, without a cryogenic protectant. Freezing of conidial suspensions may also be an option for long-term (5-10 years) storage of *N. ditissima* isolates, but further studies are required to determine optimal conditions.

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REFERENCES

- Amponsah NT, Walter M, Scheper RWA 2014. Agar media for isolation of *Neonectria ditissima* from symptomatic and asymptomatic apple tissues and production of infective conidia. *New Zealand Plant Protection* 67: 116-122.
- Barnhart EE, Terry CE 1971a. Cryobiology of *Neurospora crassa*: I. Freeze response of *Neurospora crassa* conidia. *Cryobiology* 8: 323-327.
- Barnhart EE, Terry CE 1971b. Cryobiology of *Neurospora crassa*: II. Alteration in freeze response of *Neurospora crassa* conidia by additives. *Cryobiology* 8: 328-332.
- Chehri K, Salleh B, Yli-Mattila T, Soleimani MJ, Yousefi AR 2010. Occurrence, pathogenicity and distribution of *Fusarium* spp. in stored wheat seeds Kermanshah province, Iran. *Pakistan Journal of Biological Sciences* 13: 1178-1186.
- Collins A, Okoli CAN, Morton A, Parry D, Edwards SG, Barbara DJ 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. *Phytopathology* 93: 364-376.
- Dahmen H, Staub T, Schwinn FJ 1983. Technique for long-term preservation of Phytopathogenic fungi in liquid nitrogen. *Phytopathology* 73: 241-246.
- Dubin HJ, English H 1974. Factors affecting apple leaf scar infection by *Nectria galligena* conidia. *Phytopathology* 64: 1201-1203.
- English H, Dubin HJ, Schick FJ 1979. Chemical control of European canker of apple. *Plant Disease Reporter* 63: 998-1002.
- Homolka L 2013. Methods of cryopreservation. In: Gupta VK, Tuohy MG, Awachamy M, Turner KM, O'Donovan A ed. *Laboratory protocols in fungal biology: Current methods in fungal biology*. Springer. Pp. 9-16.
- Latorre BA, Rioja ME, Lillo C, Munoz M 2002. The effect of temperature and wetness duration on infection and a warning system for European canker (*Nectria galligena*) of apple in Chile. *Crop Protection* 21: 285-291.
- Littel RC, Henry PR, Ammerman CB 1998. Statistical analysis of repeated measures data using SAS procedures. *Journal of Animal Science* 76: 1216-1231.
- Matsushima T 1961. A new medium for identification of hyphomycetes. *Transactions of the Mycological Society of Japan* 2: 118-120.
- Satou M, Fukumoto F 1993. Preservation of conidia of broccoli downy mildew fungus with cryogenic protectants by freezing at -80°C. *Annals of the Phytopathological Society of Japan* 59: 492-499.
- Scheper RWA, Fisher BM, Wood PN 2010. Pathogenicity of field and laboratory grown inoculum of *Neonectria galligena* on potted apple trees. *New Zealand Plant Protection* 63: 280 (abstract only).
- Scheper RWA, Fisher BM, Amponsah NT, Walter M 2014. Effect of culture medium, light and air circulation on sporulation of *Neonectria ditissima*. *New Zealand Plant Protection* 67: 123-132.
- Weber RWS 2014. Biology and control of the apple canker fungus *Neonectria ditissima* (syn. *N. galligena*) from a Northwestern European perspective. *Erwerbs-Obstbau* 56: 95-107.
- Xu X, Butt DJ, Ridout MS 1998. The effects of inoculum dose, duration of wet period, temperature and wound age on infection by *Nectria galligena* of pruning wounds on apple. *European Journal of Plant Pathology* 104: 511-519.