Long-term deep-freeze storage of *Neonectria ditissima* conidium suspensions does not reduce their ability to infect apple trees

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Abstract Fresh, wild-type conidium suspensions of *Neonectria ditissima* prepared from sporulating European canker lesions for use in field inoculation trials are not always readily available. The effect of freezing inoculum on the viability of spores and their ability to cause infection *in situ* was assessed using 15–19 frozen *N. ditissima* conidium suspensions during 2015–2017. Freshly prepared inoculum and water only were used to inoculate rasp wounds on ‘Braeburn’ (2015), ‘Scilate’/Envy™ (2016) and ‘Royal Gala’ and ‘Braeburn’ (2017) along with frozen inoculum ranging in age from 1–462 days at concentrations $>10^4$ conidia/mL. Lesion development was assessed at approximately monthly intervals. In all experiments, there was no substantial effect of storage time on lesion development although germination in droplets may have decreased during storage. As expected, there was a cultivar effect (2017 data only), with fewer lesions on ‘Braeburn’ compared with ‘Royal Gala’. Long-term deep-freeze storage up to 462 days of *N. ditissima* conidium suspensions ($>10^4$ conidia/mL) did not affect the ability of spores to infect apple trees. Therefore, spore suspensions can be batch-prepared and stored frozen for future experimental use.

Keywords: European canker, apple trees, *Neonectria ditissima*, storage

INTRODUCTION

Spores of the fungus *Neonectria ditissima* (Tul. & C.Tul.) Samuels & Rossman can cause European canker in apple trees, resulting in lesions that kill shoots, spurs and branches (Dubin & English 1974). Research into the pathology of *N. ditissima* requires inoculum, which is produced using fresh conidia suspended in aqueous solution, usually deionised water. However, germination can occur rapidly in solution at temperatures $>3^{\circ}\text{C}$ (Latorre et al. 2002). Therefore, the inoculum usually needs to be frozen until required (Scheper et al. 2015).

Previous research investigated the effect of freezing conidial suspensions and found that it did not affect the pathogenicity of the inoculum

(Scheper et al. 2015). However, the experiment tested a short freeze period (3 days), and inocula may need to be frozen for longer periods. The effect of long-term deep-freeze storage has not been investigated, nor the effect of multiple thawing events. If *N. ditissima* conidia can survive longer periods of deep-freeze storage then larger batches of inoculum may be stored for longer and defrosted as required. This would also reduce the need to make up fresh inoculum batches for each experiment or at times when fewer lesions are available. Therefore, the effect of long-term deep freeze and inoculum age on the viability of conidial suspensions of *N. ditissima* and its pathogenicity to produce European canker lesions in apple trees were investigated.

MATERIALS AND METHODS

Three field experiments were conducted over consecutive years 2015 (Experiment 1), 2016 (Experiment 2) and 2017 (Experiment 3) in orchards near Motueka in the Tasman region. In Experiment 1, 18 frozen and one fresh inocula of conidial suspensions were used. Slightly fewer frozen inocula plus one fresh inoculum and a distilled water control were used in subsequent years (Table 1). Each batch of inoculum had been sourced from fresh canker lesions of apple trees (e.g. ‘Scifresh/Jazz™’, ‘Scilate’/Envy™, ‘Royal Gala’, ‘Braeburn’) from orchards in the Tasman region. Inoculum was prepared in sterile distilled water as described in Walter et al. (2015). Most inoculum batches (henceforward referred to as ‘treatment’) were unique, except for six treatments that were used in both Experiments 2 and 3 (Table 2). Inocula had been placed into deep-freeze storage at -18°C.

Inocula were thawed and three 10-μL droplets of each inoculum were placed onto a glass slide, then incubated overnight at room temperature. The following morning, the number of germinated and non-germinated conidia were assessed to give the percentage germination (%germination). The ‘Fresh’ %germination was obtained at the time each inoculum batch was prepared (Table 2). The ‘Inoculation’ %germination was measured for each inoculum at the time of experimental inoculation in an apple tree, except for Experiment 2 (Table 2).

Inoculation

Experiment 1 was conducted in July 2015 in a central leader-managed, commercial ‘Braeburn’

orchard. The trees were planted (5 × 2.4 m) in the 1990s and grafted onto ‘M7’ rootstock. Nineteen conidia suspensions (treatments) of varying spore concentration and age were used (1–78 days old; Table 2). Fourteen replicate trees were available for this experiment, using one shoot per treatment per tree, with 19 shoots inoculated per tree. Treatments in a tree were allocated randomly. Each shoot had four inoculated rasp wounds. There was no water control for this experiment.

Experiment 2 was conducted in one row of a ‘Scilate’/Envy™ orchard in June 2016. The trees were planted (0.8 × 3.5 m) in 2012 and grafted onto ‘M9’ rootstock. Fifteen treatments (Table 2) between 1 and 272 days old were tested against a water control in a resolvable incomplete block design. Each block consisted of five trees, with generally three shoots per tree being inoculated (two to five shoots per tree depending on shoot availability). A single treatment was applied to each shoot (three rasp wounds per shoot), with each tree thus receiving two to five randomly chosen treatments. There were seven blocks with two buffer trees between each block.

Experiment 3 was conducted in June 2017 using two cultivars in separate orchards, ‘Braeburn’ (same as Experiment 1) and ‘Royal Gala’ planted in 2012 (0.8 × 3.5 m) grafted onto ‘M9’ rootstock. Sixteen treatments (Table 2) between 1–462 days old were tested against a water control in a resolvable incomplete block design. ‘Braeburn’: each block consisted of nine trees and three rasp wounds were made on up to two shoots per tree. A single treatment was applied to each shoot, with each tree receiving up to two treatments. ‘Royal Gala’: each block consisted of 25 trees and three rasp wounds

Table 1 Summary of all three field experiments using previously frozen inoculum.

Experiment	Cultivar	Inoculation date	No. of inocula	Max. age (days)
1	‘Braeburn’	2/07/2015	19	78
2	‘Scilate’ (Envy™)	13/06/2016	15	272
3	‘Royal Gala’	1/06/2017	16	462
3	‘Braeburn’	2/06/2017	16	462

Table 2 Spore concentration, estimated number of viable spores, age and the percentage germination rate (%germination; freshly prepared and at the time of each experiment inoculation) of *Neonectria ditissima* inoculum used in three field experiments.

Experiment	Inoculum reference	Inoculum concentration (x 10 ³ conidia/mL)	Estimated viable spores ^a (x 10 ³ conidia/mL)	Inoculum age (days)	%Germination	
					Fresh	Inoculation
1	1	150	87.0	78	nd	58
	2	150	52.5	78 ^a	nd	35
	3	150	69.0	78	nd	46
	4	70	67.2	43	nd	96
	5	100	78.0	43	nd	78
	6	80	42.4	36	nd	53
	7	80	63.2	32	nd	79
	8	50	33.0	32	nd	66
	9	80	65.6	32	nd	82
	10	80	69.6	28	nd	87
	11	80	69.6	28	nd	87
	12	60	48.6	24	nd	81
	13	80	78.4	24	nd	98
	14	40	31.2	24	nd	78
	15	50	44.0	21	nd	88
	16	70	61.6	21	nd	88
	17	100	67.0	17	nd	67
	18	150	34.5	1	nd	23
	19 (Fresh)	150	37.5	1	nd	25
2	20	250	nd	272	nd	nd
	21	170	nd	189	nd	nd
	22	92	nd	175	nd	nd
	23	70	nd	109	67	nd
	24	82	nd	104	84	nd
	25	80	nd	58	29	nd
	26	210	nd	51	99	nd
	27	10	nd	42	92	nd
	28	100	nd	35	3	nd
	29	130	nd	28	91	nd
	30	310	nd	18	58	nd
	31 (Fresh)	25	nd	1	100	nd
	32	25	nd	1	100	nd
	33	82	nd	104 ^b	84	nd
	34	100	nd	35 ^b	3	nd
	35 (C)	0	0.0	0	n/a	n/a

Table 2 continued

Experiment	Inoculum reference	Inoculum concentration (x 10 ³ conidia/mL)	Estimated viable spores ^a (x 10 ³ conidia/mL)	Inoculum age (days)	%Germination	
					Fresh	Inoculation
	53	95	2.8	457	85	3
	54	65	10.7	457	43	17
	55 ^{#25}	80	2.2	411 ^c	29	3
	56 ^{#28}	100	0.0	388 ^c	3	0
	57 ^{#30}	31	1.0	371 ^c	58	3
	58 ^{#31}	25	0.6	354	100	2
	59	25	0.3	310	93	1
	60 ^{#26}	210	28.4	309 ^c	99	14
	61	40	18.7	252	40	47
	62	2	1.0	127	90	50
	63	50	28.2	121	47	56
	64	65	41.6	106	100	64
	65	39	19.6	92	75	50
	66 (Fresh)	140	123.2	1	88	88
	67	140	90.7	1	65	65
	68 (C)	0	0	0	n/a	n/a

^a Estimated viable spore numbers based on Inoculum concentration × % Germination at inoculation

^{b,c} Number of times defrosted and re-frozen: ^b = 1, ^c = 2

Earlier reference number in Experiment 2

(C) Water control

(Fresh) Fresh inoculum control, stored overnight at 4°C

nd = Not determined

n/a = Not applicable

were made to one shoot on 17 trees. A single treatment was applied to each shoot. Therefore, each tree received only one treatment. There were 12 blocks for both cultivars. Note that six of the treatments in Experiment 3 were also used in Experiment 2 (Table 2).

For all experiments, inoculation rasp wounds were made (Walter et al. 2016) and inoculated as described in Walter et al. (2015). Each shoot was wounded with a rasp creating an approximately 6-8 mm wound between buds at least 5 cm apart. A wound was inoculated with approximately a 20-30 µL spore suspension using a camel-hair brush fixed to a plastic Pasteur pipette. Assessment of lesion development commenced after approximately 10 weeks and continued at monthly intervals for up to three months. The

number of rasp wounds which had developed canker lesions on each shoot was recorded. Shoots were cut and disposed of when one or more wound expressed symptoms (Walter et al. 2015).

Statistical analyses

Data were explored graphically as well as with formal analyses. Some initial analyses (details not shown) were carried out to assess the importance of the position of trees and whether there was more variation among trees than among shoots within a tree. For all three trials, positional effects were largely negligible. However (as would be expected), there was, in general, more variation among trees than among shoots within a tree. For simplicity, this was adjusted for by estimating

over-dispersion in the final models fitted.

Numbers of rasp wounds per shoot at the time the first lesions on a shoot were identified (when the shoot was then cut) were analysed. If no lesions were found by the final assessment, then a value of 0 lesions was used for that shoot. The number of rasp wounds with lesions per shoot out of the total wounds (%canker) was analysed with a binomial generalised linear model (McCullagh & Nelder 1989), with a logit link. The main effect assessed was 'treatment' (i.e. inoculum number), plus for the 2017 trial, cultivar ('Royal Gala' or 'Braeburn') and the cultivar $\times$ treatment interaction. Trends amongst the treatments were explored by including explanatory variables in the analyses: Variables were: control vs inoculated; inoculum age; spore concentration; %germination; and estimated viable spores (calculated as spore concentration $\times$ %germination/100 at inoculation). For Experiment 3, the interactions between these variables and cultivar were also assessed. Overall assessments of effects and relationships were made using F-tests done within the analysis of deviance carried out as part of the analysis. Results are presented as percentage wounds with canker lesions (%canker) along with associated 95% confidence limits. These were obtained on the link (logit) scale, and back-transformed for presentation. The analyses were carried out with Genstat (Genstat Committee 2016).

RESULTS

In Experiment 1, %canker did not vary substantially among the 19 treatments ($P=0.997$; mean %canker range 22.5–41.1; Fig. 1a). The %germination was negatively correlated ($r=-0.79$) with increased spore concentration, showing that germination tended to decrease with increasing conidia density (data not shown).

In Experiment 2, %canker also did not vary substantially between the 15 inoculated treatments (mean %canker range 52.4–95.2; Fig. 1b). However, the %canker in all inoculated treatments was greater than in the non-inoculated treatment (number 35) where no canker was observed ($P=0.006$). There was no

significant relationship between inoculum age, spore concentration or number of thaws on the %canker ($P>0.1$).

In Experiment 3, %canker varied substantially between the 17 treatments ($P<0.001$). The main difference was between the non-inoculated treatments (number 68; mean %canker 3.2 and 18.5 for 'Braeburn' and 'Royal Gala', respectively) and the inoculated treatments (mean %canker 28–100 and 24–100 for 'Braeburn' and 'Royal Gala', respectively; $P<0.001$ for both cultivars; Figures 1c and 1d). Overall, inoculum age was negatively correlated with %germination ($r=-0.92$), but %canker had only minimal correlation with inoculum age, or %germination or spore concentration. The %canker was not related to spore concentration and %germination ($r=0.11$), as for Experiment 1.

Overall, in Experiment 3 there were higher rates of %canker for 'Royal Gala' (85%) than for 'Braeburn' (77%). In addition, differences in %canker between the inoculated treatments varied with cultivar ($P=0.002$ for the cultivar $\times$ treatment interaction) driven primarily by treatments 55, 56 and 59 (Figure 1c and 1d). For instance, %canker was lowest in both cultivars for treatments 58: 354 days, the mean %canker 28% in 'Braeburn' was slightly higher than the 24% in 'Royal Gala' (Figs. 1c and 1d). However, for treatments 55 (411 days) and 56 (388 days), %canker was lower in 'Braeburn': 50 and 44%, respectively, than in 'Royal Gala': 97 and 93%, respectively. Whereas for treatment 59 (310 days), %canker was low in 'Royal Gala' (38%) but not in 'Braeburn' (64%; Figures 1c and 1d).

DISCUSSION

The results of the current study showed that deep-freeze storage of conidial suspensions in distilled water did not diminish the pathogenicity of *Neonectria ditissima* in apple tree shoots up to 462 days. Inoculum that had been frozen for 462 days, and which had been thawed and re-frozen twice (treatment 52), still resulted in 100% canker expression in both 'Braeburn' and 'Royal Gala' cultivars despite having only 6% germination.

Scheper et al. (2015) found that the viability

Figure 1 The percentage of rasp wounds which developed European canker lesions (%canker) in three experiments observed during a 3-month period starting 10 weeks after inoculation: (a) Experiment 1 (2015; 'Braeburn'); (b) Experiment 2 (2016; 'Scilate'); and (c)-(d) Experiment 3 (2017; 'Braeburn' and 'Royal Gala'). For each plot, the inoculated treatment reference for each *Neonectria ditissima* inocula batch (circles, described in Table 2) are shown in parentheses alongside the inoculum age in days and sorted by the average %canker. The deionised water control treatments are shown with an asterisk (none included in Experiment 1). Error bars are 95% confidence limits. Note that the lower confidence limit for 100 is difficult to obtain and is not shown.

of *N. ditissima* spores suspended in Tween® water was not affected by freezing at -20°C for a short period of 3 days and the results of the current study are consistent with this earlier work. However, results from recent work (2018) showed that germination of *N. ditissima* conidia decreased more rapidly (from 84 to 0% in an 8-week period) if they were stored frozen in a mixture of the surfactant Tween® (0.05%) and

water than if stored frozen in distilled water only (unpublished data). In contrast, conidia stored in distilled water only retained their viability (assessed by germination in droplets). A difference in cultivar susceptibility to *N. ditissima* infections is well recorded (Gomez-Cortecero et al. 2016) and a difference in %canker between cultivars was also observed in Experiment 3, with 'Royal Gala' being more susceptible than

'Braeburn'. However, there were no clear trends to explain the cultivar $\times$ treatment interaction mostly driven by treatment numbers 55, 56 and 59. For instance, the germination rate at inoculation was low in these treatments and there was a negative correlation between treatment age and %germination. However, other treatments with a low germination rate, and a greater age (e.g. the oldest treatment, number 52) resulted in 100% infection in both cultivars. In addition, the degree of canker expression was not influenced by inoculum age. Further, while a high spore count appeared to inhibit germination rate in Experiment 1, the relationship between these two variables was weak in Experiment 3 ($r < 0.5$), and spore count did not affect canker expression.

Differences in canker expression may be due, in part, to the 'hit-and-miss' application of spores during inoculation experiments. For example, there may be variation in inoculum droplet size, or spore clumping within the inoculum, or the depth of the rasp wound. All, or any, of these factors would lead to uneven application of spores to each wound. Furthermore, annual and seasonal variation in weather and temperature may be influential. For instance, the duration of a wet period and temperature may affect inoculation success (Xu et al. 1998, Latorre et al. 2002). Indeed, this may have affected Experiment 3, which was undertaken in winter 2017, which was particularly wet in the Tasman region.

Walter et al. (2016) reported that *N. ditissima* infections leading to symptom development may be initiated by just 10-30 conidia on rasp wound in 'Scilate' apple trees. Our data agrees with these results. For example, treatment 62 with 2×10^3 conidia/mL caused >88 %canker. In a 20 μ L inoculation droplet, we would have applied approximately 40 conidia. Dubin & English (1974) showed that 50 conidia were required for leaf scar infections and lesion development in 'Red Delicious' apple trees. Lower spore number increased the incubation period but did not reduce the incidence of canker (Walter et al. 2016). Thus, regardless of any loss of spore viability (assessed by germination in the laboratory in water droplets) due to deep-freeze

storage in our experiments, there remained enough spores in each inoculum treatment to infect the rasp wounds and cause symptoms in situ. Spore viability, as tested by germination in a droplet on a glass slide, was not indicative of pathogenicity potential in the field. This is well demonstrated with treatment 28 and 56 being of the same batch used in Experiment 2 (35 days old; 67 %canker; 3% germination when fresh) and Experiment 3 (388 days old; >44%canker; 3% germination when fresh; 0% germination at inoculation). The lack of correlations among %canker with viable conidium numbers; germination (both fresh and at inoculation) as well as conidium concentration further support this. Currently, to our knowledge, there is no reliable predictor for field pathogenicity, nor do we fully understand the triggers for germination.

In conclusion, conidial suspensions of *N. ditissima* can be frozen in distilled water at -18°C for one year without a significant loss in pathogenicity.

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REFERENCES

- Dubin HJ, English H 1974. Factors affecting apple leaf scar infection by *Nectria galligena* conidia. *Phytopathology* 64: 1201–1203.
- Gomez-Cortecero A, Saville RJ, Scheper RWA, Bowen JK, De Medeiros HA, Kingsnorth J, Xu XM, Harrison RJ 2016. Variation in host and pathogen in the *Neonectria/Malus* interaction; toward an understanding of the genetic basis of resistance to European canker. *Frontiers in Plant Science* 7: 1365.
- 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.
- McCullagh P, Nelder JA 1989. *Generalized Linear Models*. 2nd ed. London, Chapman & Hall. 511+xix p.

- Scheper RWA, Frijters L, Fisher BM, Hedderley DI 2015. Effect of freezing of *Neonectria ditissima* inoculum on its pathogenicity. New Zealand Plant Protection 68: 257-263.
- Walter M, Stevenson OD, Amponsah NT, Scheper RWA, Rainham d, Hornblow C, Kerer U, Dryden G, Latter I, Butler RC 2015. Control of *Neonectria ditissima* with copper based products in New Zealand. New Zealand Plant Protection 68: 241-249.
- Walter M, Roy S, Fisher BM, Mackle L, Amponsah NT, Curnow T, Campbell RE, Braun P, Reineke A, Scheper RWA 2016. How many conidia are required for wound infection of apple plants by *Neonectria ditissima*? New Zealand Plant Protection 69: 238-245.
- 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.