Effect of culture medium, light and air circulation on sporulation of *Neonectria ditissima*

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Abstract In culture, most isolates of *Neonectria ditissima*, the causal organism of European canker, do not produce conidia. Those that do, often produce single-celled conidia rather than the typical multi-celled conidia that are found in nature. A series of experiments was conducted to determine the conditions needed for conidium production. Four culture media (malt extract agar, modified Matsushima's medium (MM), apple sap amended water agar (ASAWA) and water agar), five light regimes, including darkness, near ultra violet (NUV) light and white fluorescent light, and three plate sealing patterns were tested. Subculturing onto MM in sealed plates, exposed to NUV light at 20°C for 3 weeks, resulted in 100% of isolates producing large numbers of viable conidia, of which 71% produced multi-celled conidia. Similarly, under a white fluorescent light/darkness regime, all isolates produced viable conidia on MM and ASAWA, and 100% and 97% of the isolates produced multi-celled conidia, respectively.

Keywords European canker, fungus, conidia, spore production, apple, disease, pathogen, tree.

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). In extreme cases, the main trunk can become girdled, requiring removal of entire trees.

To investigate variation in pathogenicity among strains of *N. ditissima* in different geographical areas, conidia from cultured isolates were required for inoculation. However, on standard potato dextrose agar culture, most isolates do not produce conidia and those that do usually produce single-celled conidia rather than the typical multi-celled conidia that are found in nature. This study sought to determine the culture conditions under which abundant multi-celled conidia could be produced.

MATERIALS AND METHODS

Six experiments were conducted to determine the conditions needed for conidium production. Three culture media, five light regimes and three plate sealing patterns were tested. The culture media were malt extract agar, water agar and Matsushima's medium (Matsushima 1961) as adjusted by Dubin & English (1974).

The light regimes were (1) darkness, (2) a darkness/blacklight blue fluorescent light regime (BLB; providing near ultra violet (NUV) light, 350-390 nm Sylvania F15T8/BLB), (3) continuous NUV light, (4) a white fluorescent light/darkness light regime and (5) continuous white fluorescent light. The plate sealing patterns were sealed, ventilated and non-sealed. The best method was then compared with the apple sap amended water agar method developed by Amponsah et al. (2014).

The various experiments used between four and 34 isolates (Table 1) subcultured onto potato dextrose agar (PDA, Merck) containing ampicillin (50 µg/ml) and streptomycin (100 µg/ml) or PDA. The 1- to 4-week-old PDA cultures were then subcultured (5 mm plug) onto the test media. Each plate was sealed with plastic film (Glad®wrap) and incubated at 20°C at the specified light regime for up to 3 weeks, unless stated otherwise. Plates were positioned 20 cm below the NUV light source in an incubator, unless stated otherwise. After incubation, conidial production was determined by flooding each plate with 2 ml sterile distilled water containing 0.005% Tween®20 (Tween solution) and pipetting the liquid four or five times over the culture. Conidia were enumerated using a haemocytometer (Hawksley counting chamber BN15 8TN, UK). When conidia were present, the different morphologies and sizes of conidia were recorded using a compound microscope at ×100 magnification. In some experiments, conidial germination rates were also determined by placing six drops of each spore suspension (1-2 × 10^5 conidia/ml) on glass microscopy slides. The slides were incubated at 20-22°C for up to 2 days at 100% relative humidity, and the numbers of germinated and non-germinated spores were counted using a compound microscope at ×100 magnification. Conidial morphology was compared with that of conidia collected from apple orchards in Nelson. Suspensions (1-2 × 10^5 conidia/ml) of field produced conidia were made by transferring sporodochia from cankers to Tween solution, using a binocular dissecting microscope and a sterile scalpel. Conidial production was determined using the methods described below.

Experiment 1:
Malt extract agar under NUV light
Four isolates of *N. ditissima* (Table 1), were subcultured onto malt extract agar (MEA, Merck) in 2009, four plates per isolate, and incubated using a modified method (Etebu et al. 2005; W. Molhoek, Wageningen UR, Plant Research International, personal communication). The plates were incubated for 3 weeks below NUV light, with 12:12 h light:dark. Plates were sealed for 2 weeks, but unsealed for the third week.

Experiment 2:
Matsushima's medium (MM) in darkness and under NUV light
Ten isolates (Table 1) were subcultured onto MM in 2010. There were six plates per isolate. Each plate was sealed and incubated at 18°C in the dark for 1 week, after which two plates per isolate were used to determine conidial production.

Two plates per isolate were placed at 20°C in the dark for a further 2 weeks, and in the last week the plates were unsealed. The other two plates per isolate were placed at 20°C, below NUV light at 8:16 h light:dark for 2 weeks, and in the last week the plates were unsealed. Conidial numbers were determined, and when conidia were present, the morphology and size of conidia were recorded.

Experiment 3:
MM under NUV light in ventilated and non-ventilated Petri dishes
Five isolates (Table 1), two plates per isolate, were subcultured on MM in 2010. The plates were sealed and incubated at 20°C in the dark for 1 week, followed by 2 weeks at 20°C, below continuous NUV light. One plate of each isolate was opened twice inside a laminar flow cabinet, when the cultures were 9 and 16 days old. The plates were re-sealed after 30 s. The second plate of each isolate remained sealed. The conidial numbers, morphology and sizes were determined.

Experiment 4:
MM in darkness followed by continuous NUV light
Results of Experiment 3 were further validated using 33 isolates (Table 1) subcultured on MM, two plates per isolate, in 2011. The plates were

Table 1 Isolates of *Neonectria ditissima* collected in New Zealand that were used to test different methods for conidium production in culture in six different experiments. Most isolates, except the two isolates from the ICMP collection and isolate RS62, were single-spore isolates. Isolates that were derived from a single conidium have a "c" after their number, isolates derived from a single ascospore have a "p" after their number. A number after the "c" or "p" indicates that several single-spore isolates were derived from the same sporodochium or perithecium.

Isolate	Location and year of collection	Host	Experiments
ICMP 9472	Pukekohe, Waikato before 2002	apple	1, 2, 4, 5, 6
ICMP 14098	Auckland, before 2006	apple	2, 3, 4, 5, 6
RS 51c	Riwaka, Nelson, 2007	'Scifresh'	1, 2, 3, 4
RS 52c	Riwaka, Nelson, 2007	'Scifresh'	4, 5, 6
RS 62	Riwaka, Nelson, 2008	'Sciearly'	2
RS 101p	Pukekohe, Waikato, 2006	'Royal Gala'	4
RS 110p	Pukekohe, Waikato, 2006	'Royal Gala'	1, 2, 3, 4, 5, 6
RS 117p	Pukekohe, Waikato, 2006	'Royal Gala'	1, 2, 4, 5, 6
RS 123c	Pukekohe, Waikato, 2006	'Royal Gala'	4
RS 129p	Pukekohe, Waikato, 2006	'Royal Gala'	4, 5, 6
RS 132c	Pukekohe, Waikato, 2006	'Royal Gala'	2
RS 132p	Pukekohe, Waikato, 2006	'Royal Gala'	4
RS 138c	Pukekohe, Waikato, 2006	'Braeburn'	4
RS 140p	Pukekohe, Waikato, 2006	'Braeburn'	4
RS 146p	Pukekohe, Waikato, 2006	'Braeburn'	2, 3, 4
RS 300p1	Motueka, Nelson, 2009	'Royal Gala'	4, 5, 6
RS 303p	Upper Moutere, Nelson, 2009	'Royal Gala'	4, 5, 6
RS 304p1	Lower Moutere, Nelson, 2009	'Brookfield'	4, 5, 6
RS 305p	Lower Moutere, Nelson, 2009	'Brookfield'	4, 5, 6
RS 307p1	Lower Moutere, Nelson, 2009	'Brookfield'	4, 5, 6
RS 309c	Riwaka, Nelson, 2009	'Scifresh'	4
RS 318c1	Rukuhia, Waikato, 2009	'Royal Gala'	4, 5, 6
RS 320p	Rukuhia, Waikato, 2009	'Braeburn'	4, 5, 6
RS 321c	Rukuhia, Waikato, 2009	'Pink Lady'	4
RS 322p1	Rukuhia, Waikato, 2009	'Braeburn'	2, 3, 4, 5, 6
RS 322p2	Rukuhia, Waikato, 2009	'Braeburn'	2
RS 323p1	Rukuhia, Waikato, 2009	'Braeburn'	4
RS 324p	New Plymouth, Taranaki, 2009	'Golden Delicious'	4, 5, 6
RS 325c	New Plymouth, Taranaki, 2009	'Galaxy'	4
RS 326c	New Plymouth, Taranaki, 2009	'Galaxy'	4, 5, 6
RS 330p1	Riwaka, Nelson, 2010	'Royal Gala'	4
RS 340p1	New Plymouth, Taranaki, 2010	'Golden Delicious'	4, 5, 6
RS 341c1	New Plymouth, Taranaki, 2010	'Galaxy'	4
RS 345c1	New Plymouth, Taranaki, 2010	'Granny Smith'	4
RS 347c	New Plymouth, Taranaki, 2010	'Pink Lady'	4, 5, 6
RS 370p1	Oratia, Auckland, 2010	apple	4
RS 370p4	Oratia, Auckland, 2010	apple	5, 6
RS 401p1	Upper Moutere, Nelson, 2010	'Braeburn'	5, 6
RS 401p2	Upper Moutere, Nelson, 2010	'Braeburn'	5, 6
RS 402p1	Lower Moutere, Nelson, 2010	'Brookfield'	5, 6
RS 402p2	Lower Moutere, Nelson, 2010	'Brookfield'	5, 6
MW 3c	Riwaka, Nelson, 2013	'Scilate'	5, 6
MW 5c	Riwaka, Nelson, 2013	'Scilate'	5
MW 6c	Riwaka, Nelson, 2013	'Scilate'	5, 6
MW 7c	Riwaka, Nelson, 2013	'Scilate'	5, 6
MW 12c	Riwaka, Nelson, 2013	'Scilate'	5
MW 14c	Riwaka, Nelson, 2013	'Scilate'	5
MW 15c	Riwaka, Nelson, 2013	'Scilate'	5, 6
MW 28c	Riwaka, Nelson, 2013	'Scifresh'	5, 6
MW 29c	Riwaka, Nelson, 2013	'Scifresh'	5, 6
MW 30c	Riwaka, Nelson, 2013	'Scifresh'	5, 6
MW 31c	Riwaka, Nelson, 2013	'Scifresh'	5

sealed and incubated at 20°C in the dark for 1 week, followed by 2 weeks at 20°C under continuous NUV light. The conidial numbers, morphology and sizes were determined and compared with the morphology and sizes of field conidia. Conidial germination rates were also determined.

Experiment 5:
MM and water agar under continuous NUV light and fluorescent light

In 2013, 34 isolates (Table 1) were subcultured onto MM and 1.5% distilled water agar (WA-D, Difco™), two plates per isolate on each medium. The plates were sealed. The MM plates were incubated for 3 weeks at 20°C, under continuous NUV light. The WA plates were placed on the laboratory bench at 22°C under continuous fluorescent ceiling light for 1 month. The conidial numbers, morphology, sizes and germination rates were determined for conidia produced on the MM plates. These were compared with field conidia. On the WA-D plates, the presence/absence of sporodochia-like structures was recorded. Conidial morphology and sizes were determined only for those isolates that produced few conidia on MM or had low conidial germination rates when produced on MM.

Experiment 6:
MM, water agar and apple sap amended water agar under fluorescent light

In 2014, 30 isolates (Table 1) were tested on MM, 1.5% water agar (WA-F, Fisher Scientific, Mexico) and apple sap amended water agar (ASAWA, Amponsah et al. 2014). ASAWA was prepared by adding 10 ml apple sap to 1 litre 1.5% WA-F; sap was obtained from 1-year-old shoots of 'Scilate'/ Envy™ trees, collected in March 2014.

There were two plates per isolate for each medium. Plates were laid out in a completely randomised design. The plates were not sealed and were incubated for 2 weeks at 20°C in an open room, 40 cm below three white fluorescent (PHILIPS TLD 58W/840) lights at 16:8 h light:dark. Conidia were harvested by flooding each plate with 10 ml sterile distilled water and allowed to dehisce by gently shaking for 5 min. The conidial numbers, morphology and sizes from the different media were compared with

field conidia. Conidial germination rates were also determined.

Statistical analysis

Analysis of variance (ANOVA) was used to compare overall differences between isolates and between agar media (adjusted for isolate, excluding field conidia that were not grown on media) to the media × isolate interaction. Percentage data were arc-sine transformed before analysis to stabilise the variance; count data were log-transformed before analysis to stabilise the variance. Fisher's protected LSD ($\alpha = 0.05$) was used to determine statistical differences between media and between isolates. Means presented are on the original scale.

RESULTS
Experiment 1

Of the four *N. ditissima* isolates grown on MEA and placed under NUV light (12:12 h light:dark), isolate ICMP9472 produced abundant conidia, isolate RS117p produced few conidia, and no conidia were found on isolates RS51c and RS110p. Conidia were 1- or 2-celled. The germination rate of conidia from isolate ICMP9472 was 79%. The conidial germination rate of the other isolates was not determined, as too few conidia were produced.

Experiment 2

On MM, six of the 10 isolates produced conidia after 1 week in the dark, but these were mainly 1- and 2-celled (Table 2). Approximately 10^4 conidia were collected from each plate. After 3 weeks in the dark, 70% of the isolates produced 1- and/or 2-celled conidia. Most isolates produced approximately 10^4 conidia per plate, but three isolates produced $2\text{-}5 \times 10^5$ conidia per plate. Compared with plates exposed to continuous dark, exposure to NUV light for 8 h per day increased conidial production and the number of multi-celled conidia; 90% of the isolates produced spores, of which 44% produced multi-celled conidia. Only one isolate produced approximately 10^4 conidia per plate, while the remaining isolates produced $> 10^6$ conidia per plate. Conidia varied in size from 6-15 μm-long, single-celled conidia to 50-55 μm-long, 6-celled conidia (Table 2).

Experiment 3

Under continuous NUV light on MM, all isolates produced conidia (Table 3), including isolate RS110p, which did not produce conidia in Experiment 2. When the plates remained sealed, all isolates produced multi-celled conidia, but when the plates were opened, one of the isolates produced only 1- and 2-celled conidia. Two isolates produced approximately 10^4 conidia per plate, while the remaining isolates produced $> 10^6$ conidia per plate. Conidia varied in size from 9-15 µm-long, single-celled conidia to 55-63 µm-long, 6-celled conidia (Table 3).

Experiment 4

Subculturing *N. ditissima* isolates on to MM in sealed plates at 20°C in the dark for 1 week, followed by exposure to NUV light at 20°C for 2 weeks, resulted in 91% of the isolates producing conidia, of which 27% produced multi-celled conidia, and 20% both 1- and 2-celled conidia. No conidia were observed on isolates RS101p, 138c and 140p. Four isolates (RS123c, 132p, 146p and 324p) produced $2\text{-}4 \times 10^4$ conidia per plate, while the 26 remaining isolates produced $> 10^6$ conidia per plate. Conidia varied in size from 7-10 µm-long, single-celled conidia to 50-55

Table 2 Morphology (expressed as 1, 2, 3, 4, 5 or 6 cells per conidia) for 10 isolates of *Neonectria ditissima* used in Experiment 2. Plates were either grown in the dark or under 8 h NUV light per day. The number of cells is in brackets when only a few conidia with that number of cells were observed. For each morphology group (1, 2, 3, 4, 5 or 6 cells) in the 3-week-old culture under NUV, the average length of conidia was recorded.

Isolate	1-week-old no. of cells	3-week-old (dark) no. of cells	3-week-old culture (NUV) no. of cells	Length (µm)
ICMP 9472	*1, 2, (3)	1, 2	1, 2	10, 20
ICMP 14098	*1, 2	1, 2, (4)	1, 2, (3), 4, 5	10, 20, 30, 38, 51
RS 51c	*1	*1, (2)	1, 2, (4, 6)	10, 17, 35, 50
RS 62	*1, 2	*1, 2	1, 2, 4, 6	12, 23, 45, 55
RS 110p	-	-	-	-
RS 117p	-	1	1	6
RS 132c	-	-	*1	6
RS 146p	*1, (2)	* 1, (2)	1, 2	10, 15
RS 322p1	-	-	1, 2, 3, 4	15, 20, 25, 40
RS 322p2	*1, (2)	* 1, (2)	1, (2)	10, 29

- no conidia were observed.

* approximately 10^4 conidia were collected from each plate, 100 × fewer conidia than the other plates.

Table 3 Morphology (expressed as 1, 2, 3, 4, 5 or 6 cells per conidia) and average length (µm) of conidia for five isolates of *Neonectria ditissima* used in Experiment 3. Plates were either opened twice for 30 s or remained sealed. The number of cells is in brackets when only few conidia with that number of cells were observed. For each morphology group (1, 2, 3, 4, 5 or 6 cells), the length of conidia was recorded.

Isolate	Opened plates no. of cells	Sealed plates no. of cells	Length (µm)
ICMP 14098	1, 2, 4, 6	1, 2, 4, 6	15, 23, 45, 55
RS 51c	1, 2, (4)	1, 2, (4)	12, 18, 40
RS 110p	(1, 2, 4), 6	1, 2, 4, 6	10, 19, 48, 63
RS 146p	*1, (2)	*1, 2, 4, 6	9, 18, 50, 63
RS 322p1	*1, 2, (4, 6)	*1, 2, (4)	9, 21, 47, 60

*approximately 10^4 conidia were collected from each plate, 100 × fewer conidia than the remaining plates.

μm-long, 6-celled conidia. Multi-celled conidia produced by cultures on MM had sizes similar to those of conidia collected from sporodochia on cankers in the field. Germination rates of the conidia from cultures varied between 38% and 98%, compared with 73% of the field conidia.

Experiment 5

When the conditions of Experiment 4 were modified to 3 weeks of exposure to NUV light, all 34 isolates produced conidia, of which 71% produced multi-celled conidia, 21% both 1- and 2-celled conidia, and 9% produced only single-celled conidia (Table 4). Of the field conidia, 15% were 1- or 2-celled, 35% 4-celled and 40% 6-celled.

In 35% of the cultured isolates, more than half of conidia were multi-celled and, therefore, were similar to field conidia.

The five isolates that produced multi-celled conidia in Experiment 4 also produced multi-celled conidia in Experiment 5. Of the five isolates that produced 1- and 2-celled conidia in Experiment 4, four produced multi-celled conidia in Experiment 5. Isolate RS110p produced 1- and 2-celled conidia in both experiments. Similarly, of the eight isolates that produced only single-celled conidia in Experiment 4, four produced multi-celled conidia and three produced 1- and 2-celled conidia in Experiment 5 (Table 4). All isolates produced $> 10^6$ conidia per plate.

Conidia produced by the different isolates varied in size from 6×2 μm to 11×3.5 μm single-celled conidia to 48×4 μm to 63×5 μm 6-celled conidia. The average size of single-celled field conidia was 10×3 μm. The size of the multi-celled conidia produced by the cultures on MM was similar to that of 6-celled field conidia, which had an average size of 58×4.5 μm. Conidial germination rates varied between 10% and 99%, compared with 27% germination of the field conidia. The germination rate of five isolates was between 10% and 25%. These isolates were chosen for further examination of conidial formation on WA-D.

On WA-D, 53% of the isolates produced sporodochia-like structures that were visible by eye (Table 4). Of the isolates that produced sporodochia-like structures on WA-D, 94%

produced multi-celled conidia on MM. Of the isolates that did not produce sporodochia-like structures on WA-D, 44% produced multi-celled conidia on MM. Among the five isolates on WA-D that were examined for conidia, one did not have any sporodochia-like structures, but single-celled conidia were present on the culture. In total, three of the five isolates produced only single-celled conidia on WA-D, although multi-celled conidia were produced on MM. Conidial germination rates varied between 14% and 98%.

Experiment 6

All 30 isolates produced conidia on MM, ASAWA and WA-F at 20°C under a 16:8 h fluorescent light:dark regime. On MM, all isolates produced multi-celled conidia, while 97% and 90% of the isolates produced multi-celled conidia on ASAWA and WA-F, respectively. Of the field conidia, 74-93% of the conidia were multi-celled (Table 5).

Significant differences were observed between isolates, with a range from 1% to 95% of conidia being multi-celled, depending on the isolate. The percentage of multi-celled field conidia differed significantly from that of 16 isolates, but was similar to that of the other 14 isolates (P<0.001 – overall value for difference between isolates; Table 5).

No significant differences between the mean percentage multi-celled conidia on the three media (MM, ASAWA and WA-F) was detected (P=0.116), and on each of the media, approximately half of the isolates (53%, 57% and 50%, respectively) had a similar proportion of multi-celled conidia (> 50%) to the field conidia (Table 5).

The 24 isolates that produced multi-celled conidia on MM under NUV light in Experiment 5 also produced multi-celled conidia on the three culture media in Experiment 6, except isolate RS307p1, which produced only single-celled conidia on WA-F in Experiment 6 (Table 5).

Significantly more conidia, both multi-celled and 1- and 2-celled, were produced on MM than on ASAWA, and more on ASAWA than on WA-F (P<0.001). On MM, all isolates produced $> 10^6$ conidia per plate and 30% of the isolates produced $> 10^7$ conidia per plate. On ASAWA, 87% of the isolates produced $> 10^6$ conidia per plate, but 13% of the isolates produced $4\text{-}9 \times 10^5$

Table 4 Percentage of 1-, 2- and multi-celled conidia produced by 34 isolates of *Neonectria ditissima* grown on modified Matsushima's medium (MM) under continuous NUV light, in Experiment 5. The presence of sporodochia-like structures on water agar (WA-D, Difco) under continuous white fluorescent light was recorded. Also shown are field conidia collected from cankers in Nelson – these were not grown on agar plates.

Isolate	WA-D Sporodochia	MM (NUV)					
		1-celled	2-celled	3-celled	4-celled	5-celled	6-celled
ICMP 14098[1]	N	88	5		5		2
RS 117p[1]	Y	78	5		15		2
RS 129p[1]	Y	85	10		5		
RS 307p1[1]	Y	65	10		5		20
RS 340p1[1]	Y	1	5		10	5	79
ICMP 9472[2]	Y	30	10		45		15
RS 300p1[2]	Y	86	5		2	5	2
RS 303p[2]	N	81	10		2	5	2
RS 324p[2]	Y	5	10		20	25	40
RS 110p[2]	N	95	5				
RS 52c[3]	N	60	20	5	10		5
RS 304p1[3]	Y	93	5		2		
RS 326c[3]	Y	5	30		40		25
RS 347c[3]	Y	20	15		40		25
RS 305p[3]	N	95	5				
RS 318c1[3]	N	98	2				
RS 322p1[3]	N	95	5				
RS 320p1[3]	N	100					
RS 370p4	N	96	2				2
RS 401p1	N	10	10		40	5	35
RS 401p2	N	55	20		15		10
RS 402p1	N	95	5				
RS 402p2	N	100					
MW 3c	Y	99	1				
MW 5c	N	98	2				
MW 6c	N	100					
MW 7c	Y	5	5		25		65
MW 12c	Y	25	20		40		15
MW 14c	N	88	5		5		2
MW 15c	Y	2	3		5		90
MW 28c	Y	30	10		40		20
MW 29c	Y	45	10		40		5
MW 30c	Y	25	10	5	30		30
MW 31c	Y	5	10		25		60
field conidia	-	5	10		35	5	40

[1]Multi-celled conidia were produced in Experiment 4.
[2]Both 1- and 2-celled conidia were produced in Experiment 4.
[3]Only single-celled conidia were produced in Experiment 4.

Table 5 Percentage of multi-celled conidia produced by 30 isolates of *Neonectria ditissima* in three different culture conditions in Experiment 6 (modified Matsushima's medium (MM), apple sap amended water agar (ASAWA) and water agar (WA-F, Fisher) under a 16:8 h fluorescent light:dark regime). The percentage of multi-celled conidia in sporodochia of cankers collected in Nelson orchards is also shown.

Isolate	MM	ASAWA	WA-F	Mean[2]
RS 320p	2	0	1	1.1 a
RS 305p	1	4	0	1.8 a
RS 110p	2	6	0	2.7 a
RS 304p1	8	2	5	4.9 ab
ICMP 14098	14	11	23	16.0 abc
RS 117p	37	11	7	17.9 abc
RS 318c1	24	10	27	20.5 abcd
RS 52c	18	46	7	23.5 abcd
RS 370p4	10	39	24	24.3 abcd
RS 322p1	16	19	50	28.4 bcde
RS 303p	10	51	33	31.5 bcde
RS 402p2	57	43	6	35.5 bcde
RS 129p	22	68	24	38.0 cdef
RS 307p1	48	96	0	48.2 cdefg
RS 402p1	70	40	40	50.3 cdefgh
RS 401p2	63	16	90	56.5 defgh
MW 3c	85	99	6	63.2 efghi
RS 326c	43	89	85	72.3 fghi
RS 300p1	59	94	79	77.7 ghi
RS 340p1	65	82	86	77.9 ghi
RS 401p1	56	95	93	81.4 hi
MW 6c	66	91	93	83.4 hi
RS 324p	75	96	80	83.8 hi
ICMP 9472	64	91	96	83.8 hi
MW 29c	92	79	82	84.2 hi
field conidia[1]	91	74	93	86.0 hi
RS 347c	81	94	100	91.7 i
MW 30c	92	98	88	92.5 i
MW 7c	99	83	96	93.0 i
MW 15c	89	100	93	94.0 i
MW 28c	99	95	91	95.1 i
Mean[3]	49	58	50	

[1]Field conidia were collected from cankers, not grown on agar plates.

[2]Means with the same letter are not significantly different (Fisher's protected LSD, α=0.05; P<0.001 – overall value for ANOVA).

[3]Percentage multi-celled field conidia was not included in the calculation of these means; no significant differences between the means was detected (P=0.116 – overall value for ANOVA).

conidia per plate. However, on WA-F, only 40% of the isolates produced > 10[6] conidia per plate, while 57% of the isolates produced 1-9 × 10^{5} conidia per plate and one isolate only produced 4 × 10^{4} conidia per plate.

The conidial germination rate was significantly higher for conidia produced on MM (84%) or ASAWA (77%) than on WA-F (69%, P=0.002).

Conidial germination rates varied between 47% and 98% for conidia produced on MM, 19% and 98% for conidia from ASAWA, and 0% and 96% for conidia produced on WA-F. The germination rate of the field conidia varied between 89% and 95%.

DISCUSSION

Two culture media, MM and ASAWA, were identified as excellent media to produce large numbers of viable multi-celled conidia for most isolates. MEA was not a good medium as only one isolate produced abundant conidia and these conidia were all 1- and 2-celled. WA-D was not as good a medium as MM and ASAWA because most isolates produced only single-celled conidia, and WA-F was not as good because fewer conidia were produced on the plates, fewer isolates produced multi-celled conidia, and conidia from some isolates grown on WA-F did not germinate.

Successful conidial production on MM was also reported by Dubin & English (1974) who used a single-ascospore isolate of *N. ditissima* (therein named *Nectria galligena*) to produce abundant multi-septate conidia on this agar medium. MM was modified from a medium developed by Matsushima (1961), who reported that many hyphomycetes, including *Fusarium* spp. and *Cylindrocladium* spp., which belong to the same family as *N. ditissima* (nectriaceae), produced large numbers of conidia and less vegetative growth on this agar medium.

ASAWA was developed by Amponsah et al. (2014) as a suitable agar medium for isolation of *N. ditissima* from infected woody apple tissues. Similar to this study, Amponsah et al. (2014) reported that isolates of *N. ditissima* produced numerous conidia on ASAWA that were mostly multi-celled.

The quality of the conidia produced on MM and ASAWA was comparable. On MM marginally more isolates produced multi-celled conidia compared with ASAWA, but on ASAWA, more isolates (57%) had a similar proportion of multi-celled conidia to the field conidia than on MM (53%). However, the synthetic medium MM is more reproducible (Matsushima 1961) than ASAWA, and significantly more conidia were produced on MM than on ASAWA (P<0.001). Therefore, MM is the favoured medium for conidium production.

Similar to the findings of Etebu et al. (2005), Lazarotto et al. (2014) and W. Molhoek (Wageningen UR, Plant Research International, personal communication), this study showed that light is required for abundant production of viable multi-celled conidia. Fewer isolates produced conidia in the dark compared with isolates exposed to NUV light, and isolates that were kept in the dark produced only 1- and 2-celled conidia. NUV exposure increased conidial production and the number of isolates that produced multi-celled conidia. Increasing the duration of continuous NUV exposure from 2 to 3 weeks increased the number of isolates that produced multi-celled conidia from 27% in Experiment 4 to 71% in Experiment 5. Eight of 13 isolates that produced only 1- and/or 2-celled conidia in Experiment 4 produced multi-celled conidia in Experiment 5.

Exposure to white fluorescent light for 16 h per day had an effect similar to that of continuous NUV light on conidial production of the isolates. All isolates produced conidia on all three media, with 90-100% of the isolates producing multi-celled conidia.

When the same 30 isolates were tested on MM, under either continuous NUV light or white fluorescent light for 16 h per day, all isolates produced conidia. However, under NUV, 71% of the isolates produced multi-celled conidia and 33% of the isolates produced conidia similar to field conidia. In contrast, under fluorescent light, 100% of the isolates produced multi-celled conidia and 53% of the isolates produced conidia similar to field conidia. Therefore, the conidia were of higher quality when produced under a 16:8 h fluorescent light:dark regime. The quantity of conidia was not affected. Similar numbers of spores were produced under both light regimes, with all isolates producing $> 10^6$ conidia per plate. These results differ from Etebu et al. (2005) who reported that sporulation of *Mycosphaerella fijiensis* was significantly higher under NUV light than white light. However, Lazarotto et al. (2014) reported that *Fusarium chlamydosporum* sporulated well in culture under white light, and Dubin & English (1974) reported that a single ascospore isolate of *N. ditissima* produced abundant, pathogenic, multi-septate conidia under Sylvania Gro-Lux fluorescent lights, confirming that normal fluorescent light is suitable for conidium production of *N. ditissima*.

Isolate RS110p produced $>10^6$ multi-celled conidia per plate when exposed to continuous

NUV light for 2 weeks in Experiment 3. However, this isolate subsequently produced only 1- and 2-celled conidia in Experiments 4 and 5. This isolate may either have mutated over time in culture (Waite & Stover 1960) or a sector of the colony was subcultured instead of the original isolate. Sectoring is common in many isolates of *N. ditissima*, and Xu et al. (1992) described sectors of *Fusarium graminearum* in culture that were characterised by fewer conidia than the parent isolate. Therefore, the possibility that some sectors produce conidia that are 1- or 2-celled instead of multi-celled should be examined.

In contrast to the findings of Etebu et al. (2005), in this series of experiments it was found that increasing air circulation by unsealing the plates did not increase conidial production. In contrast, when the plates remained sealed, all isolates produced multi-celled conidia, but when the plates were opened, one of the isolates did not. However, the plates in Experiment 6 were unsealed and numerous, multi-celled, viable conidia were produced on these plates. These plates were placed under a light bank in an open room, whereas the MM plates in all other trials (except in Experiment 6) were inside an incubator with a fan. Unsealed plates in the incubator were at risk of drying out, while those on the bench were not.

In the various experiments the single-celled conidia produced on the different media were 6-15 × 2-3.5 µm and the 6-celled conidia were 48-63 × 4-5 µm. These measurements were similar to those of the field conidia, where average size of single-celled conidia was 10 × 3 µm, and the average size of 6-celled field conidia was 58 × 4.5 µm. Booth (1967) reported that aseptate micro-conidia of *N. ditissima* (therein named *Nectria galligena*), were 4-8 × 2-3 µm. However, in the present study larger single-celled conidia were observed. The size Booth (1967) reported for conidia with four or more septae (45-65 × 4-7 µm) was very similar to the size of the 6-celled conidia found in culture and in field conidia in the present study.

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