Pre-infection processes of Botryosphaeriaceae species conidia

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The Botryosphaeriaceae species are important wound pathogens on grapevines, causing dieback, cankers and ultimately death of plants. This research programme investigated an unexplored aspect of the disease cycle, namely behaviour of conidia during the pre-infection stages. Adhesion properties of conidia were investigated for three species of Botryosphaeriaceae*, Botryosphaeria dothidea, Neofusicoccum luteum* and *N. parvum*. The surfaces chosen had different affinities for water, with cellulose being extremely hydrophilic, glass moderately hydrophilic, polystyrene moderately hydrophobic and parafilm (polyolefins + paraffin waxes) extremely hydrophobic. Results showed that surface wettability did not play a major role in adhesion of the conidia. However, isolates differed in their conidial adhesion properties. For *N. luteum* isolate MM558, *B. dothidea* isolate 007 and *N. parvum* isolate G652, maximum mean percent adhesion on any surface was reached after 5 min (53.1%, 54.0% and 50.6%, respectively) and for *N. luteum* isolate CC445 after 20 min (61.4%). Overall, fewer conidia of the *N. luteum* isolates adhered to cellulose than to the other surfaces, namely glass, polystyrene and parafilm. Fewest conidia of *B. dothidea* 007 adhered to parafilm and fewest conidia of *N. parvum* G652 adhered to parafilm and polystyrene. Mean percent germination for isolates MM558, CC445, G652 and 007 on the same surfaces was highest on cellulose (72.2%, 76.9%, 52.4% and 74.9%, respectively) but did not differ on the other surfaces tested. Germ tube growth for all isolates was least on cellulose; initial cellulose agar assays indicated that germ tubes might be utilising the cellulose as a food source. Germination assays on surfaces of different hardness showed no discernible patterns of germination or growth related to hardness. Further, isolate MM558 germinated equally well when continuously shaken or when in contact with a surface.

Treatment of conidia of isolate MM558 with a protease (pronase E) completely prevented adhesion, indicating that spore surface proteins play a part in adhesion. Treatment with cycloheximide, a protein biosynthesis inhibitor, reduced mean percent adhesion (25.1%) in comparison to the untreated control (72.0%) and heat treatment (110^{o}C for 10 min) reduced

the mean percent adhesion (28.0%) in comparison to the non-heated control (85.8%), further indicating a role for proteins in adhesion. Coomassie brilliant blue staining after incubation times conducive to germination was positive for all isolates; a blue 'halo', which indicated proteins, was often seen surrounding the conidia or near the germ tube emergence points. This stained matrix material also stained positive for alcian blue (mucopolysaccharides). Since this material was not seen during shorter incubation times, its production appeared to represent a second phase of the adhesion process.

Investigations into the volatiles produced upon disruption of grapevine tissues showed increases in volatile compounds known to be produced from wounded tissues. The volatiles produced after wounding stems plus leaves were collected and 13 compounds identified. Of these, nine compounds were emitted in significantly higher amounts upon wounding, with cis-3-hexenal, cis-3-hexen-1-ol and cis-3-henenyl acetate being released at the highest rates (111.52, 96.95 and 46.09 ng/gfw/h, respectively). Collection of volatiles from stems only also identified 13 compounds of which nine were emitted in significantly higher amounts upon wounding, with 2-hexenal emitted at the highest rate (48.74 ng/gfw/h).

Exposure of isolate MM558 conidia to volatiles released directly from wounded plant tissue caused a slight reduction in germination (82.9%) and in germ tube growth (33.4 μm) in comparison to the control (85.9% and 41.6 μm). Germination of isolate CC445 was unaffected by exposure to fresh plant volatiles, while mean germ tube growth was 7.3 μm less than for the control (33.8 μm). However, isolate G652 was unaffected. Exposure to wounded stem plus leaf tissue did not affect the germination of isolate MM558 conidia however germ tube growth was reduced by almost 50%. The trapped, eluted and stored liquid volatile mix had little effect on the germination and growth of isolate MM558 conidia. Exposure to 100 μL of some pure volatile compounds reduced germination of isolates MM558 and G652, with almost complete inhibition by trans-2-hexenal and cis-3-hexenyl acetate. Microscope observations of germination indicated a chemotropic response to wounds but attempts to measure the attraction to volatile sources were unsuccessful.

The new information in this thesis has shown non-specific adhesion and germination processes for conidia of some Botryosphaeriaceae species and indicated potential for further research directions which may result in innovative control methods.

Keywords: Adhesion, germination, hydrophobicity, hydrophilicity, metabolic inhibitors, wounding, volatiles

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Chapter 1

Introduction

1.1 Grapevine industry in New Zealand

The history of winemaking in New Zealand is a relatively short one, with the first record of grapevine plantings being made about 200 years ago in 1819 by Samuel Marsden, an Anglican missionary who planted about 100 vines in the Bay of Islands (Cooper, 2008). Since these early plantings the industry has slowly evolved to its current state, in which the area planted in grapes has reached about 35,182 hectares spread over 2,054 vineyards (New Zealand Winegrowers, 2013). These vineyards range in size from under 5 to over 50 ha and are spread out over ten main growing regions throughout the country (Table 1) (New Zealand Winegrowers, 2013). The growth of the New Zealand wine industry has largely been driven by the success of Marlborough Sauvignon Blanc, recognised around the world as having a distinctive character (Overton & Heitger, 2008). Not surprisingly, this has led to Marlborough being the largest wine growing region, comprising 64.9% of the national grape producing area and growing about 73% of the country's Sauvignon Blanc (New Zealand Winegrowers, 2013).

Although accounting for only a small share of the global wine production market (< 1%), New Zealand ranks tenth in the world for exports by value and eleventh for exports by volume, with France being the only country whose wines are sold at a higher average price (New Zealand Wine, 2012). New Zealand Sauvignon Blanc wines have unique flavour characters and intensity found only in cool climate viticulture (Wilson & Goddard, 2004), defined as a mean temperature of 15°C or less during the month before harvest (Jackson, 2001b). In 2012, grapes were the seventh most valuable commodity produced in New Zealand (194.3 million "international dollars"[1]), with kiwifruit being the only horticultural product of greater value (FAOSTAT, 2012).

[1] Uniform standard quoted by FAOSTAT allowing a single price to be assigned to each commodity and country.

Table 1.1 Number of vineyards by region and producing area size (adapted from New Zealand Winegrowers, 2013).

Region	Producing area (hectares)				
	0-5	**5.01 - 10**	**10.01 - 20**	**20.01 - 50**	**≥ 50.00**
Auckland/Northland	90	14	4	0	1
Canterbury	26	7	2	0	1
Gisborne	26	33	24	12	7
Hawkes Bay	81	67	64	32	22
Marlborough	203	315	214	182	101
Nelson	5	36	17	7	3
Otago	100	67	28	17	3
Waikato/Bay of Plenty/other	9	1	-	-	-
Waipara	23	21	9	8	4
Wairarapa	78	17	10	10	3
Totals	691	578	372	268	145

1.2 Cool climate viticulture

Cool climates are less forgiving than warm climates and thus viticulturists growing grapes in cooler conditions require more skill to produce grapes of high quality (Jackson, 2001b). The shorter growing season means that grapes have less time to ripen and therefore growers often select grape varieties for their ability to ripen early. Grapes grown in warm climates tend to have more fruitful buds, resulting in higher yields, and even in a cool season it is likely that ripening will still occur. In cool climates, variation between vintages can be large, generally due to cooler than average conditions resulting in grapes that have not ripened properly. Grapes grown in warm climates tend to have a high sugar content, increasing the level of alcohol in the subsequent wine produced, which results in a wine with more body.

However, these grapes tend to have low acidity and high pH levels caused by the warm conditions and the resultant juice is often unbalanced. In cool climates, grapes tend to have lower sugar levels, even in a good season, and acid levels can be high. Consequently, the wine contains lower alcohol and is light bodied, but the higher acidity is said to impart a fresh sense to the wine and the pH rarely needs to be adjusted (Jackson, 2001b).

The later ripening of grapes grown in cool climates means that careful consideration needs to be given to training and pruning practices as these can have a dramatic effect on maturity of berries (Jackson, 2001a). Vines need to be trained to ensure the canopy does not become congested and shade fruit; this could result in lower temperatures surrounding the bunch and thus cause late ripening. In addition, flower initiation requires high levels of light in the previous season so trellis systems need to be designed to encourage adequate penetration of light. Furthermore, care should be taken not to leave too many buds at pruning as this could encourage higher yield, another factor that can delay maturity (Jackson, 2001a).

Grapevines in New Zealand are usually trained onto a post and wire trellis system, on which the wires run vertically along the row (Jackson, 2001a). The base wires support the canes or cordons and foliage wires support the current season's shoots. The vertical shoot position trellis is the most common in New Zealand and in many other cool climate viticultural areas. It is well adapted to mechanisation and allows sprays to be directed onto the fruit only or onto the whole canopy. Pruning is performed in winter, with about 90% of the previous season's growth being removed. Shoot trimming (top and side) is performed two to four times a year (depending on how vigorous the growth is) to improve light penetration and aeration (Jackson, 2001a).

While pruning and trimming are essential practices that aim to improve yield by balancing the amount of intercepted light with an adequate leaf area, the wounds created from these practices can act as entry points for pathogens such as those that cause trunk diseases. Trunk diseases are responsible for significant economic losses to the worldwide wine industry (Gubler et al., 2010) and although these diseases are often associated with a number of different fungal species, Botryosphaeriaceae species are gaining recognition as important pathogens.

1.3 Botryosphaeria dieback

Trunk diseases attributed to Botryosphaeriaceae species have often been referred to as 'black dead arm' or 'Botryosphaeria canker' (Úrbez-Torres, 2011). Black dead arm was originally described in Hungary by Lehoczky (1974) and attributed to *Diplodia mutila* (Fr.) Mont., with symptoms described as leaf chlorosis and wilting, with black streaking of the wood in the xylem. Botryosphaeria canker was first named in a vineyard study in Southern California and attributed to disease symptoms caused by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Leavitt, 1990). The characteristic pie or wedge-shaped trunk cankers were similar to those produced by *Eutypa lata* but the leaf symptoms that characteristically develop with *E. lata* infections were absent. Since these original descriptions there seems to have been some confusion regarding reports of these diseases, with overlap often recorded in the described symptoms. As such, 'Botryosphaeria dieback' has been suggested to encompass the broad range of symptoms (Úrbez-Torres, 2011). The range of disease symptoms now known to be associated with Botryosphaeriaceae spp. on grapevines include dieback, wood discolouration, bud mortality, cankers, fruit rots and leaf spots (Úrbez-Torres, 2011). The effect of these symptoms on the New Zealand wine industry is as yet unknown. However, in California 'bot canker' caused by Botryosphaeriaceae species (in conjunction with Eutypa dieback) is estimated to cause economic losses to the industry of about $260 million each year (Siebert 2001, as cited in Úrbez-Torres et al., 2006b).

Species of fungi within the Botryosphaeriaceae family have a wide distribution, being found in most countries throughout the world on a wide variety of plant hosts (Slippers & Wingfield, 2007). Their importance as trunk diseases of grapevines has largely been overlooked in the past, partly due to them being considered as either saprophytic, endophytic, parasitic or weak pathogens on grapevines (Phillips, 2002). In addition, disease symptoms caused by these pathogens may have gone unnoticed due to difficulties in separating them from symptoms caused by other trunk diseases, such as Eutypa dieback and esca (Úrbez-Torres, 2011). The long incubation period between vine infection and symptom appearance is a further complicating factor for diseases such as esca and Botryosphaeria dieback (Block et al., 2013). This is believed to be one reason why apparently healthy young vines develop disease symptoms as they age. In New Zealand, Botryosphaeriaceae spp. infection levels were found to be higher in older vineyards (> 6 years old), with multiple

species far more likely to be found in single vines 21 years of age or older (Baskarathevan et al., 2012).

Within the last decade Botryosphaeriaceae fungi have been recognised as significant pathogens of grapevines, largely due to a number of surveys conducted in vineyards worldwide (Phillips, 1998; Castillo-Pando et al., 2001; Taylor et al., 2005; Úrbez-Torres et al., 2006a; Úrbez-Torres et al., 2006b; Pitt et al., 2010; Correia et al., 2013; Diaz et al., 2013; Kaliterna et al., 2013; Yan et al., 2013; Chebil et al., 2014). In New Zealand, a survey of symptomatic plant material in vineyards from six of the main grape growing regions has identified the presence of nine species of Botryosphaeriaceae which are widespread, namely *Diplodia seriata* De Not., *D. mutila* (Fr.) Mont., *Dothiorella sarmentorum* (Fr.), *Do. iberica* A.J.L Phillips, Luque & Alves, *Neofusicoccum parvum* (Pennycook & Samuels)*, N. luteum* (Pennycook & Samuels)*, N. australe* (Slippers, Crous & M.J. Wingf.), *N. ribis* (Slippers, Crous & M.J. Wingf.), and *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not. (Baskarathevan et al*.,* 2012). A survey of nine commercial grapevine nurseries throughout New Zealand confirmed the presence of six of these same species and showed them to be pathogenic on grapevines (Billones-Baaijens et al., 2013b).

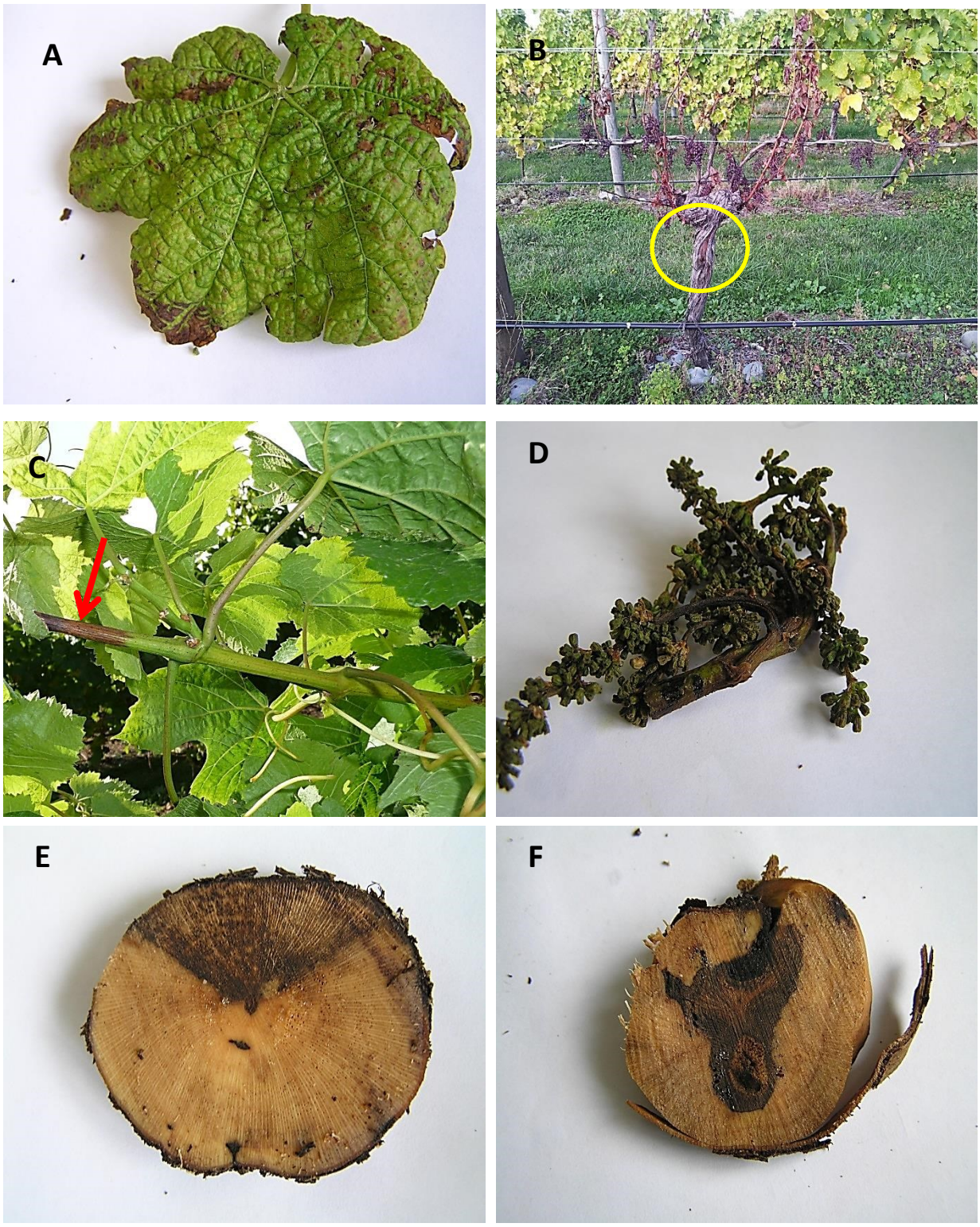

Figure 1.1 Symptoms of Botryosphaeriaceae spp. infection on grapevines. A) leaf spots; B) trunk canker; C) dieback from pruning wound; D) flower infection; E) and F) wood staining (Photographs by Marlene Jaspers).

1.4 Epidemiology

Studies have shown that 21 Botryosphaeriaceae species occur in vineyards across the world (Úrbez-Torres, 2011). The large number of species associated with grapevine trunk diseases in different countries, in conjunction with the wide range of environmental conditions in which they have been found, has complicated epidemiological studies. More recently, progress has been made in explaining the epidemiology of Botryosphaeriaceae spp. that cause disease in grapevines such as inoculum sources, environmental factors favouring the release, dispersal and germination of spores (including patterns of spore release), and the susceptibility of pruning wounds to infection (Amponsah et al., 2009; Serra et al., 2009; Úrbez-Torres et al., 2010a; van Niekerk et al., 2010; Amponsah et al., 2011; Úrbez-Torres & Gubler, 2011; Amponsah et al., 2012a).

1.4.1 Inoculum sources

Pycnidia appear to be the main overwintering structures of Botryosphaeriaceae pathogens and are commonly found in the diseased wood of vines as well as in pruning debris on the vineyard floor (van Niekerk et al., 2004; Úrbez-Torres et al., 2006b; van Niekerk et al., 2010). Although inoculum on vines and/or pruning debris is often thought to be one of the primary inoculum sources, it is also likely that propagation material plays an important role as an inoculum source. A study of commercial grapevine nurseries in Spain showed that species of Botryosphaeriaceae were isolated from rootstock cuttings taken from five of the six main production stages (Giménez-Jaime et al., 2006). Billones-Baaijens et al. (2013b) found evidence of Botryosphaeriaceae species in asymptomatic plant material received from grapevine nurseries around New Zealand. Samples received from nurseries included rootstock cuttings, scion cuttings and grafted plants (failed and healthy). In a further investigation to determine inoculum sources in New Zealand grapevine nurseries, Botryosphaeriaceous DNA was found on grafting tools, in callusing media and in hydration tanks (Billones-Baaijens et al., 2013c). However, overall results suggested that the main source of inoculum was from canes grown in the mother-vine blocks. A study of mother-vine blocks in South Africa also confirmed the presence of Botryosphaeriaceae spp. in both the pruning wounds and basal ends of two year old stubs (Fourie & Halleen, 2004). Similarly, a survey in Spain of 14 rootstock mother fields isolated a number of different Botryosphaeriaceae spp. from the basal ends of shoots (Aroca et al., 2010).

In addition to sources of inoculum from grapevines, inoculum may be produced by many other woody plants that are also hosts of Botryosphaeriaceae species. In New Zealand, Botryosphaeriaceae spp. were isolated from a number of non-grapevine hosts close to vineyards, such as blueberry, olive, pine, cherry, apple, plum, broom, oak and willow and these isolates were shown to be equally pathogenic on grapevines (Amponsah et al., 2011). In addition, *N. macroclavatum*, a species first reported on Eucalyptus in Australia (Burgess et al., 2005) has been reported in New Zealand and shown to be pathogenic on grapevines (Billones et al., 2010). An isolate of *N. ribis* from a *Ribes* species has also been shown to be virulent on grapevines (van Niekerk et al., 2004). While many non-grapevine hosts have the potential to provide inoculum for vineyard infection, efficiency of dispersal would depend on the distance between hosts.

1.4.2 Spore release and dispersal

Botryosphaeriaceae species conidia are exuded from pycnidia in 'gelatinous matrices' in the presence of high moisture (Úrbez-Torres, 2011). Dispersal over relatively short distances is then thought to be by rain splash (Úrbez-Torres et al., 2010a; Baskarathevan et al., 2013). In South Africa, van Niekirk et al. (2010) found that as little as 0.25 mm of rain, or humidity levels of 70% or above were able to promote spore release. Higher levels of spore release were found during periods of higher rainfall. They noted that spore release may be linked to a more complex set of environmental variables, with temperature also having an impact. Spore trapping studies performed in California with slides coated in petroleum jelly and attached to cordons showed conidia from Botryosphaeriaceae spp. were discharged from September to April, with release coinciding with rainfall events (Úrbez-Torres et al., 2010a). They also trapped conidia of Botryosphaeriaceae spp. with a Burkard trap, which draws air over a sticky tape and is designed to trap air-borne spores. This trap captured spores during rainfall but rarely two hours or more after a rain event. These findings suggest that spores were not wind dispersed *per se* but happened to be in the air due to being splashed by water. In extremely dry areas in California (<100 mm rain per year) spore release was related to the use of overhead irrigation. In contrast, when overhead irrigation was used as frost protection and the temperature was close to 0°C, few Botryosphaeriaceae spp. spores were captured (Úrbez-Torres et al., 2010a). In New Zealand, spores were trapped in rainwater run off but not on Vaseline® coated slides, which were designed to trap airborne spores

(Amponsah et al., 2009). The seasonal pattern of *Diplodia* spp. spore release in Alsace, France also coincided with rainfall events, with the rainy season occurring from spring through to autumn (Kuntzmann et al., 2009). In contrast to these findings, spore release events in a vineyard in Canterbury, New Zealand did not appear to have a seasonal pattern, possibly because rainfall was evenly distributed throughout the year. However, higher numbers of spores were trapped in rainfall traps in summer and early autumn, when temperatures were highest and the vines carried a large canopy of shoots (Amponsah et al., 2009).

1.4.3 Infection of pruning wounds

Pruning wounds appear to be the main points of entry for Botryosphaeriaceae species, with time of pruning and wound age considered important factors in the infection process. In South Africa, pruning wounds made in mid- and late winter were shown to be susceptible to infection for three weeks, although susceptibility declined as the wounds aged (van Niekerk et al., 2011). It was also suggested that warmer temperatures during and after pruning can speed the wound healing process and thus lessen the susceptibility window of wounds. In Italy, Serra et al. (2009) found that spurs remained susceptible to infection for up to four months when pruned in either January, February or March. In California, studies with vines pruned from November to March (dormant season) demonstrated that susceptibility to infection was significantly lower when pruning was performed in early March and highest when vines were pruned in early winter (December and January) (Úrbez-Torres & Gubler, 2011). Overall, the susceptible period decreased as the time between pruning and inoculation increased. Wounds remained susceptible to infection for up to 12 weeks when pruned early (November-January), while the duration of wound susceptibility in February and March was not ascertained. In northeast Spain, a study performed on the natural infection rates of trunk pathogens demonstrated that infection rates by *Diplodia seriata* were higher when vines were pruned in winter (February) than in autumn (November) (Luque et al., 2014).

Although progress has been made in explaining the epidemiology of species of Botryosphaeriaceae, the complete disease cycle of germination, infection, sporulation, dispersal and re-infection of grapevines by pathogenic species has not as yet been fully elucidated. For instance, infection by Botryosphaeriaceae spp. is thought to occur through

wounds and openings in the vine framework (Úrbez-Torres, 2011), but it is not clear whether these pathogens use wounds as simple entry points into the vine or whether wounding provides a chemotropic signal towards which fungi can grow. In addition, pathogenicity studies performed on other woody hosts such as peach, pistachio and apple trees have demonstrated that these pathogens can also infect through natural openings such as stomata and lenticels or even penetrate host tissue directly (Michailides, 1991; Pusey, 1993; Kim et al., 1999). Amponsah et al. (2011) also demonstrated that infection could occur on non-wounded buds and Wunderlich et al. (2011) isolated nine different species of Botryosphaeriaceae from dormant buds, flowers and berries (immature and mature). More recent experiments have shown that flower infection leads to blight and non-wounded grape berries can become rotted after infection with *N. luteum* conidia at development stages from pea-size (~5 mm, green and hard) to fully ripe (M. Jaspers, personal communication, 2014). These studies indicate that processes of infection by Botryosphaeriaceae spp. conidia may be more tissue-specific and complex than originally indicated by the wound pathogen model.

1.5 Early infection events of plant pathogens

When spores of plant pathogenic fungi are released from their source they enter a hostile environment in which they are unlikely to survive and only a small proportion arrive on a suitable host that can support growth. Spores may be dispersed by wind over long distances or by water over shorter distances, but they need to land on a susceptible host and specifically onto plant tissue that can become infected.

The plant pathogen infection process is comprised of a number of distinct phases, namely pre-penetration, penetration and post-penetration (colonisation) (Guest & Brown, 1997). During the 'pre-entry' phase, initial contact is made between the pathogen and potential host (inoculation). Other developmental phases that occur at the pre-penetration stage include spore germination, germ tube growth and, if applicable, the formation of infection structures.

Spores of many plant pathogenic fungi become attached to their host prior to or during germination processes. Attachment to a plant host confers many advantages to the pathogenic fungus (Epstein & Nicholson, 2006); it prevents spores being displaced by wind

and/or water and thus facilitates germination on the correct host. Upon attachment, the contact surface area is increased, maximising reception of signals from the host. Some fungi, such as *Uromyces appendiculatus* receive physical cues from the leaf in order to grow towards stomata (Hoch et al., 1987). This thigmotropic growth response requires surface contact in order to recognise the ridges on the leaf surface. Other fungi, such as *Colletotrichum graminicola* require surface contact in order to form appressoria. Apoga et al. (2004) reported that germ tubes required at least 4 μm of continuous contact before appressorium development was initiated. Since appressoria are specialised cells that produce penetration pegs to mechanically penetrate host tissue (Howard et al., 1991), it is surmised that firm attachment is required for these infection structures to function properly.

It has been demonstrated that conidia of *N. luteum* start to adhere to dormant one-year-old grapevine cuttings immediately after inoculation (Billones-Baaijens, 2011). Of the estimated 10,000 conidia inoculated onto one composite sample, the mean number of conidia retrieved in the wash water was 862.5 for the 0 min incubation treatment. Further decreases in the numbers of conidia retrieved occurred over time, with the mean number of conidia after 4 hours incubation being 106.2. The mechanism that spores of Botryosphaeriaceae spp. use to adhere to the surfaces of grapevine plant material is as yet unknown.

1.5.1 Attachment of spores to plant surfaces

The conditions plant pathogenic spores encounter when they land on the plant surface can ultimately determine whether attachment and subsequent germination is successful (Tucker & Talbot, 2001). Wind, rain and temperature can all affect spore displacement (Jones, 1994). In addition, the surface potential of microorganisms tends to be negative, as does that of many host surfaces, and this creates an electrostatic repulsion barrier when the two surfaces approach each other. However, cell surfaces may carry domains that are positively charged, mediating electrostatic attraction locally despite overall repulsion (Vadillo-Rodríguez et al., 2003). Opposing this repulsion barrier are van der Waal's attraction forces, as well as hydrogen and hydrophobic bonding (Douglas 1987, as cited in Jones, 1994). It is the net result of many of these forces combined that will ultimately play a part in the adhesion of spores to surfaces (Kuo & Hoch, 1996). Electrostatic repulsion and van der Waal's forces tend to operate at close distances of 1 to 10 nm, while hydrophobic interactions influence the adhesion of spores at greater distances from the surface (Kuo & Hoch, 1996). These

physiochemical aspects of adhesion have been well studied in bacteria but not in fungi. As such, the relevance of electrostatic interactions in the fungal adhesion process has been questioned with regard to the larger propagules involved (Jones, 1994). In addition, fungal spores may be sticky or carry surface appendages that aid in the adhesion process and the effects of such physical characteristics on the attraction and repulsion forces is unknown. In view of the many potential mechanisms by which attachment can occur, adhesion mechanisms for fungi have often been characterised as being either passive or active.

Passive mechanisms for spore attachment

Passive attachment of spores is often mediated by appendages and/or preformed sticky substances or may occur due to the physical characteristics of a surface (Jones, 1994). In *Cyclaneusma minus,* which causes needle cast in pine trees, polar appendages on the ascospores seem to become trapped on the irregular surface of the host. Many marine and freshwater fungi (e.g. *Trichomaris* spp. and *Aniptodera* spp.) have "hamate"-like appendages that uncoil to form long threads in water (Jones, 1994). These "viscous threads" may be sticky and wrap around the target host. Spores of *Dryosphaera navigans* have appendages that are coiled around the spore wall (Koch & Jones, 1989). Upon maturity, the thread-like appendages uncoil, each thread terminating in a pad of mucilage at the distal end; these sticky pads help spores adhere to various surfaces. Conidia of *Dilophospora alopecuri* have "finger-like setulose appendages" surrounded by a matrix material (Bird & McKay, 1987) that helps to attach the conidia to the cuticles of nematodes.

The rapid adhesion of *Stagonospora nodorum* spores is thought to be mediated by pre-formed cell surface glycoproteins, with spores also passively releasing a matrix material that may enhance adhesion (Newey et al., 2007). Pre-formed glycoproteins in the spore coat of *Colletotrichum lindemuthianum* conidia also mediate rapid attachment of spores to substrata (Hughes et al., 1999). Immediate attachment of pycnidiospores (conidia) of *Phyllosticta ampelicida* to surfaces is mediated by a 'sheath' surrounding the spores (Shaw & Hoch, 1999). Removal of the sheath by enzymatic digestion prevented the attachment of spores to surfaces. It has also been suggested that hydrophobic interactions maintain the adhesion of the spore to a surface until an adhesive material is actively released close to the time of germination. Clement et al. (1994) suggested that the spines on *Uromyces viciae-fabae* urediniospores were covered with a hydrophobic lipid sheath that might promote

adhesion through hydrophobic interactions with the leaf cuticle. Hydrophobic interactions were also thought to be involved in the attachment of spores of other plant pathogenic fungi such as *Colletotrichum musae* (Sela-Buurlage et al., 1991), *C. graminicola* (Mercure et al., 1994; Chaky et al., 2001), *C. lindemuthianum* (Rawlings et al., 2007), *Ventura inæqualis* (Schumacher et al., 2008) and *Penicillium expansum* (Amiri et al., 2005), which all showed improved adhesion to substrates with low wettability. Conidia of *Stagonospora nodorum* also adhered most strongly on hydrophobic surfaces, but adhesion rates seemed to be influenced by texture (Zelinger et al., 2006). The number of conidia (1,225) remaining attached to Teflon after washing was markedly higher than the number attached to polycarbonate (467), even though these two surfaces had similar contact angles (100.1^{o} and 100.7^{o}, respectively).

Conidia of the rice blast fungus *Magnaporthe grisea* exude mucilage immediately upon hydration, which anchors these spores to the plant surface by their apices (Hamer et al., 1988). This mucilage is preformed and appears to be located in a periplasmic space close to the spore tip. Adhesion of *Phytophthora cinnamomi* zoospores to plant roots occurs via the secretion of glycoproteins from small vesicles located in the periphery of cells (Gubler & Hardham, 1988). This process occurs during encystment, with the surface of most cells covered with adhesive material within 1 min.

Active mechanisms for spore attachment

Active attachment involves metabolic processes and thus is thought to involve any adhesive material being released over a relatively long period of time (Kuo & Hoch, 1996). Experiments performed with *Cochliobolus heterostrophus* demonstrated that conidia began adhering to glass slides after about 20 min, whereas conidia treated with metabolic inhibitors such as sodium azide did not attach to surfaces (Braun & Howard, 1994). Treatment with metabolic inhibitors sodium azide and cycloheximide also inhibited the attachment of *Nectria haematococca* microconidia, indicating that respiration and protein synthesis are required for the adhesion of these spores (Jones & Epstein, 1989). Cycloheximide treatment also reduced the adhesion of *C. graminicola* conidia by 50%, with maximum adhesion of untreated conidia reached after 30 min (Mercure et al., 1994). Conidia of *C. musae* may also require active metabolism for adhesion to occur, as treatments that killed conidia (such as UV, heat or formaldehyde) reduced the rate of adhesion (0%, 56%

and 27%, respectively) in comparison to the untreated controls (85-97%) (Sela-Buurlage et al., 1991).

Attachment mechanisms of some spores involve both passive and active stages, an example being the conidia of *Botrytis cinerea* (Doss et al., 1995). The first stage of attachment occurs when conidia are hydrated and involves hydrophobic interactions considered to be relatively weak. The second stage of adhesion involves the release of a physical film attaching the germlings strongly to surfaces. However, the adhesive appeared to be released by the germ tubes and appressoria, not by the conidia. The urediniospores of *Uromyces viciae-fabae* also appear to go through different stages of attachment (Clement et al., 1993). Attachment of dry spores is thought to be mediated by hydrophobic interactions between spore surface lipids and the target surface. This is followed by passive release of extracellular material once the spores imbibe water. A further release of adhesive material after 40 to 120 min increases adhesion rates and this correlates to germ tube emergence and growth.

Adhesive materials

Adhesion to plant surfaces is common among species of fungi but between species there appears to be variation in the composition of the materials associated with adhesion and the stimuli which induce adhesiveness (Epstein & Nicholson, 1997). In *Magnaporthe grisea*, hydration of the conidia promoted release of a pre-formed adhesive (Hamer et al., 1988). In contrast, macroconidia of *Nectria haematococca* remained non-adherent when hydrated and apparently required active metabolic processes before becoming adhesive (Jones & Epstein, 1990). Variation is also seen in the developmental stage(s) at which adhesion occurs, with different adhesive materials being associated with different fungal propagules such as conidia, zoospores (and their cysts), germlings, appressoria and infection cushions (Nicholson, 1984) . In some fungi, such as *Phytophthora cinnamomi*, adhesiveness is a transient event, occurring just after the induction of encystment and lasting less than 4 min (Gubler et al., 1989). In contrast, conidia of *C. musae* can adhere for several hours before germination occurs (Sela-Buurlage et al., 1991).

The visibility of adhesive matrices also differs between fungi. The mucilage released from the apex of *M. grisea* conidia can be readily examined under a light microscope (Hamer et al., 1988), while the matrix material associated with the propagules of the rust fungus *Gymnosporangium juniper-virginianae* is only visible with an electron microscope (Mims &

Richardson, 1989). To complicate matters further, spores of some fungal species such as *Colletotrichum graminicola* are embedded in a 'mucilaginous substance' within acervuli (Nicholson & Moraes, 1980). The purpose of this 'matrix' material is primarily for survival of conidia and has been shown to have no effect on adhesion (Mercure et al., 1994). However, the terminology used to describe all such substances, whether they appear to be involved in adhesion or not, is similar throughout the relevant literature. In addition, while progress has been made in characterising the components present in adhesive matrices, the function of the individual components is often unclear (Epstein & Nicholson, 1997). This is in part due to the many enzymes and toxins secreted into the environment as part of normal fungal activities. Such compounds may be present coincidentally during the adhesion process but play no part in adhesion. Consequently, the literature does not always show an association between the isolated compounds in matrix material and adhesion.

Many of the studies that have attempted to characterise the matrix material surrounding fungal propagules have found glycoproteins. Chaubal et al. (1991) investigated the extracellular matrix surrounding germ tubes of *Puccinia sorghi* urediniospores and found it was mainly composed of glycoproteins and glucan polymers. Germlings treated with pronase E, alkalies and laminarinase (which hydrolyses the storage glycan lamarin) detached from surfaces. As the matrix material was also removed by treatment with these same compounds, it was suggested that acidic amino acid rich glycoproteins and glucan polymers were involved in the adhesion of germlings. It has also been suggested that the 'mucilaginous materials' surrounding germ tubes and appressoria of *M. grisea* are composed of glycoproteins, which are thought to be involved in binding these structures to surfaces (Xiao et al., 1994a). The matrix material produced by *Stagonospora nodorum* conidia, present after 15 min of host contact, was found to be composed of carbohydrate and protein (Zelinger et al., 2004). After the second phase of secretion, which occurred during germ tube emergence, the matrix material also contained proteins and carbohydrates. However, these authors drew no conclusions as to the role of these compounds in the adhesion process. Schumacher et al. (2008) found proteins and carbohydrates in the 'spore tip glue' released from conidia of *Venturia inaequalis* and concluded that release of these compounds, in conjunction with surface contact and the presence of water, were essential for adhesion of these conidia. The extracellular matrix released by both germinated and non-germinated conidia of *C. graminicola* was also composed of proteins and carbohydrates,

specifically a glycoprotein containing a large amount of mannose (Sugui et al., 1998). Examination of matrix material remaining on a hydrophobic surface after removal of either germinated or non-germinated conidia led to the suggestion that this material was involved in the adhesion process. Reissing et al. (1975) showed that treatment with an endogalactosaminidase enzyme caused the removal of sporelings of *Neurospora crassa* from glass. As this enzyme cleaves galactosaminoglycan molecules, it was proposed that these molecules play a role in the adhesion of these propagules. A similar study showed that a polymer of galactosamine appeared to be involved in the adhesion of sporelings of *Bipolaris sorokiniana* to glass (Pringle, 1981).

1.5.2 Spore germination

The stimuli that may play a role in triggering spore germination include direct contact with the host, nutrient availability, absorption of material from the host surface and hydration (Agrios, 2005), although they are unknown for spores of most species. Once germination has been stimulated, spores mobilise stored food reserves to direct synthesis of materials required for germ tube formation and subsequent extension. The host surface is perceived by the germ tube but if the appropriate stimuli are not received, growth will halt once nutrients are depleted. The germ tube will also remain undifferentiated. Although the signalling process involved in the initiation of germination for most species is not yet fully understood, and the exact triggers unknown, some of the physical cues that may be involved include surface contact, surface hydrophobicity and surface hardness (Agrios, 2005).

Kunoh et al. (1988) reported that surface contact is an important stimulus for fungal development in *Blumeria graminis*. Conidia that had not come into contact with a surface showed a distinct pattern of 'reticulation', while after surface contact this distinct pattern gradually disappeared due to each conidium being covered with a 'film'. A subsequent study by Meguro et al. (2001) with the same pathogen indicated that the presence of 'matrix material' may influence the site of emergence of the primary germ tube. An absence of this material resulted in germ tubes emerging from the side of the conidium as opposed to a polar region, which they suggested might influence their ability to contact the plant surface. A study performed with *Blumeria graminis* f. sp. *hordei* conidia on leaves and artificial substrata indicated that conidia 'perceived' the site of surface contact within 1 min of deposition and that this point of contact ultimately determined the emergence site of the

first formed (primary) germ tubes (Wright et al., 2000). The response appeared to be relatively non-specific, occurring over a range of surfaces and initiated even when the surface contact interface was as little as ~0.5 μm in diameter (Wright et al., 2000). In contrast to these findings, conidia of the tomato powdery mildew fungus *Oidium neolycopersici* appear to have a predetermined site for germination which is located sub terminally on the conidia, irrespective of where the conidium makes surface contact (Takikawa et al., 2011).

Surface contact has an influence on the germination rates of pycnidiospores (conidia) of *Phyllosticta ampelicida,* which were highest (> 90%) on surfaces to which they attached firmly such as grape leaves and polystyrene (Kuo & Hoch, 1996). Germination rates were very poor (1.3%) or non-existent on surfaces to which the spores could not adhere well, such as heat-treated glass or water agar. With some species of fungi, such as *Magnaporthe grisea*, surface contact was required for development of appressoria on germ tubes (Xiao et al., 1994b). Conidia germinated whether in contact with a hard or soft surface, but on liquid agar or gel surfaces no appressoria were formed. Germ tubes only produced appressoria when in contact with a hard surface, irrespective of hydrophobicity or surface texture. With *Colletotrichum truncatum,* very few conidia (1%) germinated while in suspension and no appressoria were produced by these germlings. On different concentrations of water agar (0.025%-5%) germination rates increased as the agar firmness increased.

1.5.3 Post germination

In most sessile filamentous fungi, once germination has occurred hyphae undergo apical growth to form a network of mycelium (Brand & Gow, 2009). This filamentous growth is thought to be an adaptation for foraging for fresh sources of nutrients, giving these organisms an advantage over unicellular sessile organisms (Veses et al., 2008). In extreme cases such as that of *Armillaria bulbosa*, the hyphal aggregates (rhizomorphs) can cover in excess of 15 ha (Smith et al., 1992). During this stage of fungal growth, hyphae respond to a broad range of signals, both abiotic and biotic, such as light, temperature humidity, pH and nutrients (Read, 2007). In addition, fungal cells may respond to a range of chemicals or 'chemoattractants' such as sex pheromones, nutrients (amino acids, carbohydrates and polysaccharides) or compounds released from healthy, wounded or decaying plant tissue (Lichius & Lord, 2014). The responses of organisms to such chemical stimuli are often

classified as either chemotactic, in which the response involves movement, or chemotropic, in which a sessile organism will exhibit some kind of directional growth, either in a positive or negative direction. With regards to Botryosphaeriaceae spp., thought to infect via wounds, it is not clear whether conidia that land on a host some distance from wound sites demonstrate some kind of chemotropic response towards the volatiles released from said wounds or whether conidia can only infect if they land directly on wound sites.

1.6 Plant chemicals that affect behaviour of fungal spores and germlings

For many plant pathogens or microbial plant symbionts, growth and infection may depend on their ability to detect and recognise chemical plant signals (Morris et al., 1998). In arbuscular mycorrhizal fungi, for which colonisation of host roots is vital, diffusible chemical signals from roots enable hyphae to reorient their growth towards their symbiotic plant partner (Harrison, 2005). However, outside of these responses evidence for fungal growth orientation responses to chemicals still remains relatively limited.

The chemotactic response found in zoosporic fungi, in the context of host location, is well documented (Lichius & Lord, 2014). Zoospores typically swim in a helical path, with frequent (every 2-4 s) random changes of direction (Deacon & Donaldson, 1993); however, they exhibit directional swimming responses towards a nutrient or chemical released by the root (van West et al., 2002). When an attractant is detected, there is a reduction in the frequency of random turns, which enables the zoospores to follow a more direct path to the root. During the swimming phase, zoospores appear to be attracted to a number of substances exuded by roots (Deacon & Donaldson, 1993). Of the root exudates, it appears that the amino acids are most attractive. *In vitro* tests performed with *Pythium aphanidermatum*, *P. catenulatum* and *P. dissotocum* showed significant accumulation of zoospores in capillaries containing L-amino acids (10 mM) while responses to sugars were more variable (Donaldson & Deacon, 1993).

Zoospores of some fungi demonstrate chemotaxis towards root exudates of plants belonging to a particular family or other taxon (Deacon, 1996). A study performed with a graminicolous species of *Pythium* (*P. graminicola*) and a *Pythium* species with a broad host range (*P. ultimum*) demonstrated that in every test, more zoospores of *P. graminicola* accumulated on

the roots of grasses as opposed to other dicotyledonous plant species (Mitchell & Deacon, 1986). No such effect was seen for *P. ultimum,* for which the numbers of zoospores that accumulated were identical on the different host types. Substances exuded by roots are many and varied and different species of fungi may respond to a particular 'cocktail' of exudates that may determine host specificity (Deacon & Donaldson, 1993).

Aerial pathogens may respond to volatile chemical signals emitted from above ground plant organs. The spores of the green mould fungus *Penicillium digitatum* and the blue mould fungus *P. italicum* lie dormant on the skin of citrus fruit and become active when an injury occurs on the skin (Droby et al., 2008). Volatiles from various citrus cultivars had a pronounced effect on germination of *Penicillium* spp. The percentage of spores that germinated when exposed to the volatiles emitted from discs of grapefruit peel was 75.1% (*P. digitatum*) and 37.5% (*P. italicum*) in comparison to the germination of the controls not exposed to the citrus volatiles (6.8% and 14.7%, respectively). In addition, the germination of spores of *Botrytis cinerea*, not known to be a pathogen of citrus, was unaffected by the same volatiles, indicating pathogen specificity to citrus volatiles (Droby et al., 2008). Mendgen et al. (2006) showed that the plant/pathogen interaction may be more complex and involve differentiation of the pathogen infection structures. When broad bean plants (*Vicia faba*) became infected with the rust fungus *Uromyces fabae*, certain volatile compounds emitted by the host increased approximately ten times compared to those emitted by healthy plants. *In vitro* tests performed with synthetic equivalents of three of the main volatiles produced (decenal, nonanal and hexenyl acetate) demonstrated that these compounds induced haustorium formation in *U. fabae* (Mendgen et al., 2006). It seems possible that the plant recognises and responds to the presence of the pathogen by releasing the volatiles. However, the pathogen has also evolved to recognise the signal that these volatiles provide and once this signal has been perceived, the development of haustoria is promoted, even on synthetic membranes. Volatile compounds may also have an impact on the latent infections of plant pathogens. Neri et al. (2014) analysed the range of volatile compounds produced from ripening strawberries. They found that the volatile profile changed significantly as fruit ripened and the volatiles produced during this stage of fruit development had a stimulatory effect on the growth of *B. cinerea*. This change of volatile composition may be responsible for latent infections of *B. cinerea* in unripe fruit becoming active. A similar change in

composition of the volatiles emitted from fruit and consequent stimulation of pathogen growth was also seen after wounding.

While some of the many volatile organic compounds found within plant cells may provide a positive signal for fungal growth and/or orientation, they may also be inhibitory or toxic to fungal propagules. Some of the compounds emitted from plants contribute to the flavour in fruits and vegetables and their historical safe use as food flavouring compounds, in conjunction with their purported role in defence against decay organisms, has led to a renewed interest in their use against microorganisms that cause decay (Utama et al., 2002). Utama et al. (2002) investigated the effectiveness of a range of plant volatile compounds on the growth of a number of decay organisms, namely *Rhizopus stolonifera*, *Penicillium digitatum*, *Colletotrichum musae*, *Erwinia carotovora* and *Pseudomonas aeruginosa.* Aldehyde compounds such as acetaldehyde proved to be the most effective, completely inhibiting the growth of all five species at concentrations as low as 0.09 mmol/Petri dish. Alcohols such as benzyl alcohol and ethanol varied in their effectiveness and differed in the minimum inhibitory concentration (MIC) at which they were effective. Benzyl alcohol inhibited the growth of *P. digitatum* at a MIC of 0.48 mmol/dish and that of *C. musae* at 1.06 mmol/dish. Ethanol inhibited the growth of these same pathogens at MICs of 9.87 mmol/dish and 16.59 mmol/dish, respectively. The effectiveness of volatile compounds against pathogenic organisms may be concentration dependent (Droby et al., 2008). Studies performed with *P. digitatum* and citral demonstrated that germination was stimulated at concentrations between 0.06 and 0.15 ppm but inhibited at higher concentrations.

The above studies have demonstrated that volatile compounds can have an effect on the growth and development of various fungal species, whether pathogenic or not. With regards to pathogenic fungi, the volatiles providing signals to which they are responsive appear to be related to ripening and/or wounding. Wound volatiles are formed rapidly and many are transient in nature; it is suggested that they play a role in plant defence responses, helping to prevent microorganisms from invading plant tissue (Croft et al., 1993). In wounded green plant tissue, the compounds which are universally emitted include C_6-aldehydes and alcohols, acetate esters, acetaldehyde and methanol (Brilli et al., 2012). These volatile compounds, classed as ‘green leaf volatiles’ (GLVs), are named for their distinctive odour such as that produced when grass is cut or leaves or stems are damaged (Matsui, 2006).

GLVs have been shown to have bacteriostatic activity against both gram-positive and gram-negative bacteria (Nakamura & Hatanaka, 2002).

To date no studies have been performed to determine the effects of GLVs on trunk pathogens. Cha et al. (2008) investigated the volatile compounds emitted from unwounded potted shoots of *Vitis riparia* (riverbank grape). However, this study specifically reported those compounds that were attractive to the grape berry moth *Paralobesia viteana*, and did not provide a comprehensive list of the volatile compounds emitted from these grape plants. Little is known regarding the volatiles emitted from either wounded or non-wounded tissues of *Vitis vinifera* and no studies have investigated whether volatiles emitted from these species provide chemical signals to which conidia of Botryosphaeriaceae spp. are responsive.

1.7 Research context and objectives

While over the past ten years species of Botryosphaeriaceae fungi have finally been recognised as important grapevine pathogens, the economic cost to the industry of diseases associated with these species is yet to be fully realised. To improve understanding of the infection dynamics of these pathogens, this study will investigate some of the properties of Botryosphaeriaceae spp. conidia during the early infection process.

The objectives of this research are as follows:

1. To investigate whether adhesion by conidia is active or passive and whether it is affected by surfaces with different characteristics.
2. To investigate the effects of surface characteristics on germination and early development of conidia.
3. To investigate the effect of some volatiles associated with grapevine wounds on germination and directional growth responses of conidia.

Chapter 2

Adhesion of Botryosphaeriaceae spp. conidia: effects of surfaces and treatments

2.1 Introduction

Infection of plants by pathogenic fungi often starts when the fungal spores become attached to the plant tissues, a process that often initiates a series of physiological responses. The close contact between fungus and plant is essential for pathogenic processes, such as the secretion of enzymes and the subsequent absorption of nutrients from the plant surface (Jones, 1994). In addition, specialised structures such as appressoria, which penetrate host tissues directly, would need to be in close contact with the surface in question. It is therefore likely that adhesion plays a major role in colonisation and pathogenesis and thus it is important to understand the nature of the adhesion process when investigating the interaction between a fungus and its host. For Botryosphaeriaceae species there is no published information on the manner in which conidia adhere and the factors that may affect the adhesion process. The aim of this study was to try and elucidate some of the mechanisms involved in the adhesion of Botryosphaeriaceae spp. conidia. Artificial surfaces were used in the study because preliminary testing with grapevine tissues indicated that it would be extremely difficult to accurately count the conidia remaining on plant surfaces after washing. This was due to the topography of the shoot and leaf surfaces and also the extreme hydrophobicity of both of these tissues; these factors made staining and visualising conidia difficult. Artificial surfaces however allowed the effect of surface hydrophobicity characteristics to be investigated.

2.2 Materials and Methods

In the first adhesion experiment, conidial suspensions of Botryosphaeriaceae species were placed onto a range of surfaces where they remained for various times, ranging from 0 to 60 min, before attempting to remove them by washing. The number of conidia that remained was determined by microscopy and indicated the adhesion of conidia to that surface. One

isolate and one replicate with all surfaces and time points was assessed on a single day. The order of isolates and replicates was randomly allocated on each day.

2.2.1 Fungal isolates

The isolates of Botryosphaeriaceae spp. chosen for this study had previously been obtained during sampling in and around New Zealand vineyards (Baskarathevan et al., 2012) and in grapevine nurseries (Billones-Baaijens et al., 2013b). All isolates had been identified by amplified ribosomal DNA restriction analysis (Alves et al., 2005). Of the isolates chosen, three were obtained close to or from vineyards: *N. luteum* isolates CC445 and MM558 and *N. parvum* isolate G652 (Baskarathevan et al., 2012) while one isolate, *B. dothidea* isolate 007 was obtained from a grapevine nursery (Billones-Baaijens et al., 2013b). Isolates MM558, G652 and 007 were recovered from grapevines while isolate CC445 was recovered from *Actinidia arguta* (hardy Kiwi). Selection of isolates was also based on preliminary testing, which indicated that sufficient conidia could be produced for experimental purposes (data not shown). Unless stated otherwise, experiments were conducted using all four isolates.

2.2.2 Inoculum preparation

The method of Amponsah et al. (2008) with slight modifications was used to generate conidia from either green (soft) or semi-hard shoots. Shoots were cut from field grown Sauvignon Blanc grapevines in summer (green shoots) or autumn (semi-hard shoots). The bases of the shoot tips (~25 cm) were placed into Universal bottles containing tap water and supported with Parafilm®. On each shoot, the central internodes were swabbed with 70% ethanol and allowed to dry before a small wound (~ 5 mm) was made with a sterile scalpel. Mycelial plugs cut from the edges of young (3 day old) potato dextrose agar (PDA, Difco™) cultures were placed onto each wound. Shoots were placed into a transparent plastic container (~40 L) at room temperature either in ambient light or under lights (Osram L36W/840 Lumilux Cool white tubes) if needed in winter. Prior to use, the plastic container had been sprayed with water and contained wet paper towels in the bottom to ensure high humidity. After two days, the box lid was opened slightly to allow air flow and the shoots were sprayed with water daily to prevent drying out. When pycnidia were seen forming on the lesions, shoots were cut into ~5 cm sections containing the lesions, sterilised in 70% ethanol for 30 s, rinsed twice in sterile water and dried overnight in a laminar flow cabinet.

Shoots were stored dry in Petri dishes sealed with food grade cling film and held at room temperature until needed. When conidia were required, shoots were placed on top of sterilised plastic straws within Petri dishes, each containing a piece of sterilised moist paper towel to create a humid environment. The sealed Petri dishes were incubated at 25^{o}C (12 h light/12 h dark) until conidia were seen oozing from pycnidia (~24 h). Each shoot was then placed into a 15 mL Falcon® tube (Axygen) containing 2 mL pre-cooled (4^{o}C) sterile reverse osmosis water (SROW) and vortexed for 30 sec. Conidial concentration was determined using a haemocytometer and adjusted to ~1.5x10^{4} conidia/mL. The suspensions were not filtered prior to use to prevent any possible alteration to the adhesive properties of the conidia.

2.2.3 Microscopy

Unless stated otherwise, all microscopy work was carried out on an Olympus CX41 microscope with a DS Fi2 camera attached via a U-TVO 5XC mount adapter. Photographs were taken via a Nikon DS-L3 camera controller.

2.2.4 Contact angle measurements

The hydrophobicity/hydrophilicity of all surfaces was determined by a goniometer (CAM100, KSV). A 10 µL drop of tap water was placed onto each surface with a precision syringe (Hamilton) and imaged for 4 s (collecting one image/s). Calculation of contact angles was performed automatically and based on the Young-Laplace equation (Laplace, 1805; Young, 1805), which determined the contact angle on either side of the droplet and then the mean value. Reported values represent the mean of six replicate drops per surface.

2.2.5 Surfaces

Surfaces used in this study included glass slides (26 x 76 mm, Fronine), cellulose acetate membrane circles (Whatman 90 mm, pore size 0.45 µm), polystyrene Petri dishes (90x15 mm, LabServe, ThermoFisher) and Parafilm® M (American National Can™, Sigma-Aldrich). Surfaces were chosen to represent a range of hydrophobicities and the methods used to prepare them attempted to reduce handling to a minimum. Gloves were worn when handling surfaces: slides or slide-shaped polystyrene pieces were held by the edges when handling. The cellulose acetate membrane circles (herein known as cellulose) were cut in half and each half attached at one end to a glass slide with double sided adhesive tape

(Sellotape®). Membranes were moistened with tap water before use. Parafilm® M was cut into strips approximately the same size as a glass slide and attached to the slide at both ends with double sided adhesive tape. Polystyrene Petri dishes were cut using a cutting template made from 6 glass slides bound by masking tape. The template was placed onto the inside of the Petri dish (lid and base) then a sharp knife was used to firmly score the polystyrene. Once the scored areas were snapped off, they were wiped with 70% ethanol to remove any residues of handling.

To limit the study area, lines were drawn across the width of each surface, 3.5 cm from the top edge of the supporting slide and 2 cm further down from the first line (Fig 2.1). The area between the two lines was designated as the space within which conidia would be counted. A small marker line was drawn half way across the first line and was used as a guide for placing the drop of spore suspension and to position the microscope when starting the counting (Fig 2.1).

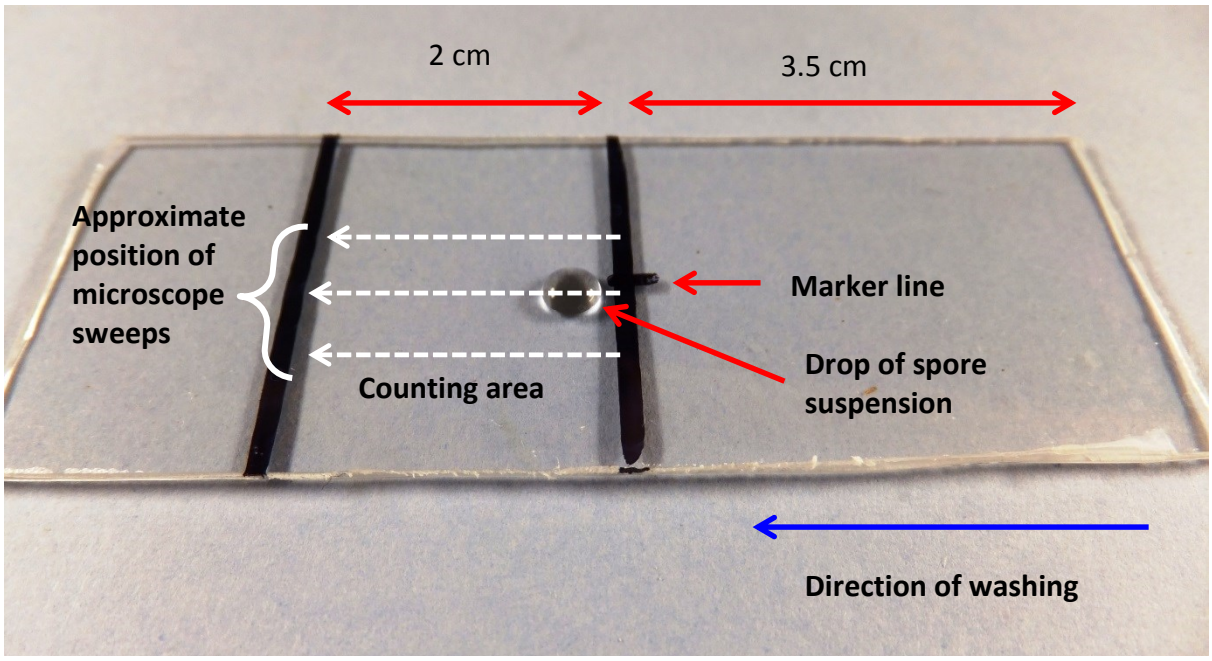

Figure 2.1 Polystyrene Petri dish cut to the size of a glass slide. Lines marking the area show where conidia were counted on this surface and other surfaces tested. Counting was performed by sweeping the microscope three times across the surface indicated by the dotted lines.

2.2.6 Adhesion of conidia to different surfaces

In order to ensure that the conidial suspensions were uniform throughout the pipetting of numerous slides, a uniform pipetting technique was required. A preliminary experiment tested different shaking methods, which consisted of a mixture of vortexing, pipetting up and down, and hand shaking. The aim was to break up clumps of conidia, preventing the conidia from settling in the tube or sticking to the sides of the tube, whilst also not mixing the suspension so much that any mucilage present could be washed off. At each time point all surfaces received a drop of conidial suspension and then the same procedure was repeated for the next time point. The order in which the surfaces were inoculated was randomly allocated at each time point. The mixing procedure that gave consistent results (as assessed by viewing a 10 µL drop of conidial suspension on a microscope slide) was achieved by vortexing briefly for a few seconds before each time point and swirling by hand between the surfaces used for that particular time point (Appendix A.2.1). To minimise conidia sticking to the inside of the pipette tip, the suspension was drawn up and down three times into the pipette before each droplet was finally placed onto a surface. For each replication, time points and surfaces were pipetted in a different order.

A support to hold the surfaces at a 45^{o} angle for washing was created from Meccano (Fig. 2.2). A small funnel was clamped in place at approximately 6 cm above the surface to be tested. This ensured that the water was delivered to the same place on each surface. A 10 µL drop of conidial suspension (~1.5x10^{4}/mL) was deposited onto each surface beside the marker line and incubated for various time points (0, 1, 5, 20 and 60 min). The surfaces were then placed onto the support and washed with tap water (50 mL) applied via the funnel, a process which took 2-3 s. At 0 min, the spore droplet was washed off immediately after application. For all other time points, surfaces were incubated in humidity chambers at room temperature prior to washing.

Humidity chambers were created by pouring water into a 5 L food storage container (Cuisine Queen) to a depth of approximately 2 cm. A piece of plastic mesh was rested onto 3 cm supports in the bottom of the container, which created a dry surface above the water onto which slides could be placed. Containers were left with their lids on for 48 h at room temperature before use to allow humidity to reach 100%.

After washing, conidia remaining on each surface were immediately stained with lactoglycerol aniline blue (Appendix A.1) and counted under a microscope (x100) with three sweeps across the slide as illustrated in Fig. 2.1. The first counting sweep began at the marker line in the centre of the field of vision, then the microscope was positioned back at the marker line and the surface was moved to show one field above, for which another sweep allowed counting. This was repeated a third time by positioning the microscope one field of vision below the marker line. Care was taken to ensure the surface was always moved beyond each field of vision to avoid counting a conidium twice. To ascertain the number of conidia initially deposited onto each surface, three 10 µL droplets of conidial suspension were placed onto a glass slide (without washing) and the number of conidia present determined. The average number of conidia in three unwashed drops of conidial suspension was used as the control. The number of conidia remaining after washing was expressed as a percentage relative to the unwashed control.

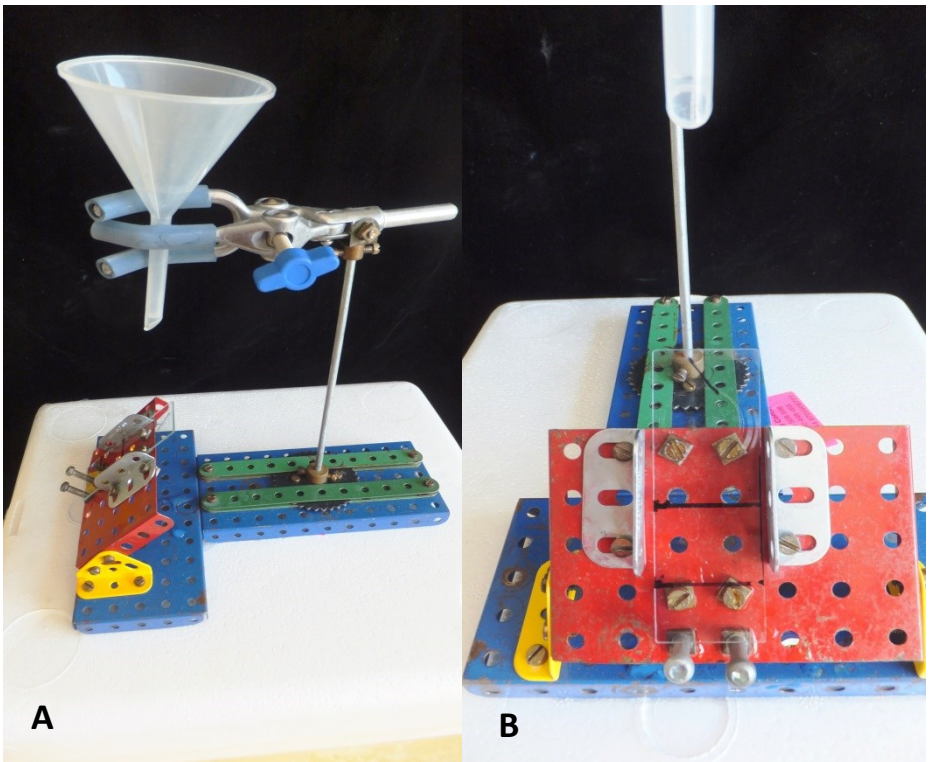

Figure 2.2 Meccano support used to hold slides in a fixed position at 45^{o}; A) side view and B) front view.

Preliminary testing showed that there were no conidia present outside of three microscope sweeps except in the case of cellulose, where the spread of spores was far greater.

Therefore, conidia were counted within five microscope sweeps on cellulose. The experiment was repeated six times for each species tested, providing six replications.

2.2.7 Adhesion of treated conidia of *N. luteum* isolate MM558

To determine whether active metabolism is involved in the adhesion process, conidial suspensions were prepared with the addition of the metabolic inhibitors sodium azide or cycloheximide. To inhibit any metabolic processes in a non-chemical manner, conidia were heat treated for 15 min at 95^{o}C to kill them. To determine if cell surface proteins are involved in the adhesion process, conidia were prepared with a protease. The adhesion assay was carried out as before (Section 2.2.6) using these treated conidial suspensions and glass slides, but not with a range of incubation times prior to washing.

Experiment 1

A conidial suspension of *N. luteum* isolate MM558 was prepared as before and the concentration adjusted to ~3x10^{4} conidia/mL. To prevent germination the suspension was placed in a cool box containing ice until use. Aqueous solutions of inhibitors were made up at double the concentration required; sodium azide (Sigma-Aldrich) was prepared at a concentration of 4 mM and cycloheximide (Acros Organics) at 2mM. Pronase E (Sigma-Aldrich) was diluted to 10 mg/mL in Tris-HCl, pH 7.6 (Rawlings et al., 2007). In each of seven 1.5 mL micro centrifuge tubes, 0.5 mL of conidial suspension (~3x10^{4}/mL) was pipetted followed by 0.5 mL of the appropriate treatment. For heat treated conidia, 0.5 mL SROW was added to the conidial suspension and the tube placed into a heat block (Stuart SBH130 block heater) at 95^{o}C for 15 min. As a control for protease treatment, 0.5 mL of protease in Tris-HCl buffer was denatured using the same heat treatment before being added to the conidial suspension. Tris HCl buffer minus the protease was also used as a treatment to ensure that it had no effect on adhesion. The control treatment comprised a conidial suspension to which SROW was added. Treatments were applied to conidial suspensions at room temperature. Slides were washed, stained and conidia counted as before. Time taken for treatment application, pipetting, incubation (20 min at 20^{o}C) and washing was ~45 min (total treatment time).

To ascertain the number of conidia initially deposited onto each surface, three 10 µL droplets of the same conidial suspension from the control treatment were placed onto a

glass slide (without washing) and the average number of conidia present determined. The number of conidia remaining after washing for each treatment was expressed as a percentage relative to the numbers present in the unwashed control. The experiment was replicated six times per treatment. To determine whether conidia were still viable after application of the treatments, two 10 μL drops of conidial suspension from each treatment were plated onto PDA and incubated at 25°C (12h light/12h dark).

Experiment 2

The experiment was repeated with a conidial suspension (~3x10^4/mL) of the same *N. luteum* isolate (MM558) and different heating times: 110°C for 10 min for heat treatment and 110°C for 20 min to denature the protease. As the previous buffer had become contaminated, a new Tris-HCl buffer was made up as before and then autoclaved before use (121°C for 15 min). In addition, skimmed milk agar plates (Appendix A.1) were used to detect if the protease was still active (casein degradation) after denaturing. The number of conidia remaining after washing for each treatment was expressed as a percentage relative to the numbers in the unwashed control as before. The experiment was replicated six times per treatment.

Adhesion of heat treated conidia

To resolve some of the questions raised from the different heat treatment results, the effect of heat alone on spore adhesion was investigated with two different temperature treatments (95°C and 110°C) and *N. luteum* isolate MM558. Into each of nine 1.5 mL micro centrifuge tubes, 1 mL of conidial suspension (~1.5 x 10^4/mL) was pipetted. The heat block was set to 95°C and four tubes of conidial suspension placed into the block. After 10, 15, 20 and 30 min, one tube was removed from the heat and placed onto ice to cool and to prevent germination. The temperature of the heat block was then increased to 110°C and the procedure repeated. The control treatment was a conidial suspension that had not been heated. The standard adhesion assay was then carried out as before with glass slides and incubation at 20°C for 20 min. To ascertain the number of conidia initially deposited onto the glass slide, three 10 μL drops of conidial suspension were pipetted from each micro centrifuge tube (each treatment) onto a glass slide and the average number of conidia present determined. The number of conidia remaining after washing for each heat treatment was expressed as a percentage of each corresponding treatment control. The experiment was replicated six times per treatment. To determine whether conidia were still

viable after heat treatment, a 20 µL drop of conidial suspension from each treatment was plated onto PDA, spread with a sterile hockey stick and incubated at 25°C (12 h light/12 h dark).

2.2.8 Adhesion of washed conidia

To determine if residual pycnidial mucilage plays a role in the adhesion process, conidia were washed in an attempt to remove the mucilage. The adhesion assay was carried out as before (Section 2.2.6) using these washed conidial suspensions and glass slides, but not with a range of incubation times.

The effect of washing on spore adhesion was investigated with two different washing treatments (twice and four times) and *N. luteum* isolate MM558. A conidial suspension was prepared as before. One mL of conidial suspension (~$5x10^4$ /mL) was pipetted into each of two 1.5 mL micro centrifuge tubes. The tubes were centrifuged for 3 min at 13,000 x*g* before supernatants were removed and the spore pellets re-suspended in 1 mL SROW. The tubes were briefly vortexed to break up the spore pellet then placed back into the centrifuge for another 3 min at 13,000 x*g*. After this time, the x2 treatment was re-suspended in 1 mL SROW and the spore concentration adjusted to ~$1.5x10^4$ conidia/mL. The x4 treatment was washed twice more and spore concentration adjusted accordingly after this time. The control treatment was unwashed. The standard adhesion assay was then carried out as before with glass slides. To ascertain the number of conidia initially deposited onto the glass slide, three 10 µL drops of conidial suspension were pipetted from each micro centrifuge tube (each treatment) onto a glass slide and the average number of conidia present determined. The number of conidia remaining after washing for each wash treatment was expressed as a percentage of each corresponding treatment control. The experiment was replicated six times per treatment.

2.2.9 Characterisation of spore surface components and spore mucilage

To investigate the composition of any spore mucilage present, conidia were stained with a range of dyes known to stain likely components (Appendix A.1.1). These were Alcian blue (Sigma Aldrich), for mucopolysaccharides (mucin), Coomassie brilliant blue (CBB) (BDH, Global Science) for proteins, Congo red (Comak Chemicals Ltd) for amyloidic carbohydrates and acid fuchsin (BDH Stains) for carbohydrates. Higgins black ink was also used as a

negative stain diluted 1:4 in SROW. The acid fuchsin and CBB dyes were filtered before each use through Whatman grade 1 (11 μm) 90 mm filter paper. Alcian blue stain was filtered before each use with a syringe (Thermo) with a millipore filter attached (0.22 μm). A conidial suspension was prepared as before and the concentration adjusted to ~$1.5x10^4$ conidia/mL. All four isolates (*N. parvum* isolate G652, *B. dothidea* isolate 007 and *N. luteum* isolates MM558 and CC445) were used in this experiment, one isolate and one stain being examined at a time to ensure sufficient time for observation. Glass slides were used due to their ease of visibility. Two 10 μL drops of conidial suspension were placed onto each glass slide and slides were incubated in a humidity chamber for various times (0 min, 5 min, 1.5 h, 2.5 h and 3.5 h) at 20°C. After each time point, slides were immediately stained with a drop of dye and examined under the microscope. When either a conidium or area immediately contiguous to the conidium was stained with a particular dye, this was considered a positive (+) result. If neither were stained, it was considered a negative (-) result. Where only some conidia were stained, both a positive and negative result was reported (±). For Higgins black ink (used as a negative stain) a positive result was characterised by a bright zone around the conidia and/or germlings and a negative result by no zone visible. Conidia that were empty of contents or shrivelled/misshapen were not included in the study. The experiment was replicated four times/isolate.

2.2.10 Statistical analysis

Data were analysed using GenStat 16th edition (VSN International). Due to high variability of data for untreated spores adhering to a particular surface, data were log transformed (log_{10}) to achieve a normal distribution before being analysed using a two-way analysis of variance (ANOVA). To account for 0 values, 1 was added to all data before log transformation occurred. The data presented in graphs are antilog transformations of the mean values generated by ANOVA analysis. For all other experiments, including treatment of spores, data did not violate the assumptions of ANOVA so did not need to be transformed. Where the ANOVA analysis reported a significant effect, differences between means were determined by using Fisher's protected least significant difference (LSD) at $P \leq 0.05$. Differences between means were considered significant within the following results only if they met this criterion.

2.3 Results

2.3.1 Inoculum preparation

The method of producing conidia from detached shoots was subject to variable results, which appeared to be associated mainly with the shoot characteristics and environment of incubation. The time taken to produce pycnidia on shoots varied over 8-10 days for isolates MM558 and CC445 and took about 10-15 days for isolates 007 and G652. Thinner shoot pieces were found unsuitable as they often broke off at the upper internodes, particularly when placed on the light bench. In all species, pycnidia varied in colour from pale brown to black, with the former appearing to be smaller in size (Fig 2.3). On any lesion section, pycnidia were often starting to ooze conidia before the lesion sections were sterilized and stored. This often resulted in a small amount of mycelial growth on the shoots. If the temperature under the light bench was too high (27-30^{o}C), plants became brown and dry and pycnidia took longer to develop. Conidial suspensions often included shrivelled or empty conidia (Figure 2.4) and frequently contained debris, likely a result of not filtering the suspension prior to use.

Production of pycnidia from *N. parvum* isolate G652 was often unsuccessful and inoculation needed to be repeated many times. Isolate *B. dothidea* 007 produced only very small lesions (~2 cm); however, pycnidia were always produced close to the inoculation point and there were always more than $1.5x10^4$ conidia/mL in the resulting suspensions. The *N. luteum* isolates gave the best results from using this technique.

2.3.2 Contact angle measurements

For the purposes of this study surfaces with a contact angle less than 90^{o} were considered hydrophilic and those over 90^{o} hydrophobic (Arkles, 2006). Glass was slightly hydrophilic, with a mean contact angle of 63.68^{o} (Table 2.1), while polystyrene was hydrophobic with a mean contact angle greater than 90^{o} (97.02^{o}), and parafilm extremely hydrophobic with a mean contact angle of 130.77^{o} (Table 2.1). It was not possible to measure the droplet placed onto cellulose as the droplet soaked into the membrane immediately upon contact. Its contact angle was thus designated as 0^{o} and it was classed as extremely hydrophilic (Table 2.1).

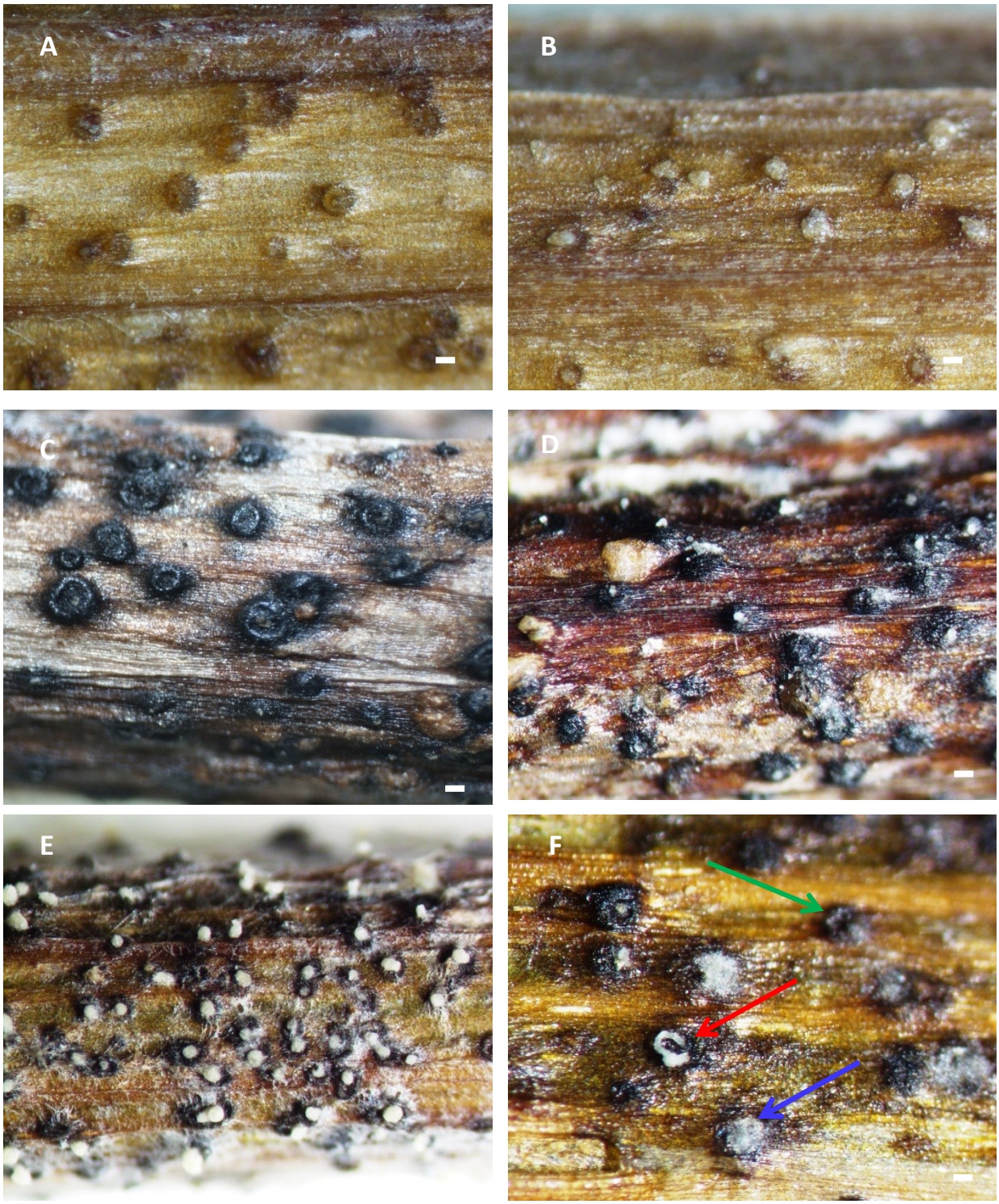

Figure 2.3 Pycnidia on grapevine shoots infected with *N. luteum* isolate MM558. A) Pale brown pycnidia; B) brown pycnidia with dried conidial ooze; C) black pycnidia with no mycelial growth; D) black pycnidia with some oozing conidia and some covered with dried ooze; E) black pycnidia with oozing conidia and mycelial growth; F) an oozing paste tendril (red arrow), dried ooze (blue arrow) and non-oozing pycnidia (green arrow). Scale bars = 80 μm.

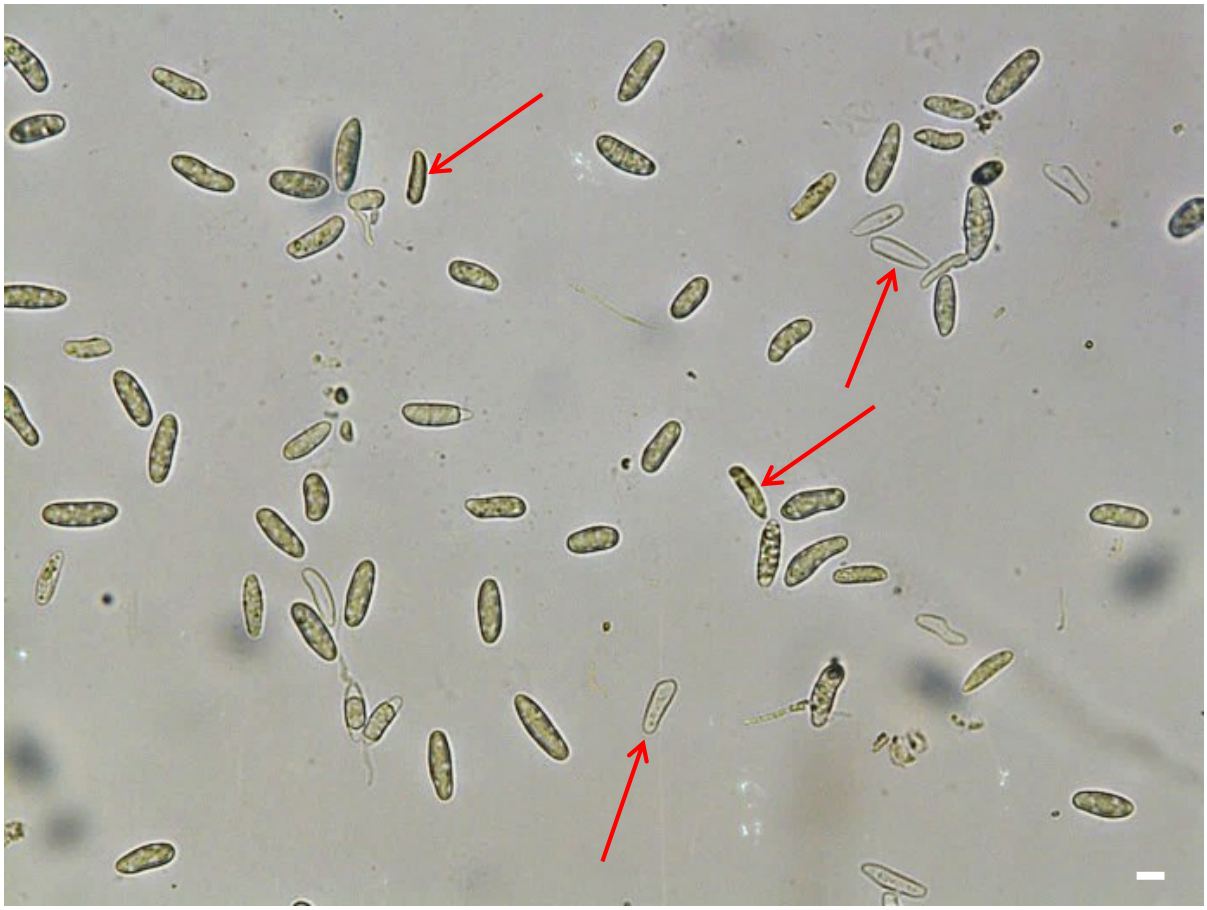

Figure 2.4 Conidia of *N. luteum* isolate MM558 produced from the green shoot technique (from unfiltered spore suspension). Examples of empty and shrivelled conidia are indicated by arrows. Scale bar = 8 μm.

Table 2.1 Mean contact angles of surfaces used to test adhesion of conidia.

Surface	Contact angle (mean degrees ±SEM)
Cellulose	0
Glass	63.68 ± 3.57
Polystyrene	97.02 ± 3.15
Parafilm	130.77 ± 6.61

2.3.3 Adhesion of conidia to different surfaces

For the non-grapevine isolate of *N. luteum* (CC445), there was a significant effect of surface type on adhesion ($P<0.001$) (Appendix A.3.1). There was a significant difference between the highest numbers of conidia that adhered to polystyrene (mean 39.3%) and the lowest numbers that adhered to cellulose (mean 12.0%). There was no significant difference

between the mean numbers of conidia adhering to either glass or parafilm. There was also a significant effect of time on spore adhesion (P<0.001) (Appendix A.3.1), with mean spore adhesion increasing significantly between 0, 1, 5 and 20 min incubation treatments (4.0%, 14.8%, 44.7% and 61.4%, respectively). Maximum adhesion was achieved after 20 min incubation with no significant increase in adhesion after this time.

There was a significant interaction between surface and time (P<0.001) (Appendix A.3.1), which was associated with conidia taking longer to adhere to some surfaces. For example, after 0, 1 and 5 min, significantly lower numbers of conidia adhered to cellulose (means 2.2%, 3.7% and 13.0%, respectively) than to the other three surfaces (Fig. 2.5). After 60 min incubation, the mean number of conidia adhering to cellulose (47.6%) was still significantly lower than the mean numbers adhering to either parafilm or polystyrene (80.2% and 79.8%, respectively) (Fig. 2.5).

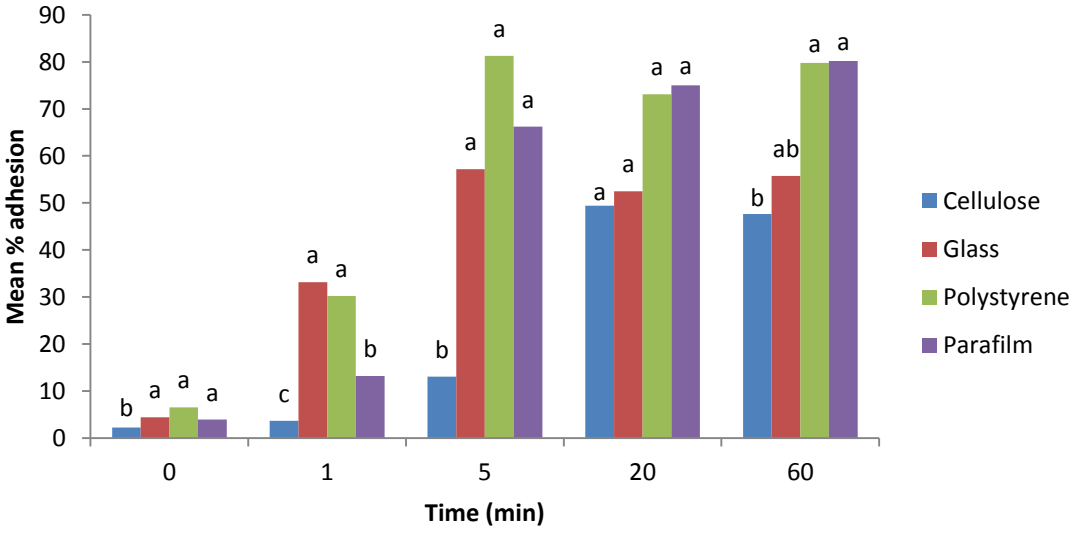

Figure 2.5 Mean percent adhesion relative to the unwashed control of non-grapevine *N. luteum* isolate CC445 conidia to different surfaces following incubation for selected times (min) then immediate washing with 50 mL water. Bars with different letters (at each time point) are significantly different at P≤0.05 LSD.

For grapevine *N. luteum* isolate MM558, there was a significant effect of surface on adhesion (P<0.001) (Appendix A.3.2), with significantly lower numbers of conidia adhering to cellulose (mean 19.9%) than to polystyrene, glass and parafilm (means 35.5%, 31.5% and 27.8%, respectively). The effect of time on adhesion was also significant (P<0.001) (Appendix A.3.2). After 0, 1 and 5 min incubation, the mean numbers of conidia adhering to a particular surface increased significantly (4.2%, 27.5% and 53.1%, respectively). Maximum adhesion was achieved after 5 min incubation with no significant increases in adhesion after this time.

There was a significant interaction between surface and time (P<0.001) (Appendix A.3.2), which was associated with conidia taking longer to adhere to some surfaces. After 0 min there were no differences between the mean numbers of conidia adhering to surfaces (Fig. 2.6). However, after 1 and 5 min significantly lower numbers of conidia adhered to cellulose (means 10.8% and 16.9%, respectively) than to the other surfaces tested at those times (Fig. 2.6).

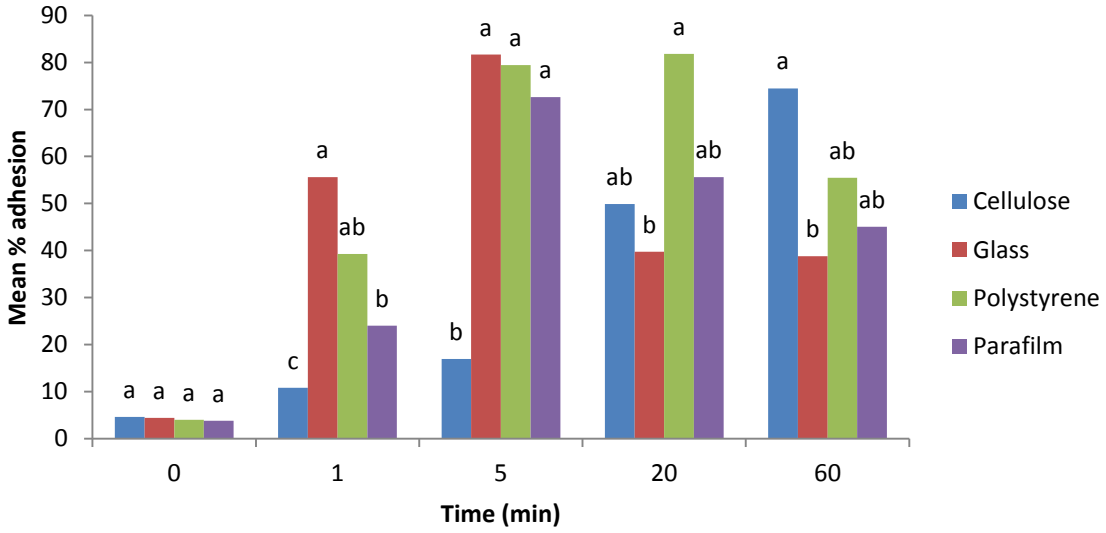

Figure 2.6 Mean percent adhesion relative to the unwashed control of grapevine *N. luteum* isolate MM558 conidia to different surfaces following incubation for selected times (min) then immediate washing with 50 mL water. Bars with different letters (at each time point) are significantly different at P≤0.05 LSD.

There was a significant effect of surface on the mean adhesion of *N. parvum* isolate G652 conidia ($P<0.001$) (Appendix A.3.3), with significantly higher numbers of conidia adhering to cellulose and polystyrene (38.7% and 29.9%, respectively). The effect of time on adhesion was also significant ($P<0.001$) (Appendix A.3.3). After 0, 1 and 5 min incubation, the mean numbers of conidia adhering to a particular surface increased significantly (5.9%, 25.9% and 50.6%, respectively), after which time no significant increase in adhesion was recorded.

There was a significant interaction between surface and time ($P<0.001$) (Appendix A.3.3), which was associated with conidia taking longer to adhere to some surfaces. After 0 min, there were significantly lower numbers of conidia adhering to glass, polystyrene and parafilm (means 4.6%, 3.7% and 2.8%, respectively) than to cellulose (mean 25.1%) (Fig 2.7). After 1 min, there was no significant difference between the numbers of conidia adhering to cellulose and glass; however, there were significantly lower numbers of conidia adhering to polystyrene and parafilm (means 18.6% and 13.2%, respectively). After 60 min there was no significant difference between the numbers of conidia adhering to any of the surfaces tested (Fig 2.7).

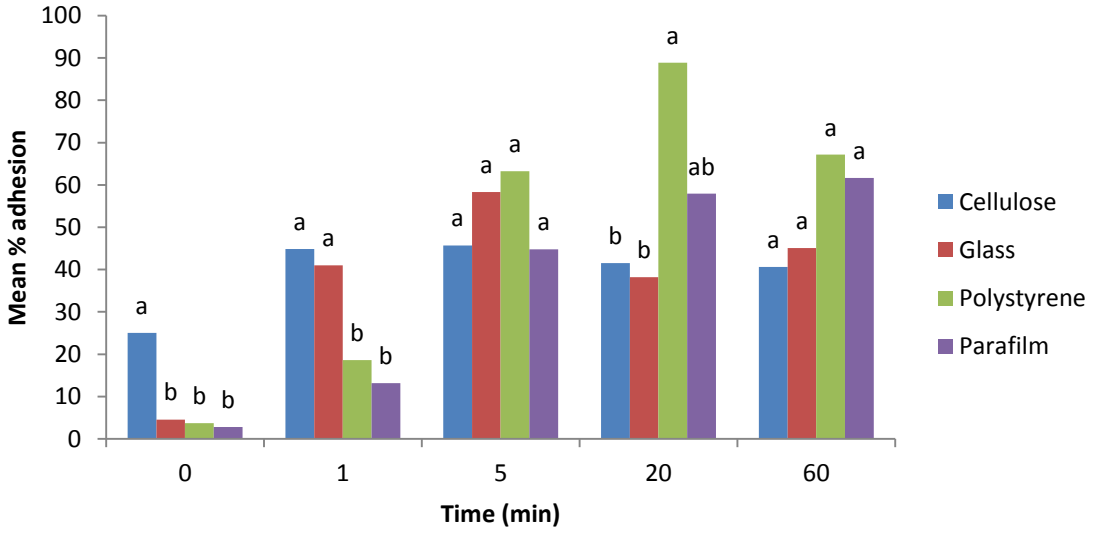

Figure 2.7 Mean percent adhesion relative to the unwashed control of grapevine *N. parvum* isolate G652 conidia to different surfaces following incubation for selected times (min) then immediate washing with 50 mL water. Bars with different letters (at each time point) are significantly different at $P \leq 0.05$ LSD.

For *B. dothidea* isolate 007, there was a significant effect of surface on adhesion ($P<0.001$) (Appendix A.3.4). Significantly lower numbers of conidia adhered to parafilm (mean 23.4%) than to glass (mean 32.7%), polystyrene (mean 33.9%) or cellulose (mean 43.2%). There was also a significant effect of time on adhesion ($P<0.001$) (Appendix A.3.4). There was a significant increase in mean spore adhesion after 0, 1 and 5 min incubation (5.8%, 29.3%, and 54.0%, respectively). Maximum adhesion was achieved after 5 min incubation with no significant increases in adhesion after this time.

There was a significant interaction between surface and time ($P=0.002$) (Appendix A.3.4), which was associated with conidia taking longer to adhere to some surfaces. For example, at 0 min there were significantly lower numbers of conidia adhering to glass, polystyrene and parafilm (means 4.7%, 3.7% and 5.6%, respectively) than to cellulose (mean 11.4%) (Fig. 2.8). After 1 min, there were no significant differences between the mean numbers of conidia adhering to cellulose, glass or polystyrene. However, there were significantly lower numbers of conidia adhering to parafilm (mean 13.0%). After 20 and 60 min, there was no significant difference in the numbers of conidia adhering to any of the surfaces tested (Fig 2.8).

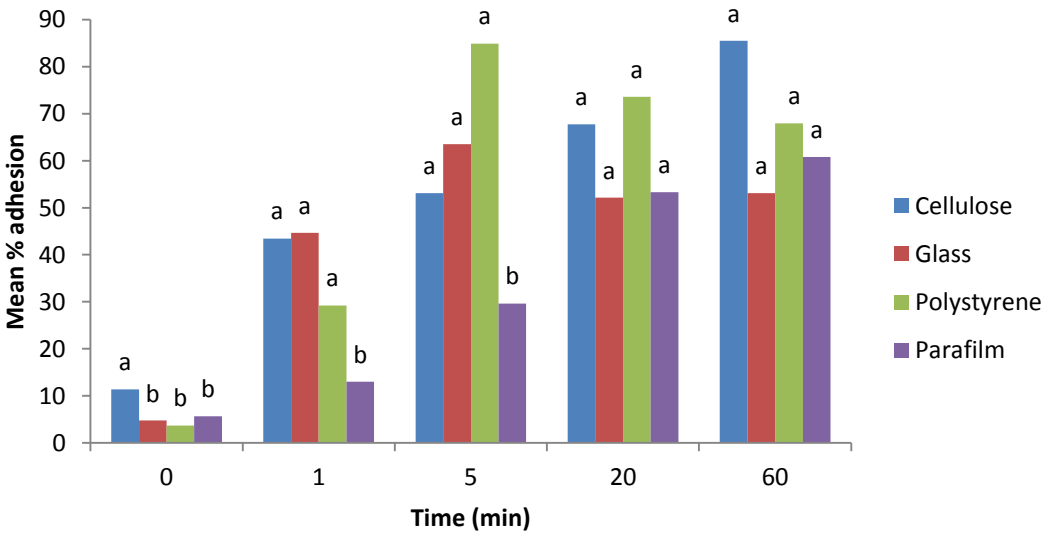

Figure 2.8 Mean percent adhesion relative to the unwashed control of grapevine *B. dothidea* isolate 007 conidia to different surfaces following incubation for selected times (min) then immediate washing with 50 mL water. Bars with different letters (at each time point) are significantly different at $P\leq0.05$ LSD.

2.3.4 Adhesion of treated conidia of *N. luteum* isolate MM558

Experiment 1

There was a significant effect of treatment on the adhesion of conidia to glass slides (P<0.001) (Appendix A.3.5). Treatment of conidia with a protease completely prevented conidial adhesion to glass slides (Table 2.2). Treatment with cycloheximide and Tris-HCl did not appear to alter the adhesion rate of conidia and the mean percent adhered conidia for these treatments (55.3% and 48.7%, respectively) did not differ significantly from the untreated control (53.1%) (Table 2.2). Treatment of conidia with the denatured protease, heat and sodium azide all reduced the mean number of conidia adhering to glass significantly (20.1%, 33.9% and 44.0%, respectively) (Table 2.2).

Experiment 2

In the second experiment there was also a significant effect of treatment on the mean adhesion of conidia (P<0.001) (Appendix A.3.6). Treatment with protease completely prevented the adhesion of conidia as before (Table 2.2). In contrast to the first experiment, treatment with cycloheximide significantly reduced the mean adhesion of conidia (25.1%) in comparison to the untreated control (72.0%), while treatment with sodium azide did not affect the mean adhesion of conidia (65.0%) (Table 2.2). Treatment of conidia with Tris-HCl significantly reduced the mean adhesion of conidia (43.0%). Heat treatment had no effect on spore adhesion in comparison to the control (Table 2.2).

Table 2.2 Mean percent adhesion of conidia of *N. luteum* isolate MM558 to glass slides after incubation with various treatments relative to the untreated control. The temperatures used for the heat treatment and denaturation of protease were 95°C for 15 min in Experiment 1 and 110°C for 10 min (heat trt) and 110°C for 20 min (protease denaturation) in Experiment 2.

Treatment	Mean Percent adhesion[a]	
	Experiment 1	Experiment 2
Control	53.1 e	72.0 d
Cycloheximide	55.3 e	25.1 b
Buffer (Tris-HCl)	48.7 de	43.0 bc
Sodium azide	44.0 d	65.0 d
Heat	33.9 c	61.4 cd
Protease (denatured)	20.1 b	59.0 cd
Protease	0.0 a	0.0 a

[a] Values with different letters in a column are significantly different at $P \leq 0.05$ LSD

Colonies developed from the spore suspension drops placed onto PDA showed that some treated conidia remained viable in all cases, except when heat treated when no growth occurred (Appendix A.4.1, A.4.2). Plating onto skimmed milk agar did not confirm the complete denaturing of the protease as the treatments containing conidia were accidentally used instead of the pure protease solutions. All plates showed a zone of casein degradation to some degree (data not shown). The experiment could not be repeated as budgets did not allow for purchase of more compounds.

Adhesion of heat treated conidia

There was a significant effect of heat treatment on the adhesion of conidia ($P<0.001$) (Appendix A.3.7). After 10 min incubation at 95°C, conidial adhesion was reduced significantly (mean 68.9%) (Fig. 2.9). After 15 min incubation, mean percent adhesion had further decreased significantly (56.0%) (Fig. 2.9). Thereafter, no significant decreases in mean adhesion were noted. Treatment at 110°C caused lower rates of adhesion than at 95°C (Fig. 2.9), with the same pattern of reduced adhesion with longer treatment time. Ten min incubation significantly reduced mean percent adhesion (28.0%) as did 15 min (7.5%), after which time no further decrease in adhesion was noted (Fig. 2.9). After 10 days incubation at 25°C no colony growth had occurred from the drops of conidial suspension placed onto PDA, showing conidia no longer remained viable after heat treatment (Appendix A.4.3).

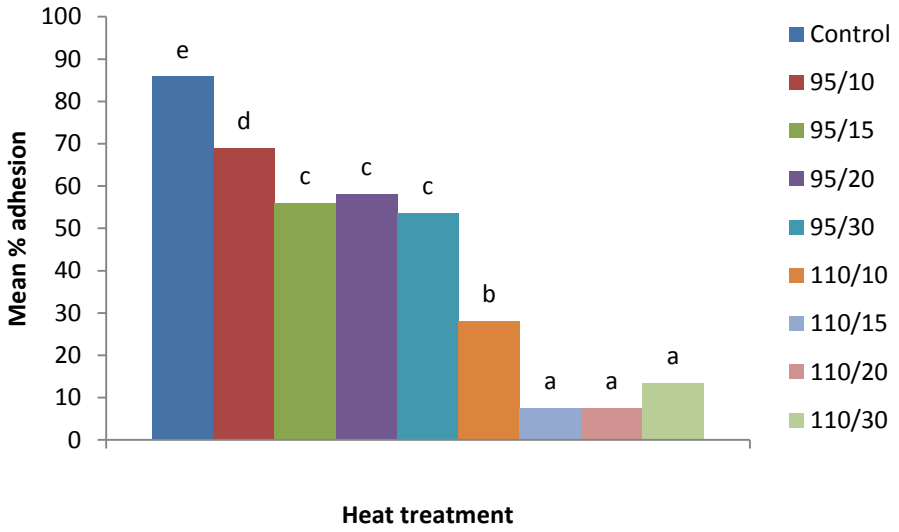

Figure 2.9 Mean percent adhesion relative to the untreated control of conidia of *N. luteum* isolate MM558 when subjected to treatment at two different temperatures (95°C and 110°C) for different times (10, 15, 20 and 30 min). Bars with different letters are significantly different at $P \leq 0.05$ LSD.

2.3.5 Adhesion of washed conidia

Washing did not affect the mean adhesion rate of conidia of *N. luteum* isolate MM558 (P=0.106) (Appendix A.3.8) with adhesion means for treatments being 70.3% (unwashed), 56.0% (washed twice) and 53.5% (washed four times).

2.3.6 Characterisation of spore surface components and spore mucilage

Conidia of all isolates stained positive with Coomassie brilliant blue after all incubation times, indicating a high level of protein in/on the spore coat and germ tube (Table 2.3, Fig 2.10 A and B) (Appendix A.5.2 G, A.5.3 D, A.5.4 E and F). In addition, there often appeared to be mucilage present at the germination point of conidia after 150 or 210 min incubation for all isolates except G652 (Fig 2.10 A and B) (Appendix A.5.2 G, A.5.3 D). For isolates MM558, CC445 and 007, alcian blue staining was negative on conidia incubated for 0, 5 and 90 min (Table 2.3). However, for 150 and 210 min incubation treatments, conidia were often seen with a distinct halo of blue, irrespective of whether they had germinated (Fig. 2.10 C and D) (Appendix A.5.1 B and C, A.5.2 A and B, A.5.3 A and B). This halo was more prominent for the *N. luteum* isolates (Fig. 2.10 B and C) (Appendix A.5.1 B and C, A.5.2 A and B) than for *B. dothidea* isolate 007 where the halo surrounded the germ tube as well as the conidium (Appendix A.5.3 A and B). This halo was not seen at any time for *N. parvum* isolate G652

either surrounding the conidium or germ tube (Appendix A.5.4 A and B). No isolate stained positive for acid fuchsin or Congo red at any time (Table 2.3). Negative staining with ink showed a clear zone around conidia incubated for 90 min or more. For isolates MM558 and CC445 the halo was only seen surrounding the conidia, even after germination (Fig. 2.10, E and F) (Appendix A.5.2 D and E). For *B. dothidea* isolate 007, the halo surrounded both the conidia and the germ tubes (Appendix A.5.3 G and H). For *N. parvum* isolate G652, the halo was very faint but appeared to also surround both the conidium and germ tube (Appendix A.5.4 H). Shrivelled, misshapen or empty conidia often stained positive for all the dyes tested, including acid fuchsin and Congo red, but as they were not considered to be viable they were not included in the results (Appendix A.5.1, A.5.2, A.5.3, A.5.4). Germ tubes were often seen with a small rounded extension near the tip of the germ tube after 210 min incubation (Appendix A.5.2 F, A.5.3 E, and A.5.4 D).

Table 2.3 Histochemical labelling of conidium surface components and mucilage of four Botryosphaeriaceae isolates with a range of dyes after incubation for various times.

Dye	Time (min)				
	0	**5**	**90**	**150**	**210**
Alcian blue (mucopolysaccharides)	-	-	-	±[a]	±[a]
Acid fuchsin (carbohydrates)	-	-	-	-	-
Coomassie brilliant blue (proteins)	+	+	+	+[b]	+[b]
Congo Red (amyloidic proteins)	-	-	-	-	-
Ink (negative stain)	-	-	±	±	±

[a] Staining isolate G652 with alcian blue was negative

[b] Isolate G652 conidia were blue but no adjacent halo was seen

Key to symbols: +all conidia were stained, ±some conidia were stained, -no conidia stained

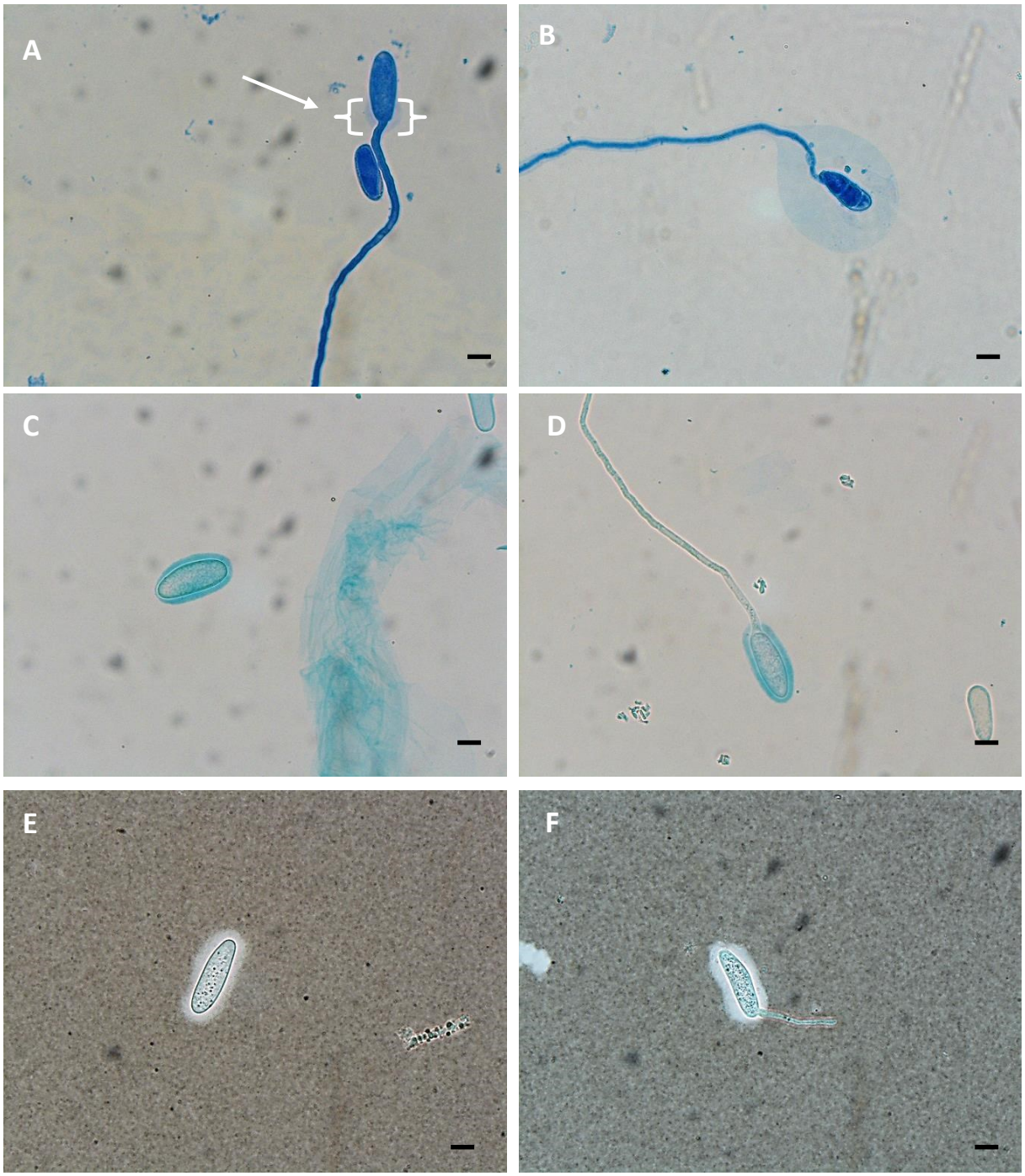

Figure 2.10 Conidia of *N. luteum* isolate MM558 incubated for various times then stained with various dyes. A) and B) Germinated conidia stained with Coomassie brilliant blue after 210 min incubation. A blue zone can be seen at the germination point (A) and totally surrounding the conidium (B); C) conidium and D) germinated conidium after 210 min incubation stained with alcian blue showing a blue halo surrounding each conidium;
E) conidium and F) germinated conidium after 150 min incubation stained with ink. A clear bright halo can clearly be seen surrounding each conidium. Scale bars = 8 µm.

2.4 Discussion

This research is the first reported attempt to elucidate the nature of the adhesion of conidia of Botryosphaeriaceae spp. and provides relevant information regarding adhesion behaviour. Although the artificial surfaces used in this study may not reflect the pathogen/host environment, it allowed for more accurate counting which was not possible with natural surfaces. The surfaces chosen differed in their hydrophobicity/hydrophilicity and as most plant surfaces are hydrophobic, it was felt that wettability was a relevant parameter with which to test adhesion.

Conidia of all the Botryosphaeriaceae isolates tested started to adhere soon after contact with all of the surfaces tested, with near maximum adhesion usually occurring after 5 min, or in the case of *N. luteum* isolate CC445, 20 min. This suggests that the wettability of a surface is not a major factor affecting the conidial adhesion of these pathogenic fungi and that the adhesion mechanism is relatively non-specific. In addition, immediate attachment to surfaces indicates a passive mechanism for adhesion, at least initially. This may reflect the biological function of Botryosphaeriaceae conidia, which is primarily for dispersal and immediate infection (Úrbez-Torres, 2011). Although green plant materials are generally hydrophobic, it would be advantageous for fungal spores to have the ability to adhere to a number of different tissues to increase the chances of infection. Although Botryosphaeriaceae spp. are thought to be primarily wound pathogens, this study shows that their conidia may have the potential to adhere to softer intact tissues such as buds and flowers, which have been shown to be susceptible to infection by Botryosphaeriaceae spp. (Amponsah et al., 2011; Wunderlich et al., 2011). In addition, the exposed tissue of fresh wounds, especially on green shoots, would be less hydrophobic than that of the cuticle, thus for wound pathogens it would be advantageous to be able to stick directly onto these surfaces. In a comparable study, Filonow (2003) showed that more conidia of wound colonising pathogens *Penicillium expansum* and *Botrytis cinerea* adhered to wounds in apple fruit (either fresh or 24 h old) than to the fruit cuticle, although it was not clear what caused this difference. For Botryosphaeriaceae species conidia it may be relevant to determine if more conidia adhere to stem wounds than to stem cuticles. However, in the natural environment, the splash dispersed conidia of Botryosphaeriaceae spp. need to 'strike a balance' between adhering to near landing sites (which may be on the host on which they

were produced) and being able to travel a certain distance to potentially colonise a fresh host. This balance is likely to favour relatively quick adherence to avoid conidia being splashed onto the soil.

This study has demonstrated that within a relatively short space of time, 50% of conidia had adhered to the surface upon which they landed, therefore repeated re-splash may not be able to disperse the spores over long distances. This concurs with the findings of Baskarathevan et al. (2013), who reported that no rain-splashed Botryosphaeriaceae spp. conidia were trapped beyond 2 m from their sources in vineyards, and with Ahimera et al. (2004) who trapped conidia of *B. dothidea* up to only 1 m from the tree canopy in pistachio orchards. As the maximum adhesion reached for any isolate (irrespective of surface) was approximately 50%, this suggests a difference in the ability of conidia to adhere, with some being more adhesion-competent than others. It is possible that the apparently similar conidia viewed under a microscope were not similar in their adhesive qualities.

Producing inoculum using the green shoot technique resulted in a variation seen in the colour of pycnidia. For example, pale brown pycnidia (Fig 2.3) may not have been fully mature and as a result oozed conidia that were less mature than those from black pycnidia. In addition, pycnidia often oozed on inoculated shoots before being harvested, sterilised and stored dry and the sterilisation method did not remove all of this ooze; the older, dried ooze was likely to have been washed into the spore suspension. However, the conidia within the dried ooze could have been dead, since Amponsah et al. (2010) showed that survival was reduced to 44% within 24 h if conidia were stored at less than 93% relative humidity, as was likely in the laboratory storage environment. The adhesive capabilities of these exposed conidia may also have been altered by the sterilisation methods or exposure to the external environment. Whether these factors could explain such a reduction in adhesion is unclear without further investigations. Mercure et al. (1994) suggested that there may be two types of conidia of *Colletotrichum graminicola*: those that are adhesion competent and those that are not, as maximum adhesion on surfaces was only 25-30%. Further, they suggested that a difference in adhesion competence raises the possibility of different genetic populations. The isolates chosen for the current study were all taken from cultures produced from single spore isolates (Baskarathevan, 2011; Billones-Baaijens, 2011). Since these isolates had been in -80^{o}C storage for two to three years there may have been some genetic changes prior to

this study, although this is unlikely given that any metabolic activity would have been slowed at this temperature. Further, instead of using these isolates directly from cold storage, they were plated onto PDA then used to infect green shoots from which fresh cultures were produced repeatedly throughout the study. It is unclear what effect this may have had.

There is no data available on the adhesion of spores of trunk pathogens in their natural environment and thus it is unclear whether the low rate of adhesion in this study is natural or induced by the experimental conditions. When Doss et al. (1995) investigated the adhesion of *Botrytis cinerea* conidia to a number of different surfaces they noted considerable variation in adhesion both between and within experiments. Unfortunately they did not speculate as to what caused these differences. It is possible that conidia of other species of plant pathogens also show high variability in their adherence capabilities, though this has not been mentioned in relevant adhesion studies in the literature. If adhesion is a two-step process and the initial adhesion phase is weak, these findings could represent only the initial weak adhesion of conidia. Conidia of Botryosphaeriaceae spp. may go through a number of adhesion phases, reaching higher levels of adhesion in phases beyond the maximum 1 h period used for this study. In a study by Apoga et al. (2001) adhesion of *Bipolaris sorokiniana* conidia occurred immediately on polystyrene, which was hydrophobic, but they were easily displaced by increasing the force of washing. It was suggested that this initial attachment was due to hydrophobic interactions, as addition of salt in the washing buffer (known to increase hydrophobic interactions) increased the rate of adhesion. The second phase of adhesion occurred after germination and was associated with the release of an extracellular matrix. This provided a much stronger bond than the initial adhesion phase and increasing the washing force did not dislodge the germlings. These findings suggest that the developmental stages of spores of some species of pathogenic fungi have an impact on the degree of adhesion. Although conidia of the Botryosphaeriaceae spp. isolates tested here started to adhere to surfaces immediately, the strength of the adhesion was not determined. If Botryosphaeriaceae spp. conidial adhesion is a two-step process, the implications are that the second adhesion phase, probably about the time of germination, would be harder to disrupt. Evidence for the possibility of a two-phase adhesion process in the Botryosphaeriaceae was supported by the histochemical characterisation of spore surface components and surrounding mucilage. For all isolates except *N. parvum* G652, there appeared to be a release of some kind of matrix material

about the time of germination. This matrix stained positive with alcian blue and Coomassie brilliant blue, implying it could be composed of mucopolysaccharides and protein. Braun and Howard (1994) demonstrated the presence of these compounds in the adhesive material surrounding conidia of *Colletotrichum heterostrophus.* They used nigrosin, a negative stain, to demonstrate a halo surrounding the germ tube. Negative staining of Botryosphaeriaceae spp. isolates with ink also showed a halo surrounding conidia and sometimes germ tubes at later incubation times when germination was being initiated. After 210 min incubation germ tubes were often seen with a small rounded extension near the tip of the germ tube and it is unclear whether these may be appressoria or the normal growth form of the mycelium and this should be investigated further.

The adhesion of conidia of *N. luteum* isolate MM558 was affected by pre-treatment with some of the inhibitory treatments but most results differed between the repeat experiments. However, the results obtained by treatment of *N. luteum* isolate MM558 conidia with pronase E were consistent across two experiments, in that adhesion was completely prevented. This suggests that spore surface proteins play a part in the adhesion process. In support of this, cycloheximide, an inhibitor of protein synthesis reduced adhesion in the second experiment (Experiment 2). This suggests that if protein interactions are involved in the adhesion of Botryosphaeriaceae spp. conidia, this process is not totally reliant on the synthesis of new proteins. As the incubation with pronase E was continuous with no washing, it was not ascertained whether conidia had the ability to regain their adhesive properties after the treatment was removed. This was demonstrated by Sela-Buurlage et al. (1991) who reported that conidia of *C. musae* treated with 100 μg mL of pronase E for one hour then washed with water showed a 22% decrease in adhesion in comparison to the heat denatured protease control (78%). However, after 90 min there were no differences between the two treatments in the numbers of conidia adhering. This suggests that the conidia of this fungus may have resynthesized new adhesive material. Spore surface proteins also appeared to be involved in the adhesion of conidia of *C. graminicola* to polystyrene (Mercure et al., 1994). When pre-treated with pronase E then washed, adhesion was reduced by 30%. In addition, when untreated conidia were allowed to adhere for 30 min and treated *in situ* with pronase E for 10 min or more, they completely lost the ability to adhere. In contrast, conidia of *B. cinerea* treated with pronase E in 10 mM Tris-HCl for 30 min then washed twice adhered just as strongly as the control (Doss et al.,

1993), indicating that cell surface proteins do not play a role in the early adhesion stage of this pathogen. It would be valuable to determine the precise role of proteins in the adhesion of Botryosphaeriaceae spp. conidia and whether treatment with a protease permanently or temporarily prevents adhesion.

The initial effects of the heat treatment on adhesion of Botryosphaeriaceae spp. conidia were contradictory. In the first experiment (Experiment 1, 95^{o}C for 15 min) heat caused a reduction in the mean rate of adhesion (33.9%) in comparison to the non-heated control (53.1%). In the second experiment (Experiment 2) the temperature was raised to 110^{o}C with an incubation time of 10 min but this had no significant effect on adhesion. Due to these inconsistencies, a further experiment was conducted with heat as the only treatment, with two temperatures (95^{o}C and 110^{o}C) and four treatment times (10, 15, 20 and 30 min). In the 95^{o}C treatment there was an initial reduction in mean adhesion after 10 min incubation, which dropped further after 15 min incubation but not thereafter. Although incubation at 110^{o}C greatly reduced the numbers of conidia able to adhere in comparison to treatment at 95^{o}C, the pattern was similar. The 95^{o}C treatments may not have been hot enough to degrade all the conidial coat proteins present but it was felt that 110^{o}C should have been hot enough to degrade most proteins. However, there were some conidia that still adhered after 15 min (7.5%), 20 min (7.5%) and 30 min (13.0%). It is possible that other physiochemical properties of the conidia that impact the rate of adhesion were not affected by heat. Previous experiments with heat treatment of spores have also demonstrated that only a proportion of the spores are affected. When Sela-Buurlage et al. (1991) 'exposed' conidia of *C. musae* to boiling water for 2 min there was a significant reduction in the numbers of adhering conidia (56%) in comparison to the untreated controls (85-97%) (Sela-Buurlage et al., 1991). In contrast, pycnidiospores (conidia) of *P. ampelicida* that had been treated for 10 min in boiling water adhered equally well as non-treated pycnidiospores (Kuo & Hoch, 1996). Conidia of *C. graminicola* that had been autoclaved for 23 min had a maximum of 10% adhesion; conidia were still intact, although plating on PDA confirmed they had been killed (Mercure et al., 1994).

The results for the other conidial treatments were contradictory between the first and second experiments. The variable results from the Tris-HCl buffer were unexpected, with no effect on adhesion in Experiment 1 but a reduction in mean adhesion (43.0%) in comparison

to the control (72.0%) in Experiment 2. There have been no reports in comparable literature of this buffer having an effect on spore adhesion (Doss et al., 1993; Newey et al., 2007; Rawlings et al., 2007) so the reason for this difference was unclear. In Experiment 1 the buffer was made up and used immediately without autoclaving, with the pH checked directly before use. In Experiment 2 the buffer was autoclaved before use, with the pH checked before autoclaving. It is possible the autoclaving may have slightly reduced the pH affecting adhesion as demonstrated by Bowen et al. (2000) with *Aspergillus niger* spores. However, given that this is a widely used buffer changes in pH would likely have been minimal as they have not been reported in the literature. The variable results from metabolic inhibitor treatments may be due to using different spore suspensions. Throughout the duration of this research it was noted that conidia of the same isolate sometimes adhered better and germinated faster than conidia produced on other days from other shoots. For example, the mean percent conidia that adhered in the control (untreated conidia) was 53.1% for the first experiment and 72.0% for the second. It is possible that some conidia were at different stages in their metabolic processes than others, due to the types of tissues used for lesion production and the age of stored lesions at the time of inducing pycnidia to ooze, which may have affected the maturity or age of the conidia. The metabolic inhibitors may therefore have had different effects on them.

There was no direct evidence in the literature for metabolic inhibitors having differential effects on spores at different stages of their metabolic processes. However, sodium azide resistance does appear to be linked to the rate of respiration of bacteria, which varied according to their developmental stage, as shown by Yadav et al. (1999) . While examining the respiration rate of cells of *Rhizobium leguminosarum* biovar *trifolii* strains resistant to sodium azide, they noted that lower respiration rates were associated with cells resistant to higher concentrations of sodium azide (15 µg/mL) and higher respiration rates with cells only resistant to sodium azide at lower concentrations (5 µg/mL). The findings of Minakshi et al. (2004) also indicated that a low level of resistance to sodium azide in strains of a *Bradyrhizobium* sp. may be associated with high respiration rates. While these studies did not speculate on why respiration rates affected sodium azide resistance, they have demonstrated a link between metabolism of the bacteria and the effectiveness of sodium azide. For fungal spores, respiration and associated reactions increase during water absorption and this process appears to be mediated by an active mechanism (Gottlieb,

1950). In addition, respiration rates appear to vary depending on the developmental stage of the spore. Goddard and Smith (1938) found the respiration rates of dormant spores of *Neurospora tetrasperma* increased up to 40 times upon being induced to germinate by heat treatment. Increases in respiration rate as measured by oxygen uptake were noted for uredospores of *Puccinia graminis* f. sp. *tritici* upon hydration (from 9 to 13 µL/mg/h), even before germination occurred (Maheshwari & Sussman, 1970). This was followed by a temporary decrease in respiration upon initiation of germination and a secondary and greater increase in the rate of respiration corresponding with germination and germ tube growth. These reports illustrate that spore respiration rates can vary depending on the developmental stage of the spore. It is therefore possible that the Botryosphaeriaceae spp. conidia used during this study varied in their rates of metabolic activity and as a result the effect of sodium azide on these conidia also varied.

There may also have been minor differences in methods used, which affected the results. In both adhesion experiments, spore suspensions were prepared and treatments were applied in one room, then slides were incubated (20 min) and washed with water in another room. Since time differences taken to complete this procedure on the different days could have been only a few min, it was unlikely to be the cause of variation. However, very small weights and volumes were used in preparation of the metabolic inhibitors to the correct concentration. Since measuring equipment has an empirical error, the proportion of error in low weights or volumes may have been quite high and this may have led to some differences in results. It is also possible that slight changes in temperature, incubation time or other variables may have a dramatic effect on results if the experimental conditions were marginal. This may have implications for future adhesion studies and indeed studies that have already been performed with metabolic inhibitors. It is possible that one repeat is not enough when running these kinds of experiments and they should be replicated several times under strict conditions where possible. The possibility of inconsistent results has not been visited in the relevant literature as researchers have not reported repeating these types of experiments. In addition, aside from their intended purpose, it is not clear what other effects the different treatments had on the properties of conidia. The effect of metabolic inhibitors on spore properties has been demonstrated by (Mercure et al., 1994) who found that sodium azide and cycloheximide added to a suspension of *C. graminicola* conidia reduced germination by 30% and 100%, respectively. Rawlings et al. (2007) also

found that these inhibitors affected the germination of *C. lindemuthianum*, with cycloheximide completely inhibiting germination. In the current study, germination of conidia of *N. luteum* isolate MM558 was also affected; when conidia treated with cycloheximide were plated onto PDA, germination rates appeared to be low, even after 5 days incubation at 25^{o}C (Appendix A.4.2) and treatment with sodium azide appeared to reduce mycelial growth (Appendix A.4.1, A.4.2). Unfortunately the simple germination test used two drops of a treated conidial suspension placed on to PDA plates, which did not allow for an accurate measure of the amount of mycelial growth inhibition.

As conidia of Botryosphaeriaceae spp. are oozed in a "gelatinous matrix" from pycnidia in the presence of high moisture (Úrbez-Torres, 2011), it is possible that this matrix may play a part in the initial adhesion of spores. However, histochemical staining of spores immediately, or at 5 and 60 min after suspending them in water did not indicate the presence of matrix material (expected to be variable in thickness) surrounding the conidia. Since this matrix material is water soluble, it is likely to be washed off when preparing the spore suspension from green shoots. Nicholson and Moraes (1980) demonstrated that the spores of *C. graminicola*, exuded in a matrix from acervuli, are composed of protein and polysaccharides. However, Mercure et al. (1994) reported that the main function of this matrix was to prevent desiccation of spores and not spore adhesion. Rawlings et al. (2007) also demonstrated that the water soluble mucilage surrounding the conidia of *C*. *lindemuthianum* upon release from acervuli played no role in adhesion. They showed that when conidia were washed twice for 10 min, followed each time by centrifugation, they still adhered to polystyrene, reaching almost 100% adhesion within 10 min. In the current study, the adhesion rate of conidia of *N. luteum* isolate MM558 to glass was also unaffected by washing, indicating that the matrix surrounding conidia upon their release from pycnidia plays no part in the adhesion process or was not removed by the washing regime used.

Variation amongst 'batches' of conidia was also observed in the experiment examining adhesion of conidia to different surfaces. The adhesion data for non-treated conidia showed considerable variation in percent conidia adhered between replicate experiments; results were not surface specific and therefore the reason for this was unclear. For example, numbers of conidia of *N. luteum* isolate MM558 adhering to glass after 20 min incubation ranged from 20% to 82% (data not shown). A superficial examination of the shoot and lesion

factors did not indicate an obvious relationship to the age of the lesions (length of time between drying and incubating for oozing spores). The variation may be explained by the nature of conidia in the pycnidial ooze. Although the conidia were suspended in water in a consistent way and attempts were made to keep them suspended in the water with frequent mixing, it is possible that they clumped together, with low or high numbers of conidia being randomly deposited onto a surface. Since values were occasionally either very low or very high (higher than the control), this theory has some merit. The clumping of *B. dothidea* conidia within the ooze was also suggested by Ahimera et al. (2004) to explain anomalies in a splash dispersal experiment with infected pistachio nuts. The individual water droplets released onto the nuts from a height of 1 m produced a number of splash droplets, with 56% of the conidia landing close to the source (15 mm). However, they reported that the expected gradient of reducing conidial numbers at further distances was interrupted by a 'spike' in conidia per droplet 55 mm from the source, which they suggested was caused by splashing parts of ooze rather than individual conidia, thus grouping more conidia together per droplet.

Production of pycnidia using the green shoot technique (Amponsah et al., 2008), did not give consistent results for any of the isolates and it is possible that this variability was related to the physiology of the shoots used. In the first year of study (2012/2013) the season was warm and shoots were produced in the vineyard over a longer period of time. This resulted in an ample supply of fleshy green shoots from which abundant pycnidia were consistently produced and that reliably oozed conidia. The following season (2013/2014) was different, with cooler temperatures resulting in fewer shoots that were harder and thinner than in the previous season. Pycnidial production on these shoots was variable, pycnidia often being brown instead of black (Fig 2.4), with fewer conidia. To provide a continuous supply of shoots during the dormant season, potted greenhouse-grown vines were also used, though they often had semi-hard shoots which produced fewer pycnidia than vineyard shoots.

The problems with producing enough inoculum on a regular basis caused delays to the study. A search of the literature found no evidence of a better method. Many pathogenicity studies performed with Botryosphaeriaceae spp. have used agar plugs of actively growing mycelium as the source of inoculum (Taylor et al., 2005; Savocchia et al., 2007; Úrbez-Torres et al., 2009; Billones-Baaijens et al., 2013a). It is unclear why these authors used

mycelium instead of conidia; it is possible that the lack of a reliable method for spore production across Botryosphaeriaceae spp. isolates may explain this. Van Niekerk et al. (2004) reported using sterilised pine needles within water agar incubated at 25°C under near-ultraviolet light for production of conidia; however, this method was slow and found to be ineffective with New Zealand isolates (M. Jaspers, personal communication, 2014). A comparative study by Amponsah et al. (2008) tested a range of media and showed that the green shoot method produced greatest numbers of conidia of *N. luteum*, *N. australe* and *D. mutila*, but did not work for the *N. parvum* isolates they selected. Later research (M. Jaspers, personal communication, 2014) showed that some isolates were better able than others to produce conidia by this method and subsequently those isolates were used in the current research. Unfortunately, the success of the method was found to be affected by the condition of the shoots. Not all apparently robust pycnidia produced the expected ooze of conidia after overnight incubation under high relative humidity. As this was not discovered until the day allocated to an experiment, it often took a further 2-3 weeks before new infected shoots were produced. In order to conduct studies in a more consistent manner while also allowing comparisons between different studies, it would be useful to develop a method of producing conidia across a broader range of species, ideally one that does not rely on a year-round supply of grapevine plant material.

Although it was outside the scope of this study, some reports have indicated a link between adhesion and virulence of some pathogens. Ding et al. (1994) showed that treatment of *Phytophthora megasperma* f.sp. *glycinea* zoospore cysts with an antibody targeting adhesion molecules prevented the pathogen from adhering to host cell walls and thereby prevented infection of wounded leaf tissue. The normal growth and germination of the pathogen was not affected by this treatment. Jones and Epstein (1989) found that for an adhesion deficient mutant of *Nectria haematococca* (a pathogen of cucurbits) the microconidia showed an approximate 50% reduction in adhesion to fruits or polystyrene in comparison to the wild type strain. Furthermore, this mutant was less virulent in unwounded fruits, producing far less macerated tissue. However, there was no difference between strains in virulence on wounded fruits, for which pathogen entry did not require adherence by microconidia. These studies, which demonstrated that alteration of the adhesion properties of spores reduced the subsequent infection rates and virulence of the respective pathogens, have indicated the

potential importance of the adhesion mechanisms of Botryosphaeriaceae spp. conidia, an aspect that to date has not been investigated.

In conclusion, this study has shown that conidia of Botryosphaeriaceae spp. adhere to surfaces immediately upon contact and that surface wettability does not play a major role in the adhesion process, which occurs in a relatively non-specific manner. Adhesion rates were variable but reached a maximum of only 50%. Proteins appeared to be involved in the adhesion mechanism as treatments that disrupted proteins (such as pronase E or heat) prevented or disrupted adhesion. The staining techniques used showed a release of matrix materials that consisted of proteins and mucopolysaccharides about the time of germination, indicating a second, active adhesion phase.

Chapter 3

Germination and development of Botryosphaeriaceae spp. conidia on different surfaces

3.1 Introduction

Once fungal propagules have adhered to a plant surface a number of developmental stages usually occur that can determine whether infection is successful or not (Tucker & Talbot, 2001). The triggers for spore germination are many and varied. Germination and differentiation can often be affected by stimuli such as nutrient availability and hydration or by the physical properties of the surface. If the appropriate signals are not received, then germination may not occur or the germ tube might remain undifferentiated and unable to penetrate when any nutrient reserves in the spore are used up. Germ tube elongation may also be reliant on a number of complex signals including those received from the plant itself (Tucker & Talbot, 2001). Signals may also be related to the surface topography, hydrophobicity or hardness of the surfaces on which the spores begin development. To date there is no information available on the cues that may trigger spore germination and germ tube elongation in Botryosphaeriaceae spp. conidia. It is possible that conidia may adhere directly to the wounds known to represent susceptible tissues in the vine framework, but equally they may adhere to some other plant tissue on the vine and then need to germinate and grow towards wounds and/or openings. These two scenarios may involve surfaces differing in their hydrophobicity or hardness, as a new shoot for example is softer than a lignified stem. This study will aim to determine if the nature of a surface, specifically wettability and hardness, has an impact on the germination and subsequent growth of Botryosphaeriaceae spp. conidia. It will also try to determine what, if any, infection structures may be produced in the early stages of conidial development.

3.2 Materials and methods

3.2.1 Inoculum preparation

Unless stated otherwise, conidial suspensions were prepared from infected shoots using the methods described in Section 2.2.2. Spore concentrations were adjusted based on haemocytometer counts.

3.2.2 Germination of conidia on different surfaces

In this experiment, conidial suspensions of Botryosphaeriaceae species were placed onto the same test surfaces as described in Section 2.2.5 where they remained for either 3 h or 6 h before germination rates and germ tube growth were assessed. The number of germinated conidia was determined by microscopy and indicated the germination rates on that surface. Germ tube growth was determined by imaging germinated conidia and measuring the length of germ tubes. One isolate with all surfaces and time points, replicated three times ('pseudoreplicates') was assessed on a single day. Each isolate/surface/time point combination was repeated six times (which represented replicates). The order of isolates and replicates was randomly allocated on each day

Germination and germ tube growth were evaluated with the same four isolates as described in Section 2.2.1. A 10 µL drop (~150 conidia) of each conidial suspension was placed onto each surface beside the marker line before being incubated for 3 h or 6 h in a humidity chamber in the dark at 20^{o}C. For each time point, three replicates of each surface received a drop of conidial suspension and the same procedure was repeated for the next time point. The order in which the surfaces were inoculated was randomly allocated for each time point. The mean percentage of germinated conidia was determined by assessing germination of 100 conidia in microscope fields of vision, starting from the marker line. Conidia were considered to have germinated when the germ tube length was at least half the width of a conidium. Reported values represent the average of three counts/surface/time point, with six average values representing true replications. To determine the growth of germinated conidia on different surfaces, photographs were taken of each surface after 6 h incubation (five for each replication). Each surface was superficially scanned under the microscope and photos taken when three or more germinating conidia were seen in one microscope view. Photos were each assigned a number and then selected by random number generation.

Starting from the top left, the germ tube length of each germling on one photo was measured. This procedure continued across photographs until 10 germ tubes had been measured per surface. Germinated conidia were imaged at x100 magnification using a DS Fi2 camera attached to an Olympus CX41 microscope. Germ tubes were measured using AnalySIS® imaging software (V 3.2, Soft Imaging System). Reported values represent the average of 10 measurements/surface/replicate after 6 h incubation.

As initial results indicated that germ tube growth on cellulose was a lot slower than on the other surfaces, it was decided to try and ascertain whether conidia of the same isolates as before were utilising the cellulose as a food source. A plate screening method (Shahriarinour et al., 2011) using carboxyl methyl cellulose (CMC) agar was chosen for this study. For each isolate, mycelial plugs were taken from the edges of actively growing (3 day old) colonies on PDA plates and each was placed into the middle of a CMC agar plate (Appendix B.1). Plates were incubated at 25^{o}C in 12 h/12 h light/dark. Isolates of *Trichoderma* sp. (LUPP5550) and *Pythium* sp. (LUPP1177), which represented genera that are known to be cellulose degraders (Pathrose, 2012), were subcultured onto PDA and mycelial plugs from the 3 day old colonies placed onto CMC agar plates to act as positive controls. After 3 days incubation, CMC agar plates were flooded with 1% w/v Congo red (Coomak Chemicals Ltd) for 30 min and then destained with 1M NaCl (LabChem) for 30 min. A clearance zone around the growing colony indicated cellulose breakdown. Variations of the CMC agar recipe were also tried by increasing the CM-cellulose concentration to 20 g L^{-1} and by adding one drop of Triton X-100 (Science Lab, USA) to try and slow down colony growth. In addition, a CMC concentration of 40 g L^{-1} was used to try and improve the distribution of the cellulose throughout the agar, which was often uneven at lower concentrations.

3.2.3 Effect of surface hardness on conidial germination

The effect of surface hardness on the germination of conidia was studied using water agar at different concentrations (1%, 2%, 4% and 8%), with 1% agar being soft to the touch and 8% being very firm. Agar (~20 mL) was poured into standard Petri dishes and allowed to set. Glass slides were used to represent the hardest surface. In order to check that the water content of the different agars was similar, water activity was measured using an AquaLab LITE (V4, Decagon Devices Inc.) water activity meter. Three readings were taken for each agar concentration. On the base of each Petri dish, three 1.5 cm x 1.5 cm squares were

drawn in random positions with a marker pen and a 10 µL drop (~150 conidia) of conidial suspension placed into the centre of each square. Plates were immediately sealed with food grade cling film, put onto a tray and covered with foil before being placed into an incubator at 20°C for three hours. After this time, plates were removed and immediately stained with lactoglycerol aniline blue (Appendix A.1) to halt any further growth. The squares of agar were then cut out with a scalpel and placed onto a glass slide. The microscope was positioned at the uppermost conidium seen on the agar square and then moved one field of vision downward. From this point, the mean percentage of germinated conidia was determined by assessing germination of 100 conidia in adjacent fields of vision. The number of germinated conidia within each agar square was expressed as a percentage. For examining the effects of hard glass slides, four counting areas were created on each slide by drawing lines across the width of the slide with a permanent marker (Fig 3.1). A drop of conidial suspension was placed into the middle of three of the four areas, which were randomly chosen. Each slide was placed into a standard Petri dish on top of a moist paper towel to maintain humidity. The dish was immediately sealed with cling film and incubated with the agar plates. After two hours, the slides were removed, immediately stained, and assessed for germination as before.

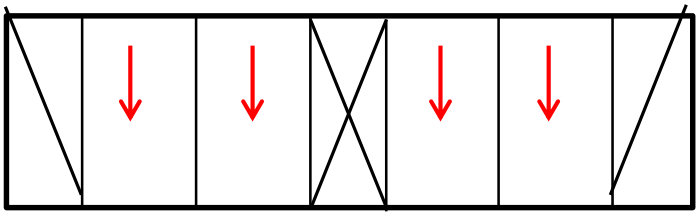

Figure 3.1 Glass slide marked with counting areas. Red arrows show approximate position of conidial suspension droplet within a counting area.

Reported values represent the average of three counts per hardness type, replicated six times. The effect of surface hardness on germ tube growth was also studied. Ten randomly selected germlings per surface hardness were imaged and measured as before. Reported values represent the average of 10 measurements/surface hardness/replicate.

3.2.4 Effect of surface contact on germination

In order to determine whether conidia require surface contact in order to germinate, an experiment was conducted with a constantly shaken conidial suspension at room temperature (20-24^{o}C). A sample of conidial suspension removed from shaking flasks after three hours was assessed to determine the percentage of germinated conidia. Three flasks were used in the experiment, with each flask being considered a replicate. Only one Neofusicoccum spp. isolate was used during this investigation.

Shoots infected with *N. luteum* isolate MM558 were incubated overnight as described in Section 2.2.2 to promote conidial oozing. A 250 mL Pyrex flask was attached to a clamp on the end of each arm of a four arm wrist action shaker. One of the flasks was filled with 120 mL of SROW and the shaker set to 450 oscillations/min. This speed was fast enough to cause good movement but not so fast as to flick the water high up the sides of the flask. Twelve oozing shoots of various sizes were gently dropped into the moving flask and left shaking for 10 min. After this time, 15 mL of conidial suspension was removed from the master flask and pipetted into each of the three remaining flasks with the shaker continuously on at the same speed as before. Flasks were stoppered with sterile sponge stoppers and the shaking continued for three hours. After this time, the contents of each of the three flasks was decanted into separate 15 mL Falcon® tubes (Axygen) and centrifuged at 4696 x*g* for 10 min. The supernatant was discarded and then the conidia were re-suspended in 2 mL aliquots of SROW. Five drops of lactoglycerol aniline blue were added immediately to each of the tubes to halt any further growth. From each tube, six 10 µL drops of conidial suspension were removed and each was placed onto a separate glass slide. The microscope was positioned at the uppermost conidia seen on the slide and then moved one field of vision downward. From this point, the mean percentage of germinated conidia was determined by assessing germination of 100 conidia in adjacent fields of vision. Reported values represent the average of six counts for each of the three replicates.

In addition to conidial suspension being placed into shaking flasks, 15 mL of conidial suspension from the master flask was also pipetted into each of three Petri dishes containing a glass slide in the bottom. After the same three hour incubation period as the shaken conidia, the slides were gently removed from the dishes, placed onto a paper towel and immediately stained with lactoglycerol aniline blue. Each slide was covered with two

coverslips which nearly covered the entire length of the slide. Within each coverslip, a vertical sweep was made on the left, in the middle and on the right, assessing 100 conidia from the top within each sweep. Values reported represent the average of six counts replicated three times. To assess whether suspended non-moving conidia behaved differently to shaking conidia, a sample of the liquid surrounding the glass slide was also assessed. The conidial suspensions left in the Petri dishes after the slides had been removed were decanted into separate 15 mL Falcon® tubes and centrifuged, re-suspended in SROW and stained as stated previously. From each tube, six 10 µL drops of conidial suspension were removed and each was placed onto a separate glass slide. Germination was assessed as before. Reported values represent the average of six counts for each of the three replicates.

3.2.5 Determination of infection structures

In this experiment, the internodes of grapevine shoots from potted vines were inoculated with *N parvum* isolate G652 conidia and incubated for various time points ranging from 6 h to 24 h. Inoculated shoot pieces were sectioned by hand (longitudinally and transversely), processed to halt further growth and to clear the plant tissue and then stained and examined to determine if any infection structures were present. Preliminary testing of methods was performed with *N. luteum* isolate MM558.

Plant material

Potted, 2-year-old Pinot noir vines were cut back to two nodes on one year old wood and left for seven weeks in the greenhouse to produce new green shoots. Eight plants, each with two healthy shoots, were chosen for the experiment. The plants were placed into a humidity chamber and laid down at an angle so that the shoots were horizontal (Fig 3.2 A and B). Humidity in the chamber was maintained by regular misting from sprinklers positioned behind the potted vines. Plastic pots (5 L) were placed upside down under each shoot for support. Shoots were loosely held in place on the pot bases with masking tape (Fig 3.2 C). Inoculation points (three per internode) were marked with a water proof marker on the two internodes closest to the shoot tip (Fig. 3.2 C). Each internode with three inoculation points represented one time point, with internodes and time points randomly allocated over the four replicate plants. Each replicate consisted of two plants, one that was non-wounded when inoculation occurred, and one that was wounded immediately before inoculation. Wounds (~5 mm) were made in the centre of each inoculation point using a sterile scalpel.

Plant inoculation

A conidial suspension of *N. parvum* isolate G652 was produced as before (~$5x10^4$ conidia/mL) and a 10 µL drop was pipetted onto each of the inoculation points. All non-wounded stems were inoculated first in a random order, followed by all wounded stems. The 24 h incubation time was inoculated first followed immediately by the 6 h time. Aliquots of conidial suspension (10 µL) were also placed onto individual glass slides and incubated at 20°C (3 slides for 6 h and 3 slides for 24 h) to check germination rates. The conidial suspension was retained on ice and then used to inoculate the 12 h treatment after a further 12 h. A germination check was also done on glass slides after this time as before. After the appropriate incubation times, shoot internodes were cut and immediately transported to the laboratory for processing. Shoots were placed into a plastic box containing plasticine to keep the shoots the correct way up and to prevent conidial displacement during transfer (Fig 3.2 D).

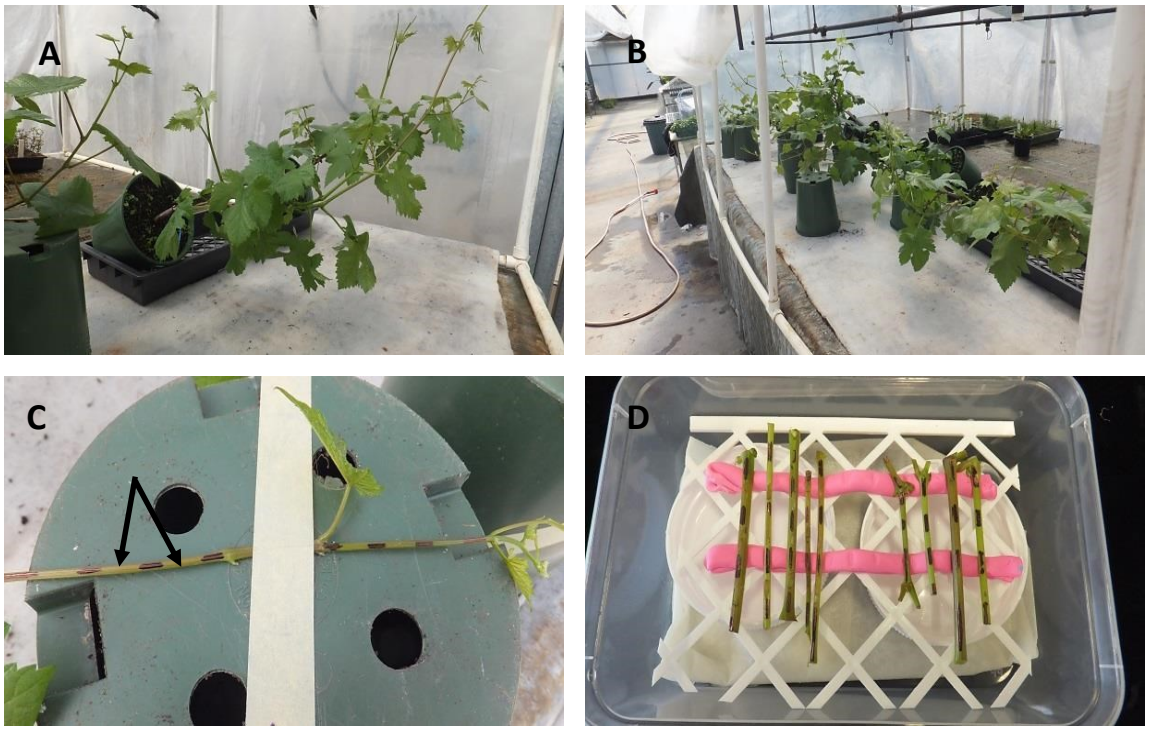

Figure 3.2 Potted Pinot noir vines in a humidity chamber. A) and B) Pots laid at an angle so that inoculated shoots were horizontal; C) shoot tips with marked inoculation points (arrows) gently taped in place onto the bottom of a 5 L pot; D) shoot pieces placed onto plasticine in a humid container for transport to the laboratory.

Hand sectioning of stem tissue

Shoot sections were cut using a double edged razor blade (Astra Superior Platinum), each being cut in half lengthways before use. For transverse shoot sections, shoot pieces were placed lengthways onto a glass cutting board and held in place at one end by hand. Sections were cut vertically, with cutting initiated just outside the inoculation zone and continuing to just past the inoculation point. The razor blade was moistened before use by dipping into water to facilitate cutting. When tissue sections became stuck to the razor blade they were gently rinsed off into a Petri dish containing water. For longitudinal sections, cutting of shoot pieces began about 1 cm above the inoculation point. The shoot pieces were positioned vertically on the cutting board and held upright with a pair of tweezers. A 0.5 cm cut was made in the top end of the shoot next to the inoculated side. While still holding the shoot upright, a pair of tweezers was used to take hold of that sliced piece and it was pulled downward past the inoculation point to the base of the shoot. The result of this was a thin peel of the cuticle. The thicker end of the longitudinal piece was cut off. A diagonal cut was made in the top left corner of the section to ensure it was oriented correctly on the microscope after processing (Fig. 3.3).

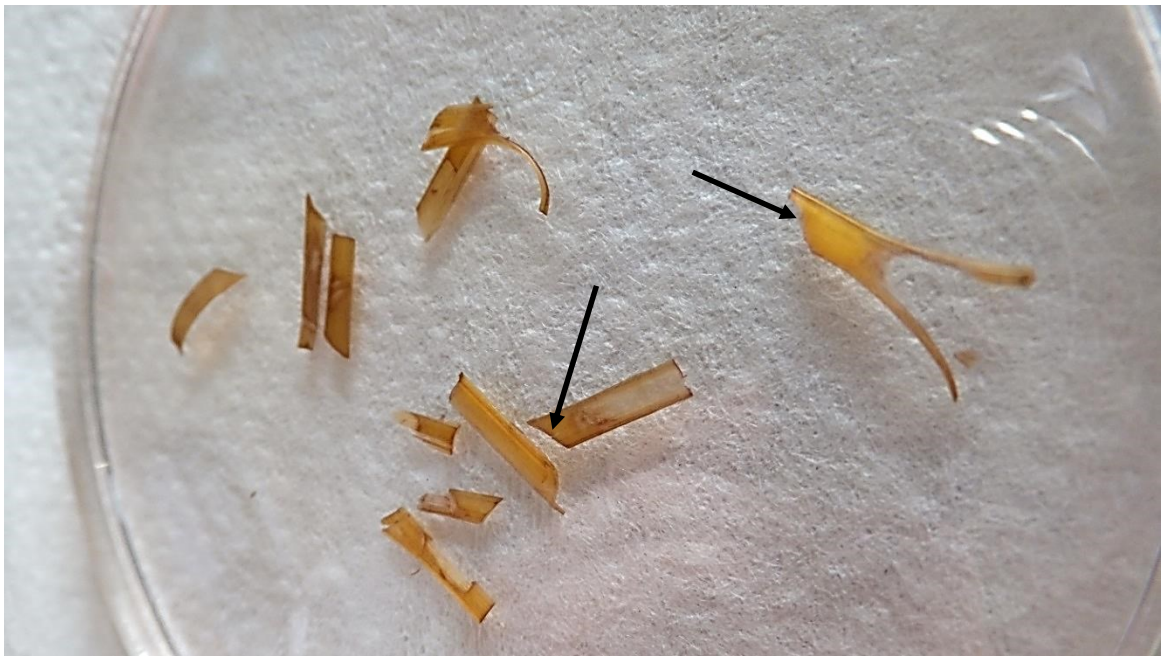

Figure 3.3 Longitudinal sections of wounded Pinot noir stem tissue inoculated with *N. parvum* isolate G652. Tissue sections had been cleared by autoclaving in 1M KOH. Diagonal cuts at the corner of tissue (indicated by arrows) enable correct orientation of tissue for examination under the microscope.

Processing of sectioned plant tissue

The method of Hood and Shew (1996) was used with minor modifications. Transverse and longitudinal shoot sections were placed into Universal bottles containing ~20 mL 1M KOH, autoclaved (121^{o}C for 15 min), and then left to cool. As the shoot tissue was fragile after autoclaving, it was gently decanted into a Petri dish and the KOH pipetted off into a waste container. The shoot tissue was washed twice by gently flooding the Petri dish with SROW, agitating for a minute and then pipetting off the waste SROW. Shoot pieces were left in a small volume of SROW until staining was performed (1-2 h). Shoot pieces were gently removed from the SROW with a soft paintbrush and then placed onto a glass slide. Excess water surrounding the shoot piece was blotted with a paper tissue. Staining was then performed with a drop of 0.05% aniline blue (J.T Baker® brand) in 0.067 M K_2HPO_4 at pH 9.0 (Hood & Shew, 1996) or with a drop of Calcofluor White (Fluka Analytical, one drop of undiluted Calcofluor White being followed by one drop 10% w/v potassium hydroxide (LabServe). Photos were taken with an Olympus BX51 microscope with a camera attached (Olympus DP70). The camera was equipped with a U-MWU2 filter (excitation filter 330-385 nm, emission filter 420nm, and dichromic filter 400nm). Photos were taken with Cell^F imaging software (Olympus).

3.2.6 Statistical analysis

Data were analysed using GenStat 16th edition (VSN international). For germination on different types of surface, analysis was performed using a two-way analysis of variance (ANOVA). For germination on surfaces of different hardness, and for germination without surface contact, a one-way ANOVA was performed. Where the ANOVA analysis reported a significant effect, differences between means were determined by using Fisher's protected least significant difference test (LSD) at $P \leq 0.05$. Differences between means are called significant within the following results only if they meet this criterion. The data presented in tables and graphs were those generated by the ANOVA analysis.

3.3 Results

3.3.1 Germination of conidia on different surfaces

For *N. luteum* isolate MM558, there was a significant effect of surface on mean percent germination ($P<0.001$, Appendix B.2.1). Mean percent germination on cellulose (72.2%) was significantly higher than on the other surfaces tested (Fig. 3.4). On glass, polystyrene and parafilm, there were no significant differences in the mean number of germinated conidia (65.9%, 62.8% and 63.3%, respectively) (Fig. 3.4). There was no significant effect of time on mean percent germination ($P=0.421$, Appendix B.2.1), with similar mean germination rates after 3 h and 6 h on all surfaces. There was no significant interaction between surface and time ($P=0.951$, Appendix B.2.1). There was a significant effect of surface on mean germ tube growth ($P<0.001$, Appendix B.2.5). Mean germ tube growth was significantly slower (86.3 µm) on cellulose, than on the other surfaces tested (Table 3.1). On glass, polystyrene and parafilm, there was no significant difference in the mean length of germ tubes (141.7 µm, 152.1 µm and 142 µm, respectively).

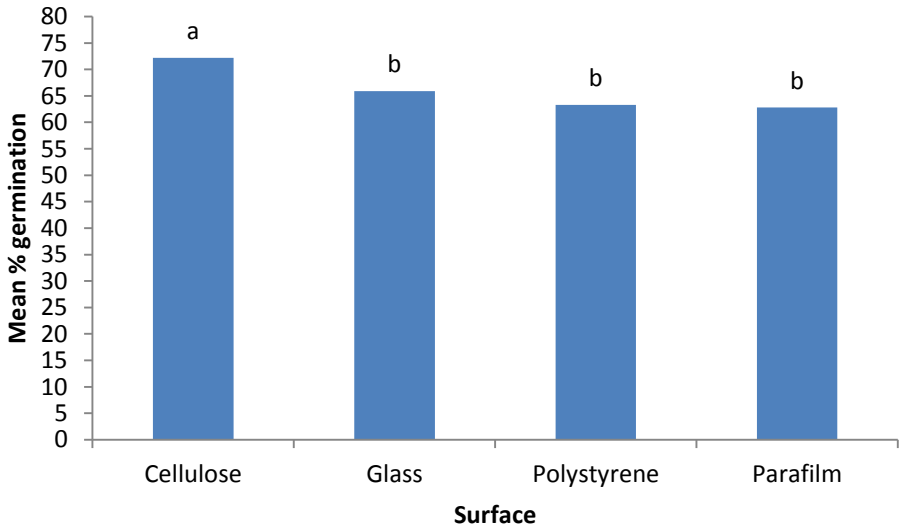

Figure 3.4 Mean percent germination of *N. luteum* isolate MM558 on different surfaces averaged across both 3 and 6 h. Bars with different letters are significantly different at $P\leq0.05$ LSD.

For *N. luteum* isolate CC445, there was a significant effect of surface on mean percent germination ($P<0.001$, Appendix B.2.2). Mean percent germination on cellulose (76.9%) was significantly higher than on the other surfaces tested (Fig. 3.5). On glass, polystyrene and parafilm, there were no significant differences in the mean number of germinated conidia (52.9%, 49.4% and 47.8%, respectively) (Fig. 3.5). There was no significant effect of time on mean percent germination ($P=0.078$, Appendix B.2.2), with similar germination rates after 3 h and 6 h on all surfaces. There was no interaction between surface and time ($P=0.864$, Appendix B.2.2). Mean germ tube length was significantly less ($P<0.001$, Appendix B.2.6) on cellulose (mean 81.4 µm), than on the other surfaces tested (Table 3.1). On glass, polystyrene and parafilm, there were no significant differences in the mean length of germ tubes (123.2 µm, 113.6 µm and 124.7 µm, respectively).

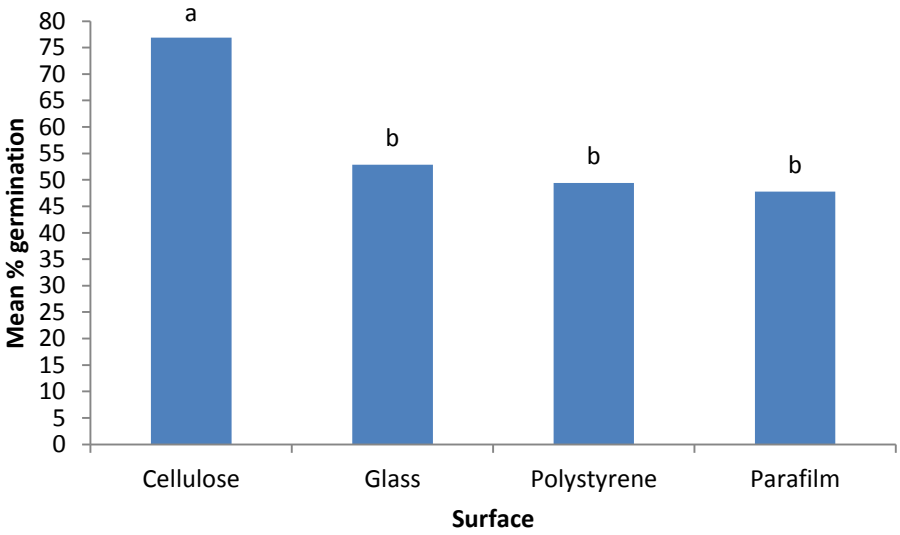

Figure 3.5 Mean percent germination of *N. luteum* isolate CC445 on different surfaces averaged across both 3 and 6 h. Bars with different letters are significantly different at $P\leq0.05$ LSD.

For *N. parvum* isolate G652, there was a significant effect of surface on mean percent germination (*P*=0.035, Appendix B.2.3). The mean germination rate on cellulose (52.4%) was significantly higher than on glass (47.5%), polystyrene (46.1%) or parafilm (47.3%) (Fig. 3.6). There was also a significant effect of time on mean percent germination (*P*<0.001, Appendix B.2.3), with more conidia having germinated after 6 h (51%) than after 3 h (45%). There was no significant interaction between surface and time (*P*=0.783, Appendix B.2.3). There was a significant effect of surface on mean germ tube growth (*P*=0.010, Appendix B.2.7). The mean length of germ tubes on cellulose (60.9 μm) was significantly less than on glass (73.7 μm), polystyrene (77.8 μm) or parafilm (73.0 μm), for which there were no differences in the mean germ tube lengths (Table 3.1).

For *B. dothidea* isolate 007, there was a significant effect of surface on mean percent germination (*P*=0.024, Appendix B.2.4). The mean germination rate on cellulose (74.9%) was significantly higher than on glass (64.2%), polystyrene (62.2%) or parafilm (61.9%) (Fig. 3.7). There was also a significant effect of time on mean percent germination (*P*=0.004, Table B.2.4), with more conidia germinating after 6 h (70.9%) than after 3 h (60.8%). There was no significant interaction between surface and time (*P*=0.936, Appendix B.2.4). There was a significant effect of surface on mean germ tube growth (*P*=0.029, Appendix B.2.8). The mean length of germ tubes on cellulose (67.8 μm) was significantly less than on glass (95.2 μm), polystyrene (87.2 μm) or parafilm (85.9 μm), on which there were no differences in mean germ tube lengths (Table 3.1).

Table 3.1 Mean lengths of germ tubes (μm) of four Botryosphaeriaceae species isolates on different surfaces. Incubation time on each surface was six hours.

Surface	Isolate[a]			
	***N. luteum* MM558**	***N. luteum* CC445**	***N. parvum* G652**	***B. dothidea 007***
Cellulose	86.3 a	81.4 a	60.9 a	67.8 a
Glass	141.1 b	123.2 b	73.7 b	95.2 b
Polystyrene	152.1 b	113.6 b	77.8 b	87.2 b
Parafilm	142.0 b	124.7 b	73.0 b	85.9 b

[a] Mean values with different letters within columns are significantly different at *P*≤0.05 LSD

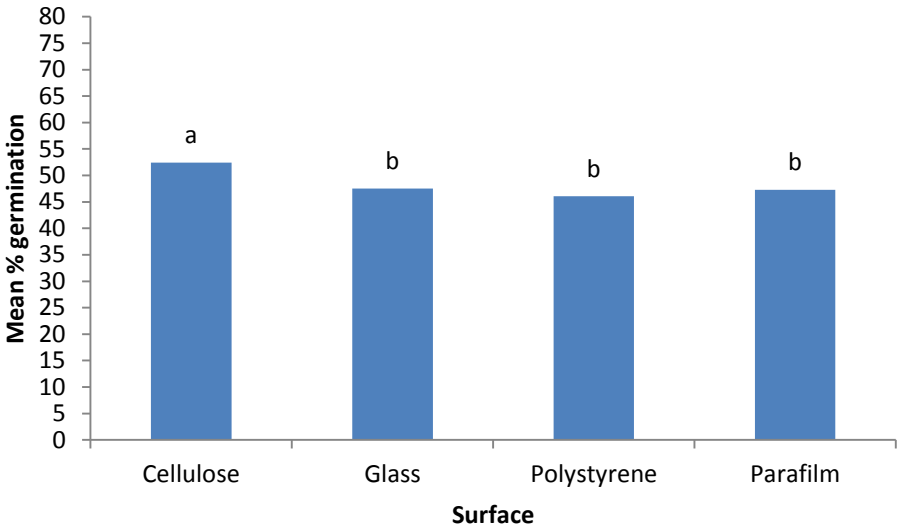

Figure 3.6 Mean percent germination of *N. parvum* isolate G652 on different surfaces averaged across both 3 and 6 h. Bars with different letters are significantly different at *P*≤0.05 LSD.

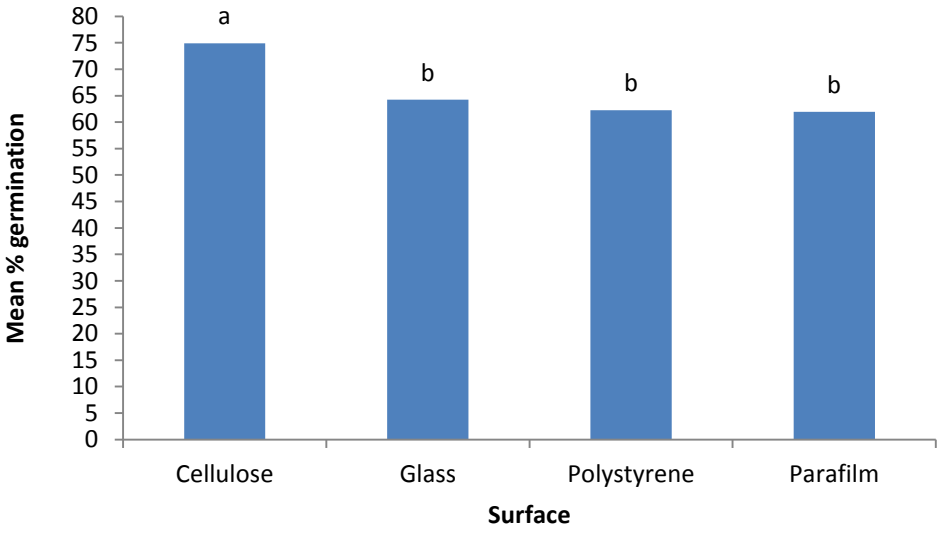

Figure 3.7 Mean percent germination of *B. dothidea* isolate 007 on different surfaces averaged across both 3 and 6 h. Bars with different letters are significantly different at *P*≤0.05 LSD.

Although the pattern of mycelial growth on different surfaces was not quantified during this study, germ tubes growing on cellulose were often observed to begin branching after 6 h incubation (Appendix B.4.1, B.4.2) and this pattern was not seen on the other surfaces used during the study.

The cellulose plate assay did appear to create a small zone of clearance around the colonies of all four Botryosphaeriaceae isolates tested, but this was only visible on agar with a CMC concentration of 40 g L^{-1} (Fig. 3.8). However, the halo could only be seen when Petri dishes were backlit with the light of a colony counter (Labserve). The *N. parvum* isolate G652 appeared to have a distinct clearance zone surrounding the agar plug and then another zone surrounding the edge of the growing colony (Fig. 3.8). All other isolates tested had a halo surrounding the colony and occasionally small clear patches within the colony (Fig. 3.8). The positive controls did not produce any visible zones of clearance. The addition of Triton X to the agar did slow growth however it took 10 days for the colonies to reach a few cm in width and no halo was seen.

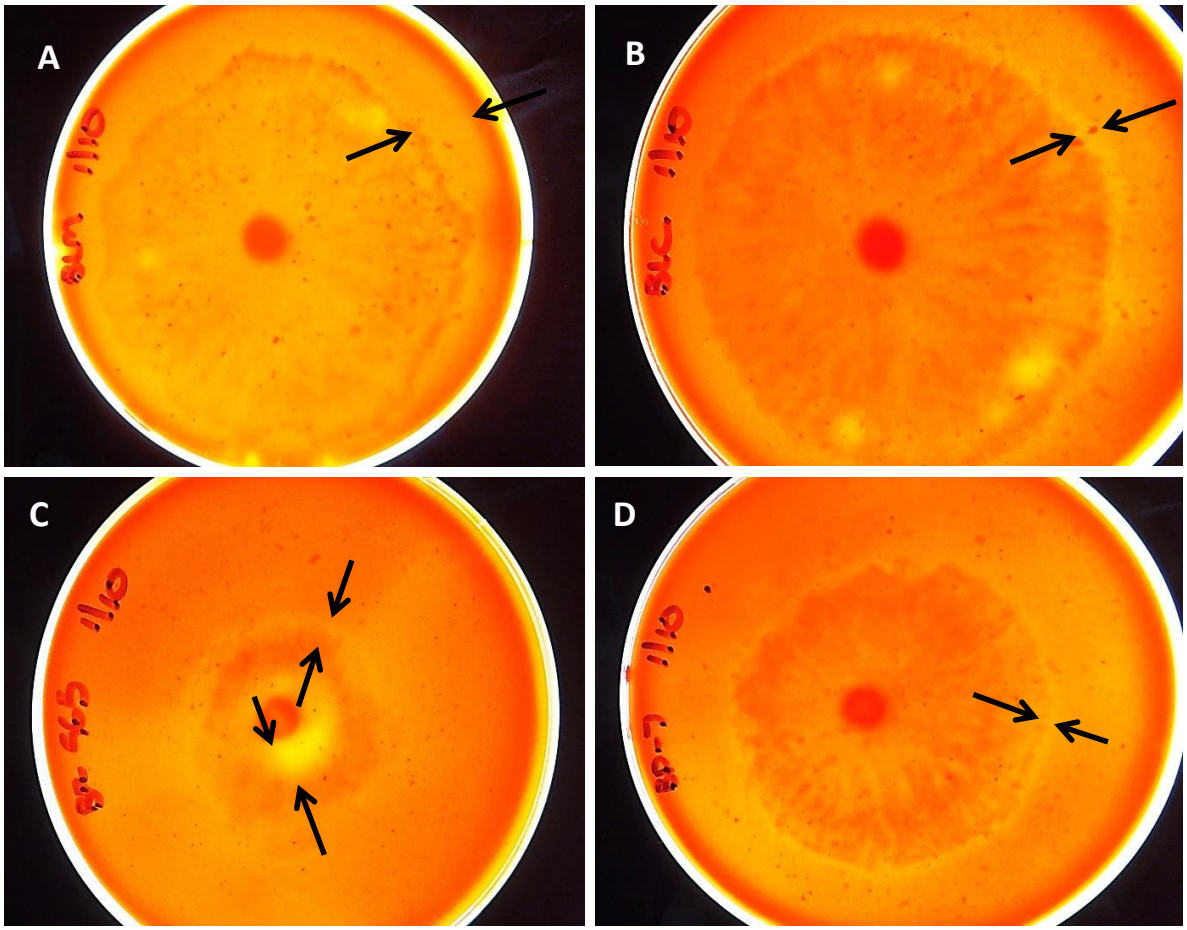

Figure 3.8 CMC agar plates inoculated with agar plugs from 3 day old colonies and then incubated at 25°C 12/12 light/dark for 3 days. Zones of clearance are indicated by arrows. A) *N. luteum* isolate MM558; B) *N. luteum* isolate CC445; C) *N. parvum* isolate G652; D) *B. dothidea* isolate 007.

3.3.2 Effect of surface hardness on conidial germination

Water activity measurements

The mean water activities (a_w) of 1%, 2%, 4% and 8% water agar were just over 1 a_w (data not shown) indicating maximum availability of water for all agars tested, with no differences between different agar concentrations.

Surface hardness and conidial germination

There was a significant effect of surface hardness on mean percent germination of conidia of *N. luteum* isolate MM558 (*P*=0.002, Appendix B.2.9). Significantly more conidia germinated on surfaces representing intermediate hardness, namely 2% and 4% agar (means 81.8% and 83.0%, respectively) (Table 3.2). There were no differences in germination rates between 1% agar (mean 76.8%) representing the softest surface and the two hardest surfaces of 8% agar and glass (means 73.5% and 73.5%, respectively) (Table 3.2). There was a significant effect of surface on mean germ tube length (*P*=0.027, Appendix B.2.13). Germ tubes were significantly shorter on 8% agar (mean 14.0 μm) than on either 2% agar or glass (means 18.1 μm, and 17.3 μm, respectively) (Table 3.2).

Surface hardness did not affect the mean germination of conidia of *N. luteum* isolate CC445 (*P*=0.064, Appendix B.2.10) (Table 3.2). Surface hardness had a significant effect on germ tube growth (*P*=0.016, Appendix B.2.14). Germ tubes were significantly longer on 2% agar (mean 21.2 μm) than on either 8% or 1% agar (means 17.0 μm and 17.7 μm, respectively) (Table 3.2).

For *N. parvum* isolate G652, there was a significant effect of surface hardness on mean germination (*P*<0.001, Appendix B.2.11). Significantly fewer conidia germinated on glass (mean 10.8%), than on any of the other surfaces tested, for which germination rates were not significantly different (Table 3.2). There was no significant effect of surface hardness on mean germ tube growth (*P*=0.196, Appendix B.2.15) (Table 3.2).

For *B. dothidea* isolate 007, there was a significant effect of surface hardness on mean germination (*P*=0.011, Appendix B.2.12). Significantly more conidia germinated on the softer surfaces, namely 2% (mean 64.6%), 4% (mean 61.6%) and 1% agar (mean 59.4%), than on the harder surfaces, 8% agar (mean 52.2%) and glass (mean 55.4%) (Table 3.2). There was a

significant effect of surface hardness on mean germ tube growth (P<0.001, Appendix B.2.16). Germ tubes were significantly longer on glass (mean 17.6 µm) than on all the other surfaces tested (Table. 3.2). Germ tubes were shortest on 8% agar and 1% agar (means 8.5 µm and 10.1 µm, respectively) (Table 3.2).

Table 3.2 Mean percent germination of conidia (Germ) and mean growth (µm) of germ tubes (GTGr) of four Botryosphaeriaceae isolates on surfaces representing different levels of hardness after three hours incubation.

Surface	Isolates[a]							
	***N. luteum* MM558**		***N. luteum* CC445**		***N. parvum* G652**		***B. dothidea 007***	
	Germ	**GTGr**	**Germ**	**GTGr**	**Germ**	**GTGr**	**Germ**	**GTGr**
1% agar	76.8 ab	16.1 ab	84.3 a	17.7 ab	35.6 b	9.0 a	59.4 bc	10.1 ab
2% agar	81.8 bc	18.1 b	84.7 a	21.2 c	32.0 b	9.2 a	64.6 c	12.0 b
4% agar	83.0 c	16.5 ab	87.7 a	19.8 bc	35.2 b	9.4 a	61.6 bc	11.4 b
8% agar	73.5 a	14.0 a	87.2 a	17.0 a	31.3 b	9.0 a	52.2 a	8.5 a
Glass	73.5 a	17.3 b	80.3 a	19.6 bc	10.8 a	7.2 a	55.4 ab	17.6 c

[a] Mean values with different letters within columns are significantly different at P≤0.05LSD

3.3.3 Germination and surface contact

For *N. luteum* isolate MM558 there was no significant difference in the germination of conidia which had been in constant contact with a surface and those which were shaken for the duration of incubation (P=0.244, Appendix B.2.17). Mean percent germination of unshaken conidia was 61.3% for those on the glass slide and was 60.7% for those in the liquid surrounding the glass slide. The mean germination rate of conidia in the shaken flasks was 58.6%.

3.3.4 Determination of infection structures

The mean germination rates of conidia of *N. parvum* isolate G652 placed onto glass slides to serve as a germination check were 45% after 6 h, 60% after 12 h and 78% after 24 h.

Calcofluor White stained plant tissue very well (Appendix B.3.1) but no conidia or germlings were seen with this dye, so it was deemed unsuitable for the purposes of this study. Transverse tissue sections stained well but no conidia were seen when stained with either Calcofluor White or aniline blue dye (Appendix B.3.1). Some conidia and germlings were visible on longitudinal sections when stained with 0.05% aniline blue (Fig. 3.9), but tissue sections were never truly clear and had brown specks throughout (Fig. 3.9 B). When viewed under the microscope, conidia and germlings were very faint and so were rarely found on

non-wounded tissue sections. On wounded tissue sections, conidia were easier to find when the microscope was positioned at the edge of the wound site (Fig. 3.9). No infection structures were seen. During preliminary testing of the tissue processing and staining procedure, germ tubes of *N. luteum* isolate MM558 close to the edge of wound sites appeared to be oriented towards the direction of the wound site, although this was not quantified (Appendix B.5.1, B.5.2).

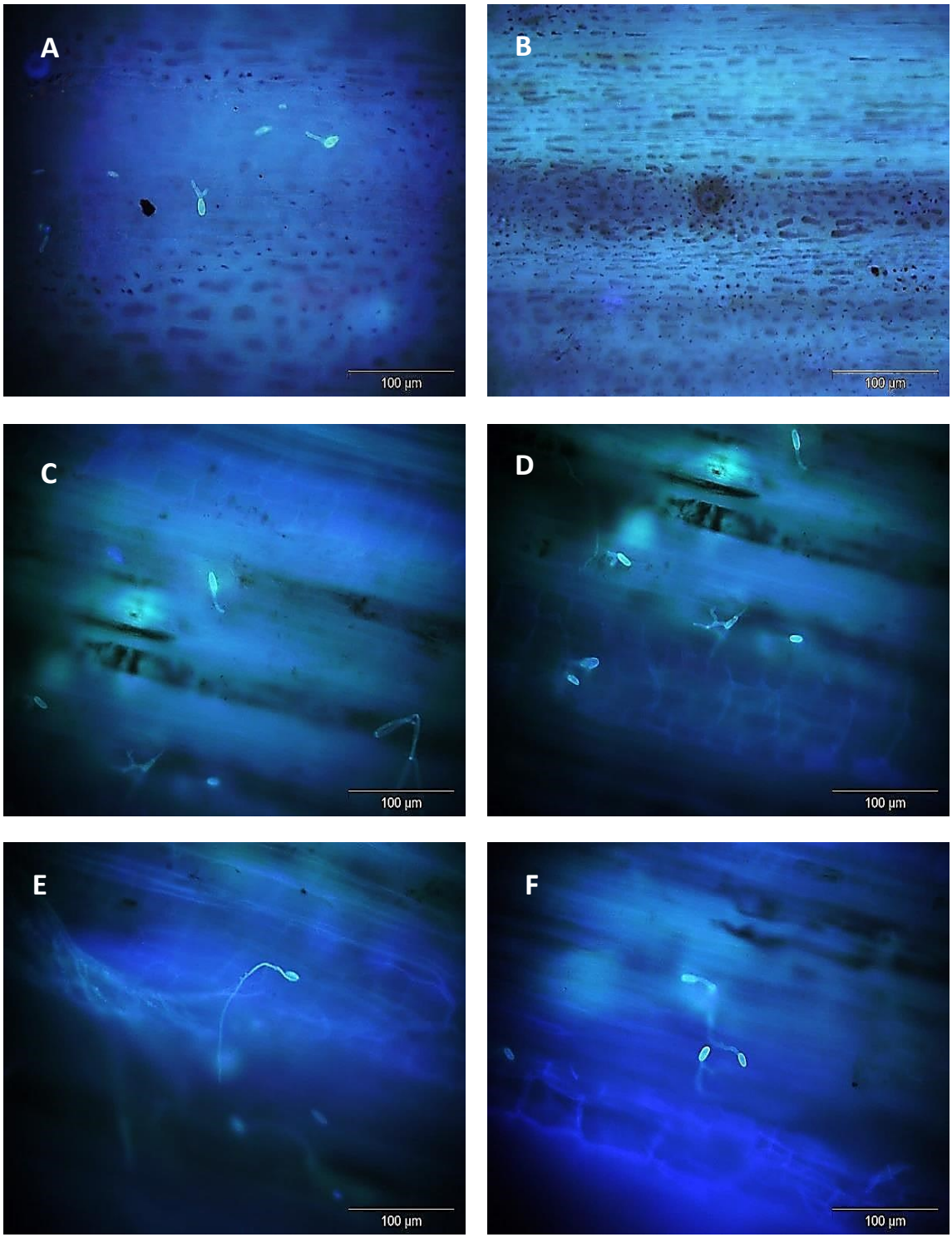

Figure 3.9 Longitudinal sections of Pinot noir shoots inoculated with conidia of isolate *N. parvum* G652. Shoot sections were autoclaved in 1 M KOH, rinsed and then stained with 0.05% aniline blue in 0.067 M K_2HPO_4. A) Germinating conidia on non-wounded tissue after 12 h incubation; B) shoot section showing tissue not completely clear; C), D), E) and F) germlings visible on wounded shoots after 24 h incubation.

3.4 Discussion

This study, the first of its kind conducted with Botryosphaeriaceae spp., investigated some factors which may impact the germination of conidia, such as surface wettability and hardness and surface contact. For all isolates tested, the mean germination rates were highest on cellulose. For example, for *N. luteum* isolate MM558, mean percent germination on cellulose (72.2%) was 6.3% higher than on glass (65.9%) with no difference in germination rates between glass, plastic and parafilm. This indicates that although cellulose had a slight impact on the rate of germination, the effect does not appear to be strongly related to the wettability of a surface and may be a result of some other property related to that surface. Exactly the same pattern of germination was noted for the other isolates tested. For *N. luteum* isolate CC445, the difference between the mean percent germination for cellulose (76.9%) and glass (52.9%) was a lot higher (24%). The differences between mean percent germination on cellulose and glass were 5.1% for *N. parvum* G652 and 10.7% for *B. dothidea* 007. Although these figures represent significant differences in germination it is not possible to interpret their relevance to grapevines because aside from a grape leaf having an advancing contact angle of 95.8^{o} (Kuo & Hoch, 1996), the hydrophilic or hydrophobic natures of other grapevine tissues are unknown. However, the wounds which have been reported to be necessary for infection of green stems (Úrbez-Torres, 2011), clearly allow conidia to get beyond the hydrophobic cuticles and give access to the internal tissues which are high in cellulose. With regards to disease development, it would be an advantage for conidia to be able to germinate and grow on many different types of surfaces

For some species of plant pathogenic fungi, surface wettability is a parameter that has an impact on germination. Kuo and Hoch (1996) reported that the germination rates of pycnidiospores (conidia) of *Phyllosticta ampelicida*, the pathogen causing black rot of grape berries, were greater on more hydrophobic surfaces, namely those having an “advancing” contact angle of > 80^{o} such as grape leaf, polystyrene and Teflon. No germination occurred on surfaces with a contact angle of 15^{o} or less. Chaky et al. (2001) reported that for *C. graminicola*, falcate and oval conidia responded differently to surface wettability. For falcate conidia, the highest germination rates (> 70%) were on surfaces with an advancing contact angle of >55^{o} and reduced significantly (<20%) when the advancing contact angle fell below 50^{o}. In contrast, the smaller oval conidia germinated equally well on all the surfaces tested,

irrespective of surface wettability. They concluded that because oval conidia are produced within plant tissue and are not thought to play a role in dispersal between plants, surface wettability would not be an important factor for their germination. Germination of wet conidia of *B. cinerea*, another pathogen of soft fruits such as grape berries, was also found to be induced by surface hydrophobicity, only occurring on surfaces with contact angles above 60-75^{o} (Doehlemann et al., 2006). In contrast, germination rates of conidia of *Penicillium expansum*, a pathogen of fruits and grains, were similar on a range of contact angles created by treating polystyrene sheets with different radiation intensities (Amiri et al., 2005). The ability of these fungi to infect a range of different tissue types could be compared with the abilities of Botryosphaeriaceae species. They have been shown to infect soft tissue in wounds and fruits as well as hard stem tissues (Amponsah et al., 2011; Wunderlich et al., 2011).

Analysis of germ tube growth in this study provided further evidence for cellulose having an effect on early pathogen development. For all isolates, the mean germ tube growth on cellulose was significantly less than on the other three surfaces, on which there were no differences in growth rates (Table 3.1). The reduction in germ tube growth when germination rates had been shown to increase on cellulose was unexpected and the reasons for this were unclear. However, if cellulase enzymes were produced by these fungi then the presence of a growth substrate might affect the rate and/or growth pattern of mycelial extension. On cellulose, the earlier branching of germ tubes could indicate a reaction to the presence of nutrients not available on the other substrates. The growth form of fungi enables them to adopt a foraging strategy, with growth tending to be sparse and explorative in regions of sparse resources and branched and dense when nutrients are readily available (Ritz & Young, 2004). The best examples of this can be seen in basidiomycetes such as *Hypholoma fasciculare* and *Phanerochaete fasciculare* that decompose wood (Dowson et al., 1986). Many such species form specialised cords to enable extensive foraging. When these cords encounter a piece of wood, absorptive growth is switched on, producing densely branching mycelium to exploit the available nutrients (Ritz & Young, 2004). This growth strategy is not restricted to soil fungi, Newton and Guy (1998) showed that colony growth in the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* was denser and more branched on barley grown under high nitrogen conditions, indicating an exploitation of the nutrient source. Under low nitrogen conditions, growth was sparser and more spread out. It is

possible that the apparent initiation of branching of all isolates of Botryosphaeriaceae spp. used in this study when incubated on cellulose (Appendix B.4.1, B.4.2) indicated a switch from explorative to exploitative growth in the presence of a food source. It would be useful to incubate conidia on this substrate over a longer time period to confirm if this was the case.

Testing for cellulose degradation with the CMC plate assay appeared to show that the Botryosphaeriaceae isolates did break down cellulose. However, the effect was not sufficiently clear to allow for any quantitative assessment. Furthermore, the positive controls used during the study did not show zones of clearance. This may be a result of them having been retained in ongoing culture conditions over many years which may have affected their ability to degrade cellulose. As such, a similar assay should be conducted to confirm the results using positive and negative controls. The CMC product used in this assay did not appear to dissolve well and thus the resultant agar was very grainy, often with an uneven spread of cellulose which made staining and subsequent interpretation of the results difficult. Since Botryosphaeriaceae spp. also grow very quickly on nutrient agars, mycelium from a central point of inoculation filling a ~9 cm agar plate in 4-6 days, the induction and activity of any enzymes produced may have lagged behind the mycelium perimeter, thus producing a clearance zone which was difficult to see. Although this method has been used, particularly for bacteria, difficulties in differentiating the areas containing CMC from those that have been cleared (lysed by cellulose enzymes) have been reported (Hankin & Anagnostakis, 1977; Kasana et al., 2008; Jo et al., 2009). Since cellulose is a major component of plant material, the extent to which fungi can break down cellulose may be a significant factor in their ability to attack their host (Ilmén et al., 1997). It could also play a role in determining whether they have the capability to grow intracellularly or intercellularly (ten Have et al., 2002). Botryosphaeriaceae species pathogens are commonly found on woody hosts and it is likely that they contain multiple enzymes which enable them to breakdown the many components of stem tissues. It has already been demonstrated by Baskarathevan (2011) that isolates of *N. parvum* produce laccase, a cell wall degrading enzyme. It would therefore be relevant to identify other enzymes that may be produced by these pathogens that enable the degradation of plant tissue.

Although the ability to utilise cellulose as a food source may account for the reduced mycelial growth of Botryosphaeriaceae spp. conidia on cellulose, availability of water may also have had an effect. Although slides were incubated in a humidity chamber, opening the lid of the chamber in order to place the surfaces within would have lowered the relative humidity levels. Further, the water droplet of conidial suspension completely soaked into cellulose as opposed to glass, parafilm or polystyrene, where the water droplet did not dry out and was still visible after two hours incubation. This may have resulted in the conidia on cellulose being more subject to influences by matric water potential as opposed to osmotic water potential. Some fungi are sensitive to changes in matric potential, particularly in water stressed soil systems, and the suggested reason for this is that it affects transport of nutrients to the fungi and by-products away from fungi (Adebayo & Harris, 1971). Magan and Lynch (1986), who investigated the decomposition rate of various cellulolytic fungi on both straw residues and straw agar over a range of water potentials, found that growth of most fungal species tested was less on straw than on agar. They suggested that this was a consequence of the matric control of water potential on straw as opposed to the osmotic control on agar. Adebayo and Harris (1971) also reported that the growth of both *Alternaria tenuis* and *Phytophthora cinnamomi* were less tolerant of matric as opposed to osmotic stress at decreasing water potentials, a result also reported for *Fusarium roseum* (Wearing & Burgess, 1979). In addition, the growth rate of strains of *Aspergillus flavus* and *A. parasiticus* were found to be more susceptible to changes in matric potential than osmotic potential (Nesci et al., 2004), and germination and growth of *Coniothyrium minitans* isolates were more sensitive to low matric potential as opposed to low osmotic potential, with germination being more sensitive than growth (Jones et al., 2011). If conidia of Botryosphaeriaceae spp. were subject to influences from matric potential when incubated on cellulose, germination appears to be less sensitive to this influence than the subsequent growth of mycelium.

Surface hardness does not appear to be a major factor affecting the germination or germ tube growth of Botryosphaeriaceae spp. conidia as no overall patterns directly relating to surface hardness were established. With regards to mean percent germination, isolates MM558 and 007 appeared to prefer slightly softer surfaces (Table 3.2). Isolate CC445 had no preference for any surface and germinated equally well on all of them. Isolate G652 showed the lowest mean percent germination on glass (10.8%) but showed no difference in

germination rates on the other surfaces. Mean germ tube growth for this isolate was not significantly different on any of the surfaces tested supporting the theory that surface hardness alone does not trigger germination and growth processes. If germination of Botryosphaeriaceae spp. conidia occurs equally well on soft and hard surfaces, this flexibility is likely to be advantageous to these pathogens. This may account for the reported abilities of *N. luteum* conidia to germinate and infect woody and green stems, buds, leaves and mature berries (Amponsah et al., 2012b).

For the entomopathogenic fungus *Erynia conica,* surface hardness was also shown not to influence the germination rates of spores, with no differences in mean germination rates on 1.5%, 3% or 6% water agar (33.9%, 32.4% and 20.2%, respectively) (Nadeau et al., 1995). In contrast, conidial germination of *C. graminicola* was significantly higher (96.3%) on glass coverslips than on silicone grease (13.2%) (Chaky et al., 2001). As these two surfaces had similar wettabilities, it was concluded that the rigidity of the surface had influenced germination. The conidia of *B. cinerea* conidia also require a hard surface for optimal germination (Doehlemann et al., 2006). After 24 h incubation in 10 mM fructose, germination rates were higher on a glass coverslip (97.7%) than in suspension (14.3%). These authors also showed that surface hardness was required for hydrophobicity induced germination. Interestingly, Staples and Hoch (1997) who reviewed relevant literature on cues for spore germination and appressorium formation by fungal pathogens noted that hardness is a physical cue that has rarely been "evaluated objectively". Many investigations may have evaluated hardness and hydrophobicity together in such a way that does not clearly differentiate the effects. A particular effect that is often not considered is the reduced porosity of harder surfaces which is likely to limit the loss of nutrients (ions, sugars etc.) from the contact surface. This might result in a reduction in membrane flux which could be an important signal for appressorium formation (Staples & Hoch, 1997). In the present study, agars of different concentration were used to try and minimise the effect of porosity.

In this study, there were similar rates of germination for *N. luteum* MM558 conidia when being continuously shaken or resting in continuous contact with a surface. Conidia of *C. graminicola* can also germinate without surface contact (Chaky et al., 2001) as can conidia of *M. grisea* (Jelitto et al., 1994). In contrast, spores of some pathogenic fungi require surface contact in order to germinate, an example being the pycnidiospores (conidia) of *P.*

ampelicida that rarely germinated when free in suspension (Kuo & Hoch, 1996; Shaw et al., 2006). Apoga et al. (2001) also demonstrated that significantly higher numbers of conidia of *Bipolaris sorokiniana* germinated when in contact with a solid surface such as glass or polystyrene than when suspended in bulk medium. Egley (1994) also reported that only 1% of conidia of *C. truncatum* germinated while suspended in water in comparison to 94% on chromatography paper and Warwar and Dickman (1996) found that conidia of *C. trifoli* did not germinate under continuous agitation.

Surface contact may also be required for differentiation of hyphae after germination, as reported by Jelitto et al. (1994) who used a hanging drop technique to demonstrate that surface contact was essential for appressorium development in *M. grisea*. Contact with hard hydrophobic surfaces induced the formation of appressoria in *C. trifoli*, while contact with a hydrophobic surface that was soft (mineral oil) resulted in the germ tubes remaining undifferentiated (Warwar & Dickman, 1996).

The current investigation of infection structures on grapevine tissue was unsuccessful. It was the last experiment in this program and due to exhaustion of conidium producing infected shoots of the preferred *N. luteum*, *N. parvum* was used instead. The tissue of the red grapevine cultivar Pinot noir was never completely clear irrespective of the method used and this exacerbated the difficulty in finding the smaller conidia of *N. parvum*. It is possible that a white grapevine variety such as Sauvignon Blanc may have shown less residual pigment in the cleared tissue. Although use of aniline blue caused the conidia to fluoresce, they were never really bright and it was unclear why this was the case. The stain used was quite old (11 years) and it is possible that the shelf life has had an impact of the effectiveness of this dye. In future studies of this type, it would be recommended to use Sauvignon Blanc shoots and conidia of *N. luteum* or other species with larger conidia. The observations made during this investigation that germ tubes appeared to be growing towards wound sites may be of relevance to these wound pathogens and will be investigated in more detail in the next chapter.

Of the Botryosphaeriaceae species reported to infect plant stem tissues, infection structures (appressoria) have been reported only for *B. dothidea* and only on apple fruits (Kim et al., 2005). To-date formation of appressoria has not been reported for this species on grapevines (van Niekerk et al., 2004) or in culture (Phillips et al., 2013). Furthermore, a

scanning electron microscopy study that assessed development of *N. luteum* conidia and germlings on green leaves and stems did not find evidence of appressoria (Amponsah et al., 2012a). It therefore seems likely that not all Botryosphaeriaceae species have the potential to produce appressoria and that the developmental signals required for their production may be complex and vary between species. This should be explored with an in-depth study that tests a greater range of substrates and isolates than was possible in this study.

Throughout these Botryosphaeriaceae spp. germination studies, rates of germination varied for any one isolate and it was unclear whether this was related to environmental or experimental conditions. It appeared as if infected shoots that were stored dry for more than 2-3 weeks frequently produced conidia that had lower germination rates than shoots stored for shorter periods of time and it was unclear why this was the case. Initial trials with the green shoot technique for producing conidia found that infected shoots that had been dried and stored at room temperature were still capable of oozing conidia after 3 months storage (Amponsah et al., 2008). However, as the purpose of that particular study was to develop a method for producing sufficient conidia for infection studies, no testing was performed on the effect of such a method on germination rates. As such, it would be useful to investigate the effects of storage time on germination rates.

The apparent lack of germination during the short incubation times used during this study may have just been related to delayed germination. Germination rates in such cases are often highly variable. If a longer incubation had been used, this difference may not have been as noticeable. A review on the germination rates of representative fungi conducted nearly a century ago reported that the spores of a number of species will often germinate over a broad time frame (Doran, 1922). Even under optimal conditions for germination, a period of 10 h elapsed before all the ascospores of *Sclerotinia fructicola* were observed to germinate (Gottlieb, 1950). The conidia of *Glomerella rufomaculans* could take between 6 to 24 h to germinate (Taubenhaus, 1912), those of *Plasmopara viticola* 3 to 12 h (Gregory, 1912) and those of *Colletotrichum lagenarium* 6 to 24 h (Stoneman, 1898). In all these cases, the rate of germination reported might have been artificially low and subject to more variation had germination been assessed during early time points. Germination studies performed by Úrbez-Torres et al. (2010b), with eight species of Botryosphaeriaceae known to infect grapevines, found that germination rates tended to increase with time from 2 h to

12 h, with no significant increases after that time for most species. Amponsah (2010) reported that the mean maximum germination rate of conidia of four species of Botryosphaeriaceae at the optimum temperature of 25^{o}C occurred after 24 h (100%), with the highest mean germination rate after 12 h being recorded for *N. luteum* (88.7%). These studies demonstrate that even at optimal temperatures, conidia of Botryosphaeriaceae spp. may germinate over a long time period. The assessment of germination after the short incubation times used during this study may have resulted in higher variability in the rates of germination recorded, and this variation may have differed between replicates and similar treatments. However, in concentrated spore suspension the rapid growth of Botryosphaeriaceae spp. can result in germ tubes appearing tangled if left for longer periods of time, making assessment of germinated conidia difficult. It was this factor which drove the decision to assess germination after a few hours.

Another factor that may have affected germination rates of the Botryosphaeriaceae spp. used in this study is the maturity of the conidia. With regards to the relevant literature, the concept of 'maturity' has been ill defined, often quoted merely in relation to the ability of spores to germinate. As such, the morphology of mature and immature spores may not differ greatly (Doran, 1922). With regards to this study, perhaps the most relevant definition of this term was made by Melhaus and Durrell (1919). They referred to spore maturity in the context of development within sori, with immature spores being produced last in the sorus and not able to germinate; spores produced first in the sorus were likely to be fully mature and possibly even dead by the time they were removed from the host. Therefore, the maturity of spores used in any study may depend on the timing of spore collection and the method by which the spores were collected. Melhaus and Durrell (1919) examined the germination rate of spores of *Puccinia coronata*, which causes crown rust of oats. Germination rates were examined in relation to the age of sori. It was noted that germination rates of spores gathered two days after sori first appeared on host leaves were low. However, on the third day after sori appeared, germination rates were consistently high and remained so until the leaf died. Jones (1919, as cited in Gottlieb, 1950) found that ascospores of *Pseudopeziza trifolii* readily germinated if naturally discharged from the ascus but not if they were obtained by crushing the ascus, a method which extracted mature and immature ascospores. When the conidia of *Venturia inequalis* were obtained by brushing apple lesions only a relatively low number geminated ("25"). When conidia were obtained by

washing lesions under a stream of water from a pipette, over “100” conidia germinated (Doran, 1922). This suggests that where possible, spores used for experimental purposes should be obtained in a manner that reflects their natural release into the environment. For the purposes of this study, conidial suspensions were obtained by washing shoot pieces with oozing pycnidia in water. Often 3-4 shoots would be placed into a single tube with 2 mL of water and then briefly vortexed. The vortexing was usually at maximum speed, causing the shoots to bang against each other and also against the sides of the plastic tube. It is therefore possible that this method of washing encouraged the release of conidia from the pycnidia before they were fully mature. In addition, it would have removed conidia that were present in the dried ooze often seen on infected shoots during this study. It is possible that the conidia within the dried ooze had lost their viability during prolonged storage. A similar effect was reported by Gough and Lee (1985) who examined the effects of relative humidity on the viability of *Septoria tritici* conidia within cirrhi that had exuded from pycnidia. They found that 100% of conidia remained viable for 15 days when held at 35-75% relative humidity, which reduced to 65% viability after 15-20 days at 65% RH or above and by the 30th day viability of conidia was less than 2%.

In hind sight, factors affecting the variability of methods on conidial germination should have been assessed in more detail. Preliminary testing was performed for all Botryosphaeriaceae spp. to determine the incubation temperature (20^{o}C or 25^{o}C) at which all species would germinate (data not shown). As the purpose of this testing was to ensure that all species could germinate after the same period of incubation, the variability in germination rates of a single species were not assessed. The chosen incubation temperature of 20^{o}C used throughout the germination studies may have affected the observed variability in germination rates. This temperature was chosen instead of the usual 25^{o}C in an attempt to reduce the speed of germination and so allow time for assessment while taking into account the different rates of germination and germ tube elongation between different species. In one experiment, Amponsah (2010) noted that the mean germination rate of conidia of *N. luteum* and *N. parvum* at 20^{o}C was less than 20% whereas the germination rate for both these species was over 50% at 25^{o}C, both after 3 h incubation. Úrbez-Torres et al. (2010b) found the germination rate of *B. dothidea* conidia at 20^{o}C after 2 h incubation was less than 30% and after 4 h was over 80%. At 25^{o}C, germination rates were over 70% after 2 h and over 90% after 4 h. For *N. parvum*, germination rates at 20^{o}C were also less than 30% after

2 h and just over 50% after 4 h. At 25^{o}C, the germination rate for this species was just over 60% after 2 h and about 75% after 4 h (Úrbez-Torres et al., 2010b). These studies demonstrate the variability in germination rates at different temperatures assuming other conditions were optimal. However, humidity is also an important factor affecting spore germination, as it interacts with the temperature range over which germination and growth occurs (Gottlieb, 1950). The humidity chambers used during this study were opened at one corner in order to place the test surfaces inside. It is likely that this would have resulted in a drop in humidity within the chamber and it is unclear whether the short incubation times used during the study would have allowed humidity to get back to optimal (100%). In addition, it is likely that test surfaces placed in the corner furthest away from the box opening point may have been in a more humid area of the chamber than those placed directly beside it. Humidity was shown to be an important factor for Botryosphaeriaceae spp. conidial germination by Amponsah et al. (2010). At 100% humidity, mean germination rates were 91.8% after 3 h. When the relative humidity dropped only 3% to 97%, it took 6 h for the germination rate to reach 97%. When humidity levels were dropped further to 93% no germination occurred after 6 h. This demonstrates that the small reductions in humidity levels likely from opening the humidity chamber, could have affected germination rates in this study.

In summary, these germination assays showed that conidia of Botryosphaeriaceae spp. had slightly higher germination rates on cellulose, a hydrophilic surface, although there was no trend for higher germination rates on surfaces with high levels of hydrophilism than on the hydrophobic surfaces. In contrast, germ tube growth on cellulose was significantly less than on the other surfaces tested, which may have indicated the biological relevance of this material for the pathogen. Preliminary studies suggested that germlings could utilise the cellulose in the cellulose acetate membranes as a food source which may reflect the slower advance across that substrate. Surface hardness does not appear to play a role in the germination or germ tube growth of these conidia and indeed contact with a surface is not required in order for germination to occur. No infection structures were seen during this study however to date no appressoria have been reported for these fungi on grapevines.

Chapter 4

The role of plant volatiles on conidial germination and germ tube development

4.1 Introduction

Although host features such as surface wettability, surface hardness and topography are all potential cues involved in the pre-infection development of plant pathogens, other host cues may involve chemical signals such as the production and release of volatile compounds (Deacon, 1996). Volatiles produced from ripening fruits such as tomato and banana have been found to induce germination and formation of appressoria in *C. gloeosporioides* (Flaishman & Kolattukudy, 1994) as do the chemical signals from the skin of avocado fruit, a host for this pathogen (Podila et al., 1993). Volatile compounds such as 'green leaf volatiles' (GLVs), six carbon alcohols, aldehydes and derivatives, are released from plant tissues almost immediately after wounding (Fall et al., 1999). The mode of action of GLVs towards plant pathogens is as yet unclear. However, changing the levels of GLV biosynthesis in plants has been reported to either increase or decrease susceptibility to infection from plant pathogens (Shiojiri et al., 2006). In grapevines, GLVs may be emitted when tissues are wounded but to date no studies have been performed to determine whether grapevine wounds emit volatile signals to which conidia of Botryosphaeriaceae spp. respond. This could be advantageous to conidia that land and germinate on non-wounded parts of the vine which they cannot directly penetrate. This study aims to determine whether volatiles are produced from wounded green grapevine tissues and whether said volatiles have an effect on germination and/or directional growth responses of Botryosphaeriaceae spp. conidia.

4.2 Materials and methods

In these experiments, conidia of two *Neofusicoccum* species were exposed to grapevine wound volatiles which were released directly from fresh plant tissue or those collected by trapping the volatile compounds on a polymer adsorbent for later use. The effects of these volatiles on germination rates, growth rates and directional growth responses were examined.

4.2.1 Plant material for GCMS analysis

Dormant cuttings were taken from Sauvignon Blanc vines in the Lincoln University vineyard in the winter (July) of 2013. Canes were cut to one internode length with one bud at the tip of the cutting. To encourage shoot development, the basal ends of the canes (~ 1.5 cm) were inserted into holes cut into a polystyrene sheet which was floated in a tray of water. The tray was placed under fluorescent light (Osram L36W/840 Lumilux Cool white tubes) with a diurnal light regime (12 h: 12 h). Once the first roots were seen, the water in the tray was replaced by a hydroponic nutrient solution (NFT General Blended Nutrient, NFT Group). The water was then continuously aerated by two air cylinders (15 mm, Blue Planet, Bubbilo) placed inside the water tray at opposite sides. The cylinders were attached to air tubing connected to a twin air pump (TW300, Blue Planet, Bubbilo). Nutrient stock solutions A and B were made up as per manufacturer's instructions and then added to the trays containing the grapevine canes at the rate of 3 mL each /L water once per week after they had developed significant root systems.

4.2.2 Volatile collection

Dynamic headspace collection of shoot and leaf volatiles was carried out with a modified push-pull system using glass odour source vessels (Turlings et al., 2004). Each vessel consisted of a bottom section (11 cm high, 5 cm wide) with a ground glass joint (male, 50-55 mm) which fitted into the main section (~28.5 cm long) (Fig. 4.1 A). The main section of each vessel contained three glass 'ports', one situated towards the base of the main section and two at the other (top) end of the main section towards the neck (Fig. 4.1 A). Air from a compressed air cylinder was pushed into the odour source vessel via one of the ports at the top of the main section of the vessel at a rate of 225 mL min^{-1} (Fig. 4.1 C), after first being filtered through an activated charcoal filter (400 cc, Alltech, Deerfield, IL, USA). The bottom port was used to connect the volatile trapping filter, containing Super-Q® adsorbent (30 mg, ARS Inc., Gainesville, Florida) to the vessel. Filters were attached to tubing that was connected to a vacuum pump (ILMVAC GmbH, Germany) which pulled air out of the vessel at a rate of 220 mL min^{-1} (Fig. 4.1 C).

Before each experiment, empty odour source vessels were cleaned by rinsing with 1 mL hexane (HPLC grade (95% purity), Ajax Finechem) followed by rinsing with distilled water. Each plant (with a green shoot of 20-25 cm) was carefully removed from the polystyrene

support and gently placed into the base of an odour source vessel containing water, making sure the roots were not damaged in the process (Fig. 4.1 B). In order to position the base of the shoot close to the volatile trapping filter, the vessel base also contained a ball of compressed aluminium foil (cooking grade) (Fig. 4.1 B). Volatile collection was performed first on non-wounded plants, then those same plants were wounded and the volatiles collected. Two negative controls contained only a ball of aluminium foil and water. For the non-wounded plants, volatiles were collected for 60 min then the adsorbed volatile compounds were eluted from each filter into a separate glass vial with 100 µL of hexane. Octane (5 µL) was also added to each vial to act as an internal standard (concentration 20 ng/µL). The glass vials containing the volatile compounds were immediately placed into -80^{o}C storage until use. The apparatus allowed for a maximum of six collection vessels to be set up at one time (Fig. 4.1 C).

Due to the leaves being slightly flaccid after volatile collection, the non-wounded plants were left in the bases of the glass vessels with water overnight to recover and then these same plants were used for collecting wound volatiles. Stem internodes were wounded by quickly scraping four times along 3-4 cm of the stem with a sharp scalpel and leaves were rubbed briskly with fingers. The top of the vessel was then quickly placed over the plant and the air flow tubes reconnected. Volatiles were collected for 20 min and then each plant was briefly removed from the air flow, wounded again on a different stem area, and volatiles collected for another 20 min using the same volatile trapping filter. This process was repeated a third time. Volatiles were eluted and an internal standard added as previously described. Volatiles were collected from six replicate plants (stems and leaves). After volatile collection was complete, each plant was weighed (without roots) to determine fresh weight. Volatiles were also collected from stems only. Four plants with stems of approximately equal diameter had all their leaves removed and were left overnight to recover. Volatiles were collected from non-wounded stems first for 60 min, left to recover for 3 h and then volatiles were collected from wounded stems three times over a 60 min period as described above. The glass vials containing the volatile compounds were immediately placed into -80^{o}C storage until use.

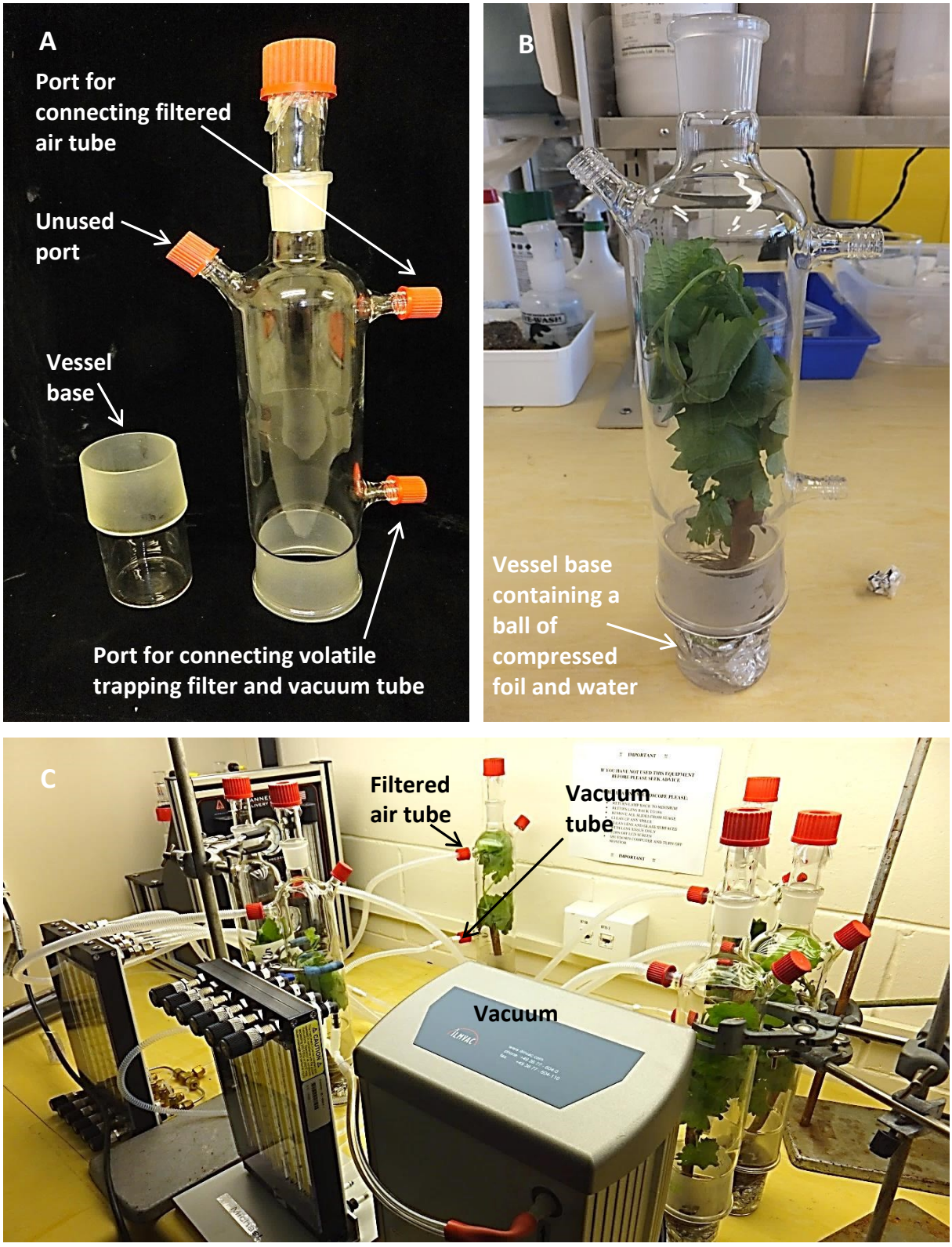

Figure 4.1 Volatile collection equipment. A) Glass odour source vessel; B) odour source vessel with grapevine shoot placed into water and positioned onto a ball of foil; C) equipment for trapping volatiles showing airflow in and vacuum out of each odour source vessel.

4.2.3 GCMS analysis

The grapevine shoot volatiles were removed from -80°C and were analysed with a Shimadzu GCMS-QP2010 gas chromatograph/mass spectrometer (GCMS) (Shimadzu Corporation, Japan). The GCMS was fitted with a Restek Rxi-1ms capillary column (fused silica, 30 m x 0.25 mm i.d. x 0.25 µm film thickness, Bellefonte, USA). From each grapevine sample, 3 µL was injected in pulsed splitless mode (temp 220°C, pulse 168kPa for 40 s). The GC oven temperature was held at 35°C for 3 min and then increased by 8˚C min^{-1} to 320°C for 8 min. The carrier gas was helium (constant flow rate 1.5 mL min^{-1} and linear velocity of 44 cm s^{-1}). Compounds were identified using GCMS solution v. 2.72 (Shimadzu Corporation, Japan) software with Wiley 11 and NIST 11 mass spectral libraries. Quantification was obtained by comparing the area of the identified compounds to the area of the internal standard octane. Identified compounds are expressed as ng/g^{-1} fresh shoot weight (fw) per hour (ng/gfw/h). For stems+leaves (wounded and non-wounded) volatile collection and analysis was repeated eight times and included two negative controls of foil+water. For stem only volatiles (wounded and non-wounded) collection and analysis was repeated six times and included two negative controls as described.

4.2.4 Exposure of *Neofusicoccum* spp. conidia to volatile compounds emitted directly from fresh plant tissue

Conidial suspensions of isolates *N. luteum* MM558, *N. luteum* CC445 and *N. parvum* G652 were produced as described in Section 2.2.2. Fresh plant tissue (green shoots) was always cut on the morning of the day on which the experiment was to be conducted. Green shoots (~60 cm long) were cut from Sauvignon Blanc vines in the Lincoln University vineyard and immediately placed into water. From each shoot, three internode sections (~50 mm) were cut from the three internodes nearest the apex. The cut ends were immediately sealed with melted candle wax (National Candles, Wellington) (Fig. 4.2). The three wax-sealed shoot pieces were gently pressed onto pieces of double sided adhesive tape (Sellotape®) on the inside of the lid of a 25 mm deep Petri dish for stability. The Petri dish base had four squares drawn on the outside to mark where the conidial suspensions would be placed (Fig. 4.2) and contained 2% water agar. A 10 µL drop of conidial suspension (~150 conidia) was pipetted onto the agar in the centre of each square. Shoots were wounded by quickly scraping along 3-4 cm of each shoot piece with a sharp scalpel four times. The base was immediately placed

over the lid and the dish was sealed with food grade cling film. Control plates contained non-wounded shoots. Plates were arranged randomly onto a tray, covered with foil and then incubated for 2 h at 20^{o}C. After this time, plates were opened and conidia immediately stained with lactophenol aniline blue to halt any further development. The agar squares were cut out and placed onto a glass slide for microscopic examination. For each agar square, the microscope was positioned at the topmost conidia seen and then moved one field of vision downwards. The mean percentage of germinated conidia was determined by assessing germination of 100 conidia in adjacent fields of vision. A conidium was considered to have germinated if the germ tube was half the width of the conidium. Reported values represent the average of four counts in each of six replicate Petri dishes per isolate. In addition to percent germination, germ tube growth was also assessed. Four photos of germlings were taken from each replicate. Germlings were superficially scanned under the microscope and photos taken when seven or more germlings were seen. Photos were each assigned a number and then selected by random number generation. Starting from the top left, the germ tube length of each germling on one photo was measured. This procedure continued across photographs until 20 germ tubes had been measured per replicate. Germinated conidia were imaged at x100 magnification using a DS Fi2 camera attached to an Olympus CX41 microscope. Germ tubes were measured using AnalySIS® imaging software (V 3.2, Soft Imaging System). Reported values represent the average of 20 germ tube measurements replicated six times.

For isolate *N. luteum* MM558 the above experiment was repeated with stems plus leaves. Stems were secured to the Petri dish lid and wounded as before. Then the fifth leaf from the tip of the same grapevine shoot from which the shoot pieces originated was removed, rubbed briskly by hand, and then placed beside the wounded shoot pieces. The Petri dish was closed and sealed immediately and the dishes incubated for 2 h as before. Conidial germination counts and germ tube lengths were assessed as previously described.

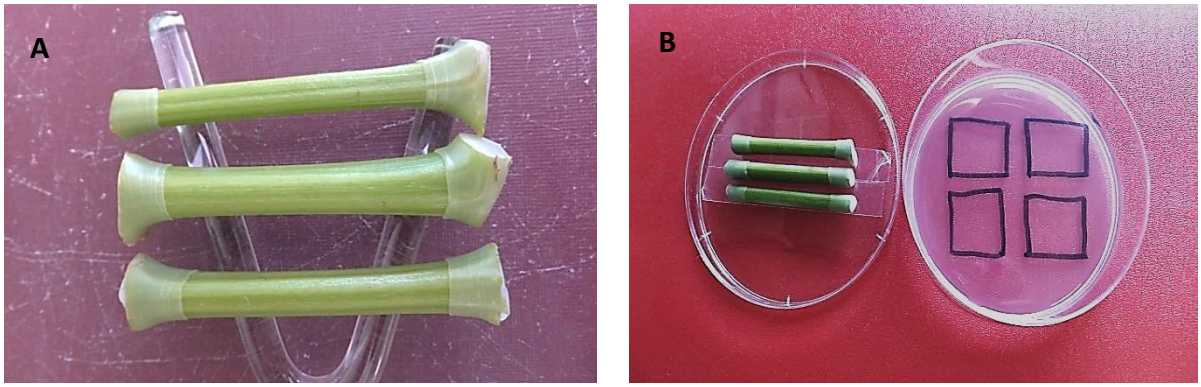

Figure 4.2 Pieces of green shoot used during volatile experiments. A) Shoot internodes cut and sealed with candle wax; B) shoots fixed to the lid of a Petri dish with double sided adhesive tape prior to wounding, squares marked on the base indicate points for placement of conidial suspensions.

4.2.5 Exposure of *Neofusicoccum* spp. conidia to liquid volatile mix obtained from headspace sampling of grapevine tissues.

Isolate *N. luteum* MM558 was used in these experiments, with conidial suspensions being produced as before. The eluted volatiles from the collection of wounded stem+leaf grapevine tissue (Section 4.2.2) were combined into one glass vial to maximise the volume of liquid available for use. Available volumes of elutant were much lower than expected, resulting in 30 µL of the volatile mixture being used per replicate and only allowing for four replicates. Each Petri dish was marked on its base as before and contained 2% water agar. A 10 µL drop of conidial suspension (~150 conidia) was pipetted onto each agar square (Section 4.2.3). A piece of filter paper (~1 cm x 2 cm) was placed into the centre of the lid of a deep Petri dish. The liquid volatile mixture was pipetted onto the filter paper and then the base of the dish was immediately placed onto the lid and sealed as before. Control plates contained 30 µL hexane as this was the volatile elutant. Sealed plates were randomly arranged onto a tray and covered with foil before being incubated at 20^{o}C for two hours. After this time, the conidia were stained with lactoglycerol aniline blue, with the agar squares being cut out and assessed as before. This experiment was repeated with volatiles collected from wounded stems and also with volatiles collected from non-wounded stems. In addition, an experiment was conducted with pure hexane as the treatment, with the control in this case being dry filter paper. All other conditions remained the same, with four replicates per experiment.

The concentration of each compound within a Petri dish was estimated in order to make comparisons to relevant studies. These estimations were based on the assumption that all of the 100 µL of hexane was retained after elution had occurred. The reported concentrations of compounds obtained from GCMS analysis based on analysis of 3 µl of elutant were used to calculate the final concentrations. As such, these concentrations can be assumed to be maximum concentrations, given that the volume of volatile mix available for experimentation was far less than the original 100 µL per plant used for elution.

4.2.6 Exposure of *Neofusicoccum* spp. conidia to pure volatile compounds

As exposure to stem+leaf volatiles from fresh plant tissue appeared to have an effect on germ tube growth, four of the volatile compounds that were produced in the highest concentrations from wounded stem+leaf grapevine tissue, and were readily available at Lincoln University, were chosen for this experiment, namely cis-3-hexen-1-ol (Fluka, Sigma Alrich), cis-3-hexenyl acetate (SAFC, Sigma Aldrich), trans-2-hexenal (Acros Organics) and 1-hexanol (Fluka, Sigma Aldrich). Plate assays were conducted as described previously (Section 4.2.4) using filter paper and water agar. For each deep Petri dish, 100 µL of pure volatile compound was pipetted onto the filter paper placed into the lid as before. The base of the dish contained water agar and was marked for inoculation with four 10 µL drops of conidial suspension (each ~150 conidia). Isolates *N. luteum* MM558 and *N. parvum* G652 were chosen for this study, with conidial suspensions being produced as before. Control plates contained filter paper only. Mean percent germination was assessed as before. Reported values represent the average of four counts per replicate, with four replicates.

4.2.7 Directional growth responses of Neofusicoccum spp. germlings to grapevine wound volatiles

In this experiment, conidia of *N. luteum* isolate MM558 were incubated to induce germination and then the germlings were exposed to volatiles produced from freshly wounded grapevine tissue. After three hours exposure the germlings were imaged and the direction of growth of germ tubes was assessed.

A 650 mL plastic food storage container was used for setting up each volatile experiment. A long narrow opening (~0.7 cm X 75 cm) was cut at the end of the container away from the plant material (Fig. 4.3) which allowed volatiles to escape and so create a directional

gradient of volatiles. Preliminary testing with a larger opening (2 cm X 75 cm) resulted in some of the spore suspensions completely drying up. The bottom of the container was lined with two paper towels which were saturated with water. A plastic mesh was placed onto the paper towels to support the glass slides used in this study. Fresh green shoots (~45 cm) were cut from grapevines from the Lincoln University vineyard as before. Four shoot pieces (~52 mm) were cut from the apex of the shoot and immediately sealed with candle wax as before. Two shoot pieces were each secured to a glass slide by placement onto double sided adhesive tape for stability. The two slides, each with two shoot pieces, were placed into the container at the end farthest away from the opening (Fig. 4.3).

A conidial suspension of *N. luteum* MM558 was prepared as before. Glass slides were each marked with two inoculation areas, created by drawing lines across the width of the slide with a permanent marker (Fig. 4.3). An arrow was also marked onto each slide to show the direction from which the volatiles were coming (Fig. 4.3). A 10 µL drop of conidial suspension (~150 conidia) was placed into the centre of each inoculation area. Slides were then placed into a humidity chamber in the dark at 20^{o}C for 3 h in order for conidia to germinate. After this time, slides were removed and placed into the volatile container, with four slides in the base of the container beside the wounded grapevine shoots, and four on the lid (secured with double sided adhesive tape). The slides on the lid were positioned to be directly above those in the base (Fig. 4.3). The shoots in the container were wounded by quickly scraping along 3-4 cm of each shoot piece with a sharp scalpel four times immediately before replacing the container lid. The container was left at room temperature (21-23^{o}C) for three hours. The control slides were placed into a container without shoots. Slides placed in the base were named B1-B4, with B1 being closest to the shoots. Those attached to the lid (top) were named T1-T4, with T1 being closest to the shoots.

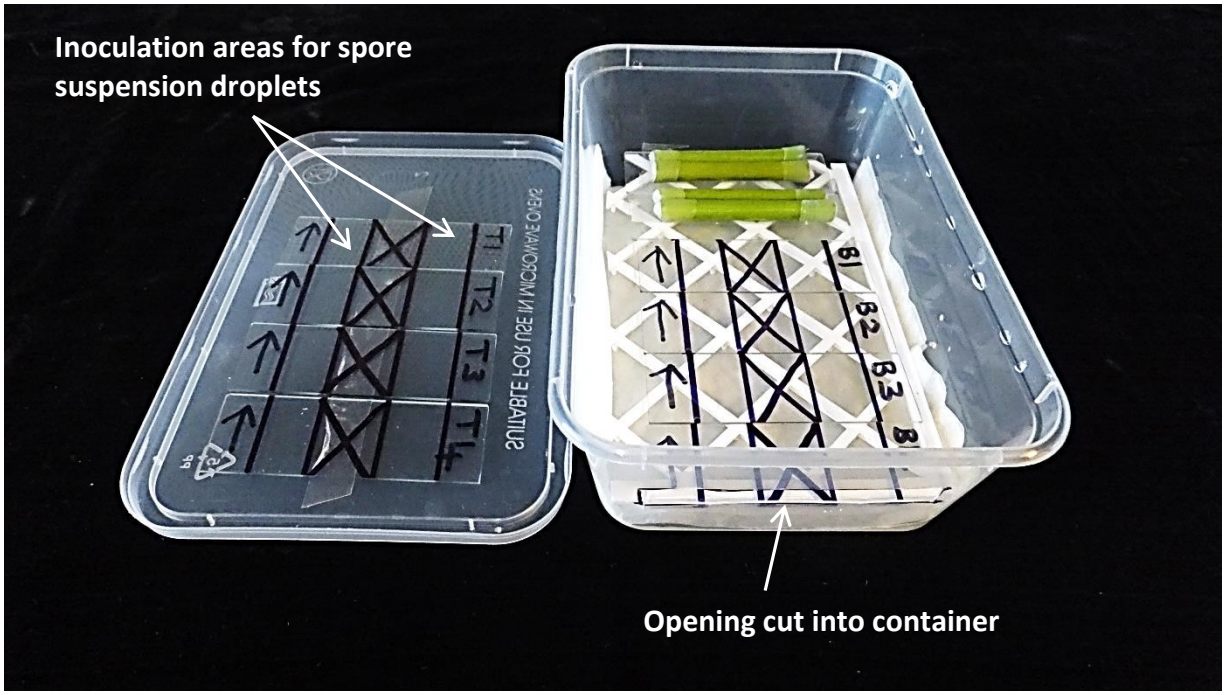

Figure 4.3 Experimental setup for determination of directional growth responses to shoot volatiles. Shoot pieces were secured onto glass slides with double sided adhesive tape and placed furthest away from the container opening. Slides on the base were labelled B1-B4 with B1 closest to the shoots. Slides on the top (lid) were named T1-T4, with T1 closest to the shoots once the lid was closed. Slides were incubated for 3 h to initiate germination before being placed into the box.

From each inoculation point on each slide, two images were taken at x100 magnification (four images per slide position). Each image was magnified to the same size as the A4 paper onto which it was printed. The directional growth of 25 germ tubes was assessed using the four images per slide. The first six or seven germ tubes seen from the top left of each page were assessed before moving onto the next image until 25 germ tubes had been assessed. For assessment, it was assumed that conidium orientation was likely to determine the initial direction of a germ tube, for the first 4-5 conidium lengths. The later direction of germ tube growth was considered likely to be affected by external influences. Thus only the final third of the germ tube was assessed for direction of growth. A transparency made by photocopying graph paper was placed over the top of each image. For each germ tube, the graph paper was positioned over the final third of the germ tube and the directional growth was recorded as either towards the volatiles (top left or right) or away from the volatiles (bottom left or right) (Fig. 4.4).

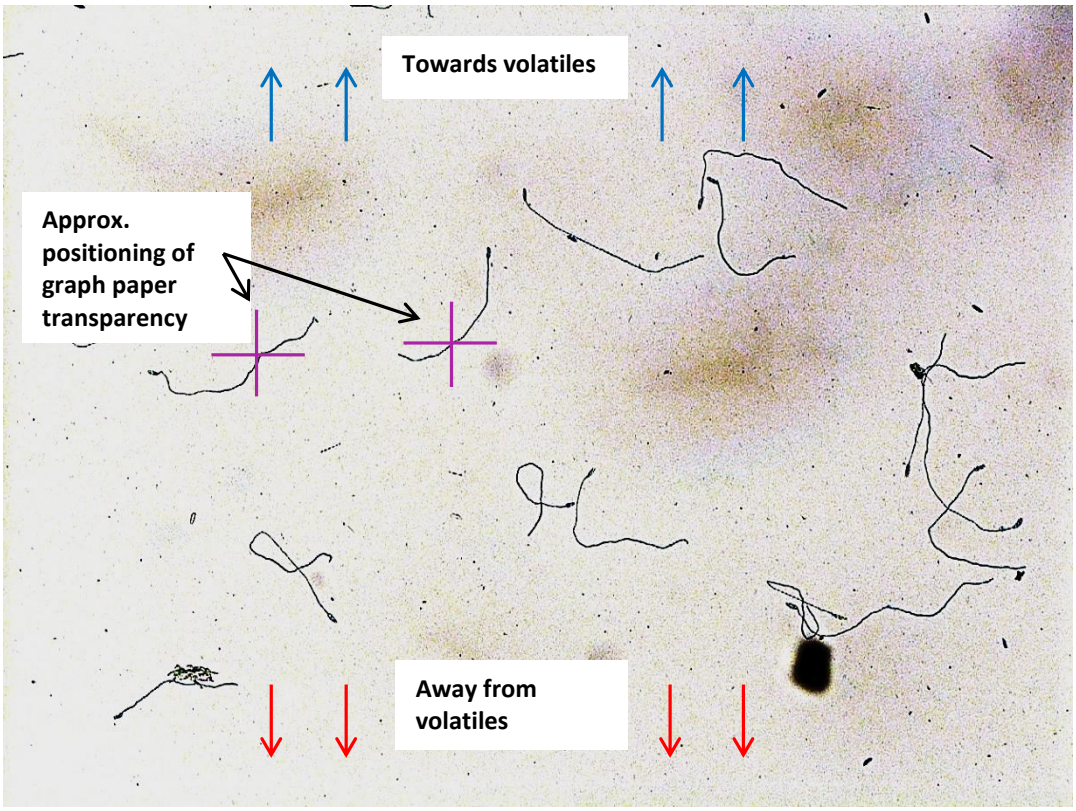

Figure 4.4 Directional growth of *N. luteum* isolate MM558 germlings after incubation for 3 h in the presence of wounded grapevine shoots. The direction of reducing volatile concentration is shown by red arrows, that of increasing volatile concentration is shown by blue arrows and examples of approximate positioning of graph paper for analysis by purple crosses.

4.2.8 Statistical analysis

Data were analysed using GenStat 16th edition (VSN international). For the plant volatiles analysed by GCMS, the concentrations of each compound were compared using a paired t-test at $P \leq 0.05$ (wounded versus non-wounded treatments). For the experiments on effects of plant wound volatiles, data of percent germination and germ tube length were analysed using a one-way ANOVA. For the experiments with pure volatiles, data were log transformed ($\log_{10}$) before analysis. To account for 0 values, 1 was added to all data before log transformation occurred. The data presented in graphs are antilog transformations of the mean values generated by ANOVA analysis. Where the ANOVA analysis reported a significant effect, differences between means were determined by Fisher's protected least significant difference (LSD) at $P \leq 0.05$. Differences between means are called significant within the following results only if they meet this criterion. Data from the experiment that

assessed directional growth of germ tubes were analysed using a Chi-square test ($P \leq 0.05$) as follows: data from all slides in the box of wounded shoots compared to all slides from the control box; data from slides closest to volatiles (T1+B1) (wounded versus control), and data from slides furthest from the volatiles (T4+B4) (wounded versus control).

4.3 Results

4.3.1 Volatile collection

Fresh weight (minus roots) of the six plants used for stem+leaf volatile collection were 13.08 g, 13.53 g, 13.3 g, 11.5 g, 14.21 g and 16.65 g. For stem only plants, fresh weights were 1.94 g, 2.47 g, 2.49 g and 3.11 g.

GCMS analysis identified 19 compounds in the volatiles collected from stem+leaf tissues (Appendix C.1.1, C.1.2). However, under advice from the GCMS technician (J. Breitmeyer, personal communication, 2014), six of these compounds which showed very inconsistent results were considered likely contaminants so were omitted from further analysis. Of the 13 remaining compounds, nine compounds were released in significantly different amounts between wounded and non-wounded plants (Table 4.1). The compound released in the highest amount from wounded plants was cis-3-hexenal (mean 111.52 ng/gfw/h), which was significantly different to that released from non-wounded tissues (mean 4.62 ng/gfw/h). A significant difference in emission rate was also noticed for cis-3-hexen-1-ol and cis-3-hexenyl acetate (means 96.65 and 46.09 ng/gfw/h for wounded tissues, respectively) as opposed to that emitted from non-wounded tissues (means 0.69 and 0.21 ng/gfw/h, respectively). All the other compounds were released at rates of less than 10 ng/gfw/h when wounding occurred (Table 4.1).

Table 4.1 Plant volatile compounds emitted from wounded and non-wounded stems +leaves of grapevine shoots. Volatiles were collected for 1 h then analysed with GCMS. Plants were weighed and emission rate expressed as ng/g fresh weight (fw) per hour (ng/gfw/h).

Compound	Mean concentration of compound (ng/g fw/h) (±SEM)		*P* value[a]
	Wounded	Non-wounded	
cis 3-Hexenal	111.52 (±37.53)	4.62 (±1.48)	0.036*
cis-3-Hexen-1-ol	96.65 (±28.25)	0.69 (±0.16)	0.019*
cis-3-Hexenyl acetate	46.09 (±15.35)	0.21 (±0.13)	0.030*
2-Hexenal	26.42 (±10.26)	0.12 (±0.02)	0.051
1-Hexanol	8.73 (±2.84)	0.24 (±0.06)	0.029*
trans-2-Hexen-1-ol	5.48 (±1.93)	0.07 (±0.02)	0.037*
cis-3-Hexenyl butyrate	4.96 (±1.94)	0.003 (±0.001)	0.051
Hexyl acetate	1.20 (±0.43)	0.03 (±0.02)	0.039*
cis-3-Hexenyl hexanoate	0.38 (±0.15)	0.014 (±0.009)	0.054
Geraniol	0.32 (±0.11)	0.00	0.031*
trans-2-Hexenyl butyrate	0.27 (±0.11)	0.003 (±0.001)	0.070
cis-3-Hexenyl 2-methylbutanoate	0.10 (±0.04)	0.01 (±0.003)	0.102
Decyl acetate	0.07 (±0.04)	0.00 (±0.0002)	0.031*

[a] Asterisks indicate statistically significant differences ($P \leq 0.05$, Student's paired t-test)

For collection of volatiles from stems only, 13 compounds were identified by GCMS (Appendix C.1.3, C.1.4) and the emission rates of nine of these compounds were significantly different between wounded and non-wounded shoots (Table 4.2). The compound released in the highest amount from wounded shoots was 2-hexenal (mean 48.74 ng/gfw/h), which was significantly different to the amount released from non-wounded shoots (mean 5.34 ng/g fw/h). A significant difference in emission rate was also noticed for trans-geraniol from wounded tissue (mean 8.98 ng/gfw/h) as opposed to that emitted from non-wounded tissue (mean 1.32 ng/gfw/h). All other compounds were released at rates of less than 6 ng/gfw/h when wounding occurred (Table 4.2).

Table 4.2 Plant volatile compounds emitted from wounded and non-wounded stems of grapevine shoots. Volatiles were collected for 1 h and then analysed with GCMS. Plants were weighed and emission rate expressed as ng/g fresh weight (fw) per hour (ng/gfw/h).

Compound	Mean concentration of compound (ng/gfw/h) (±SEM)		*P* value[a]
	Wounded	Non-wounded	
2-Hexenal	48.74 (±7.87)	5.34 (±1.13)	0.016*
cis-3-Hexenal	42.91 (±11.6)	8.56 (±0.54)	0.086
Hexanal	17.78 (±3.70)	8.50 (±0.55)	0.101
trans-Geraniol	8.98 (±1.66)	1.32 (±0.76)	0.042*
1-Hexanol	5.24 (±1.05)	1.60 (±0.14)	0.060
trans-2-Hexen-1-ol	3.75 (±0.54)	1.61(±0.15)	0.035*
Styrene	3.47 (±0.39)	0.67 (±0.07)	0.007*
D-Limonene	2.88 (±0.27)	0.91 (±0.06)	0.006*
alpha-Pinene	1.99 (±0.25)	1.09 (±0.13)	0.109
1,2,4-trimethyl Benzene	1.53 (±0.16)	0.56 (±0.05)	0.008*
trans-Citral	1.22 (±0.16)	0.17 (±0.04)	0.022*
Toluene	1.17 (±0.13)	0.35 (±0.03)	0.007*
Citronellol	1.08 (±0.13)	0.23 (±0.04)	0.010*

[a] Asterisks indicate statistically significant differences ($P \leq 0.05$, Student's paired t-test)

4.3.2 Exposure of *Neofusicoccum* spp. conidia to volatile compounds emitted directly from fresh plant tissue

There was a significant effect of volatiles from wounded grapevine stems on mean percent germination of conidia of *N. luteum* isolate MM558 (P=0.044, Appendix C.4.1). Mean germination of conidia in the presence of wounded grapevine stems was 82.9% while mean germination in the presence of non-wounded stems was 85.9% (Table 4.3). There was also a significant effect of wound volatiles on germ tube growth (P<.001, Appendix C.4.4), with the mean germ tube length in the presence of wounded grapevine stems being 33.4 µm and 41.6 µm in the presence of non-wounded stems (Table 4.3).

There was no significant effect of volatiles from wounded grapevine stems on mean percent germination of conidia of *N. luteum* isolate CC445 (P=0.068, Appendix C.4.2) (Table 4.3). There was a significant effect of wound volatiles on germ tube growth (P=0.025, Appendix C.4.5), with the mean germ tube length in the presence of wounded grapevine stems being 26.5 µm and 33.8 µm in the presence of non-wounded stems (Table 4.3).

There was no significant effect of volatiles from wounded grapevine stems on mean percent germination of conidia of *N. parvum* isolate G652 (*P*=0.064, Appendix C.4.3) (Table 4.3). There was also no significant effect of these same wound volatiles on germ tube growth (*P*=0.640, Appendix C.4.6) (Table 4.3).

Table 4.3 Mean percent germination of conidia (Germ) and mean length (μm) of germ tubes (GTGr) of three *Neofusicoccum* spp. isolates after two hours exposure to volatiles from wounded and non-wounded grapevine stems.

Source of volatiles	Isolates[a]					
	***N. luteum* MM558**		***N. luteum* CC445**		***N. parvum* G652**	
	Germ	**GTGr**	**Germ**	**GTGr**	**Germ**	**GTGr**
Wounded stems	82.92 a	33.42 a	75.92 a	26.5 a	57.5 a	17.10 a
Non-wounded stems	85.96 b	41.56 b	80.29 a	33.8 b	53.38 a	16.75 a

[a] Mean values with different letters within columns are significantly different at *P*≤0.05 LSD

The volatiles from wounded stems plus leaves had no significant effect on mean percent germination of conidia of *N. luteum* isolate MM558 (*P*=0.121, Appendix C.4.7). The volatiles from wounded stems and leaves had a significant effect on germ tube growth (*P*=0.003, Appendix C.4.8), with mean germ tube length in the presence of wounded grapevine stems plus leaves being 22.8 μm, and 43.4 μm in the presence of non-wounded stems

4.3.3 Exposure of *Neofusicoccum* spp. conidia to liquid volatile mix eluted from headspace sampling of grapevine tissues.

The liquid volatile mixture collected from non-wounded shoots for GCMS analysis had no effect on mean percent germination of isolate *N. luteum* MM558 (*P*=0.835, Appendix C.4.9) or on mean germ tube growth (*P*=0.859, Appendix C.4.10).

The liquid volatile mixture collected from wounded shoots for GCMS analysis had a significant effect on mean percent germination of isolate *N. luteum* MM558 (*P*=0.044, Appendix C.4.11). Mean percent germination in the presence of the liquid volatile mix was significantly lower (84.9%) than in the presence of the control hexane (87.4%). This same liquid volatile mix had no effect on germ tube growth (*P*=0.462, Appendix C.4.12).

The liquid volatile mixture collected from wounded shoots+leaves for GCMS analysis did not have an effect on mean percent germination of isolate *N. luteum* MM558 (*P*=0.809, Appendix C.4.13). However, this same mixture had a significant effect on mean germ tube growth (*P*=0.015, Appendix C.4.14) with mean germ tube length in the presence of the stem+leaf liquid volatile mix being 36.9 μm, and 40.8 μm with the hexane only control.

Hexane had no effect on germination of conidia of *N. luteum* isolate MM558 (*P*=0.389, Appendix C.4.15) however mean germ tube growth was affected by the presence of hexane (*P*=0.013, Appendix C.4.16), with significantly less growth (mean 26.5 μm) in comparison to the dry filter paper control (mean 30.8 μm).

Maximum possible concentrations of each compound present within each Petri dish, based on elution with 100 μL of hexane and no loss of volatiles are shown in Appendix C.5.

4.3.4 Exposure of *Neofusicoccum* spp. conidia to pure volatile compounds

The pure volatile compounds had a significant effect on the mean percent germination of *N. luteum* isolate MM558 (*P*<.001, Appendix C.4.17). All of the compounds caused a significant reduction in mean percent germination, with greatest inhibition of germination occurring in the presence of cis-3-hexenyl acetate (mean 1.3%) and trans-2-Hexenal (mean 1%) in comparison to the control (mean 89.6%) (Fig. 4.5).

The pure volatile compounds had a significant effect on the mean percent germination of *N. parvum* isolate G652 (*P*<.001, Appendix C.4.18). All of the compounds caused a significant reduction in mean percent germination, with the greatest inhibition of germination occurring in the presence of cis-3-hexenyl acetate (mean 1.5%) and trans-2-Hexenal (mean 1.4 %) in comparison to the control (mean 69.2%) (Fig. 4.6).

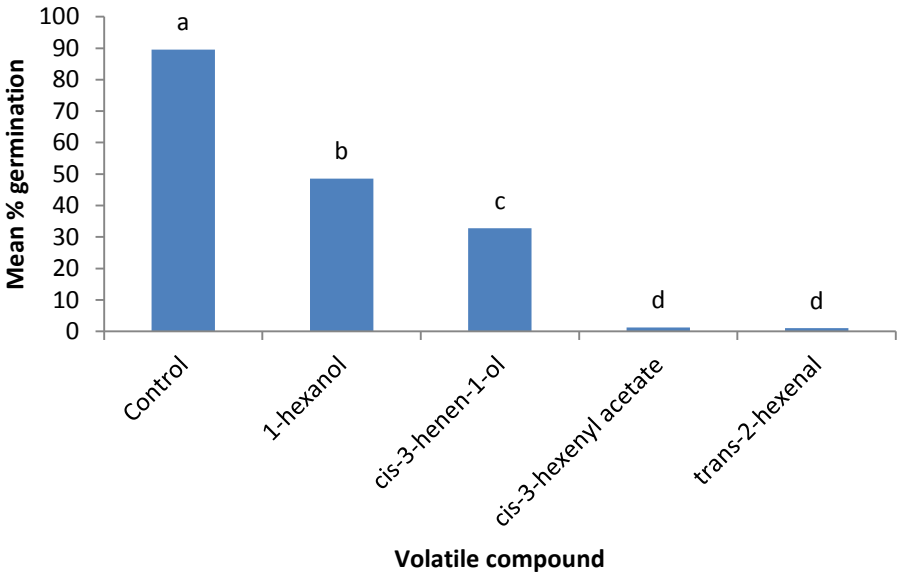

Figure 4.5 Mean percent germination of grapevine *N. luteum* isolate MM558 conidia incubated at 20°C for 2 h in the presence of pure volatile compounds (100 µL) or in the presence of filter paper (control). Bars with different letters are significantly different at $P \leq 0.05$.

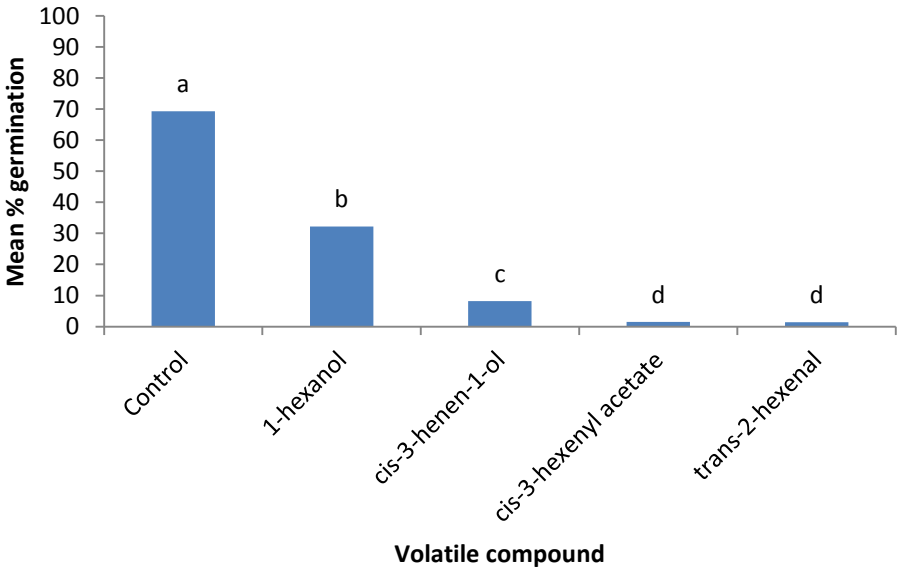

Figure 4.6 Mean percent germination of grapevine *N. parvum* isolate G652 conidia incubated at 20°C for 2 h in the presence of pure volatile compounds (100 µL) or in the presence of filter paper (control). Bars with different letters are significantly different at $P \leq 0.05$.

4.3.5 Directional growth responses of *Neofusicoccum* spp. germlings to grapevine wound volatiles

Raw data from assessment of directional growth responses are presented in Appendix C.7. Analysis of growth of germ tubes of *N. luteum* isolate MM558 in the presence of wounded grapevine shoots indicated that there were no directional growth responses occurring (Appendix C.6). Comparison of data from all slides showed no differences (P=0.161) between germ tubes in the box of wounded tissues and those in the control box; data from slides closest to volatiles (T1+B1) showed no differences (P=0.688) between wounded and control treatments, as did data from slides furthest from the volatiles (T4+B4) (P=0.065).

4.4 Discussion

This research is the first attempt of its kind to sample the different volatile compounds that were released from both non-wounded and wounded grapevine tissues and to investigate if said compounds have an effect on the germination and growth of Botryosphaeriaceae spp. conidia. As these pathogens are primarily thought to penetrate their hosts via wounds (Úrbez-Torres, 2011), it was felt to be a relevant aspect of the disease cycle to investigate in more detail. It was not the aim of this study to provide a comprehensive analysis of each individual compound released from wounded grapevine tissue, rather to determine if said volatiles may play a role in the Botryosphaeriaceae infection process. .

A number of compounds were emitted in significantly higher amounts from wounded tissues in comparison to the equivalent non-wounded tissues. Cis-3-hexenal was emitted at the highest concentration from stem+leaf volatile collection and at the second highest concentration from stems only. This compound is reported to be the first to be synthesised upon wounding, then being quickly converted into a number of six-carbon-atom alcohols, aldehydes and esters commonly known as the “green leaf volatiles” (GLVs) (Shiojiri et al., 2006). GLVs are thought to play important roles in signalling and show both antibacterial and fungicidal activity (Matsui, 2006). GLVs that are commonly found in plants include n-hexanal, trans-2-hexenal, cis-3-hexenal, trans-3-hexenal, cis-3-hexenyl acetate, n-hexanol, trans-2-hexenol, cis-3-hexenol and trans-3-hexenol. Many of these compounds are transient in nature and are formed via oxylipin metabolism, specifically the hydroperoxide lyase pathway (Matsui, 2006). During the collection of stem+leaf volatiles, many of the compounds associated with the metabolism of GLVs showed significantly increased emission

rates after wounding occurred, namely, cis-3-hexen-1-ol, cis-3-hexenyl acetate, 1-hexanol and trans-2-hexen-1-ol. Collection of stem only volatiles also demonstrated that the emission rates of the GLVs 2-hexenal and trans-2-hexen-1-ol increased significantly after wounding. In addition, the stem only volatile collection showed that emission rates of compounds such as toluene, citral, geraniol (isomer of citral) and limonene increased significantly after wounding. Emission of toluene has been shown to increase after wounding of sunflower and pine plants (Heiden et al., 1999), while the other compounds have often been associated with ripening or wounding of citrus fruit (Droby et al., 2008). It therefore appears that mechanical wounding of grapevine tissues caused release of a number of compounds known to be associated with wounding or ripening of other plant tissues. However, there were other GLV related compounds that appeared to be emitted in higher quantities upon wounding, for which results were not significant possibly due to the variability seen between some replicates.

It was unclear whether differences seen in emission rates between replicates were caused by the sampling method, natural differences in emission rates or both. When sampling wounded tissues, stems were scraped three times and leaves were rubbed on all replicate plants. However, the need to get the wounded plants re-attached to the volatile trapping equipment as quickly as possible may have meant that there was slight variation in the length of scraped wounds or in the amount of leaf rubbing. Fall (1999) demonstrated that the emission rate of hexenal compounds from wounded aspen leaves was directly related to the degree of wounding, with greater wounds emitting more compounds. It is thus possible that slight differences in the wounding technique caused some variation in the emission rates of compounds between replicate plants. In addition, the time taken to reconnect the odour source vessels to the trapping equipment after wounding may have contributed to variation in amounts of collected volatiles as some may have been lost during the reconnection process. Fall (1999), who investigated the volatile compounds released from aspen leaves after wounding, found that (*Z*)-3-hexenal (=cis-3-hexenal) was produced within 1-2 s of wounding the leaves but was transient and short lived, either through release into the air or conversion into other derivatives. In the current study, the variability between replicates did not appear to be related to plant fresh weight as during the collection of stem+leaf volatiles, replicate plants two and three had lower emission rates for certain compounds than the other four replicate plants (Appendix C.1.1, C.1.2). In order to reduce

variability of volatiles emitted from replicates, future studies should use a more precise wounding technique while employing a volatile collection method that is easier to resume after wounding has occurred.

None of the liquid volatile mixes from headspace sampling of grapevine tissues appeared to have a major effect on the germination or growth of conidia of *N. luteum* isolate MM558. The volatile mix from non-wounded stems did not affect germination rate or growth whereas that from the wounded stems did cause a slight reduction in germination (mean 2.4%) but not in germ tube growth rate compared to the control. The liquid volatile mix from wounded stems+leaves did not affect germination rate but there was a slight reduction in germ tube length (mean 3.83 μm). However, as the volatile filter elutant was pure hexane, which also reduced mean germ tube growth by 4.28 μm, it seems likely that it was hexane that caused this reduction in growth. The lack of effect on the germination and growth of species of Botryosphaeriaceae conidia may have been because the volatile compounds were present at concentrations too low to affect conidia. For stem+leaf volatiles the maximum amount of cis-3-hexenal present in one Petri dish was 15.45 μg and that of (*Z*)-3-hexen-1-ol (=cis-3-hexen-1-ol) was 13.12 μg, with other compounds being present at much lower concentrations. Given that the calculations for maximum concentrations were based on the use of 100 μL hexane elutant these estimates are generous at best. The volatiles may have been lost during elution or during the experiment. Volatiles were eluted into a glass storage vial and the base of the filter was about the same diameter as the neck of the glass vial. When hexane was injected into the filter the drop of liquid did not always fall directly into the vial and so a proportion may have been lost before closure of the vial. Also, the volatiles from each replicate were combined into one vial before being pipetted into each Petri dish. This procedure was performed in a fume hood and it is likely that even more volatiles were lost during this stage. Comparisons to similar studies in the literature were difficult to make as commercial preparations of pure (>95% purity) compounds were frequently used and analysed singly, not as a mix of compounds (Nakamura & Hatanaka, 2002; Droby et al., 2008; Cleveland et al., 2009; Neri et al., 2014).

The concentrations of plant volatiles have been reported to have differential effects on pathogenic microorganisms. Neri et al. (2006) reported that trans-2-hexenal inhibited germination of *P. expansum* conidia at 24.6 μL L^{-1} (minimum inhibitory concentration).

However, they also stated that germination was enhanced at low concentrations (5 µL L^{-1}) although this data was not presented. Neri et al. (2014) also reported that this same compound enhanced the growth of *Botrytis cinerea* at 0.0062 uL L^{-1} but inhibited growth at 12.3 uL L^{-1} as did trans-2-hexenyl butanoate and hexyl butanoate. Droby et al. (2008) reported concentration effects of citrus volatiles on conidia of *Penicillium digitatum* and *P. italicum.* Germination of *P. digitatum* conidia was stimulated at citral concentrations of 006-0.015 ppm but completely inhibited at higher concentrations. Germination of *P. italicum* was stimulated from 0.06 to 1.5 ppm and completely inhibited at 15 ppm. These same authors also found limonene and alpha-pinene were both released in appreciable concentrations from wounded clementine, orange and grapefruit (Droby et al., 2008). Both of these compounds tended to have a stimulatory effect on germination and growth of *P. italicum and P. digitatum* and an inhibitory effect on germination and germ tube elongation of *P. expansum* and *B. cinerea*. The authors concluded that a combination of volatiles acted as a signalling mechanism for host recognition for citrus pathogens but inhibited non-citrus pathogens, indicating a pathogen specific nature of wound induced volatiles. Limonene and alpha-pinene were sampled from wounded grapevine shoots during this study and limonene emission rates increased significantly upon wounding of the stems. In addition, the emission rates of trans-citral and citronella increased upon wounding and these compounds have been shown to reduce *Geotrichum candidum* spore germination by more than 60% at concentrations of 1 µL/mL (Suprapta et al., 1997).

During this study, the volatiles emitted directly from freshly wounded stem tissue had differential effects on the isolates used. Small to moderate reductions occurred in germination and germ tube growth of *N. luteum* isolate MM558 and in germ tube growth of isolate *N. luteum* CC445, while *N. parvum* isolate G652 was unaffected by these volatiles. The inhibition of growth of isolate MM558 was more pronounced in the presence of fresh stem+leaf volatiles in comparison to the stem only volatiles, being reduced by almost a half in comparison to the control. The greater inhibitory effect seen in the presence of leaf volatiles may account for the observations made by Amponsah et al. (2012b) when investigating the susceptibility of different grapevines tissues to infection. They reported that disease symptoms did not develop more than 2-3 mm from the inoculation point on leaves inoculated with Botryosphaeriaceae spp. conidia. In the current experiments the possible effects of hexane were eliminated as fresh plant tissue was used directly. Therefore,

it seems clear that volatiles from wounded grapevine tissue can affect the germ tube growth of some Botryosphaeriaceae isolates and that growth seems more sensitive to volatiles than germination. In addition, tissues with different volatile profiles may have the potential to prevent disease development. It would therefore be useful to design an inoculation experiment with different grapevine tissues and Botryosphaeriaceae spp., in which the levels of certain volatiles present in the air space above the tissues are measured and effects correlated with the disease incidence on said tissues.

The positive results from investigating the effects of volatiles emitted directly from fresh tissue prompted a further study with pure volatile compounds. The volatiles chosen for analysis in their pure form, namely cis-3-hexen-1-ol, cis-3-hexenyl acetate, trans-2-hexenal and 1-hexanol were based on the compounds produced in the highest concentrations from the stem+leaf volatile collection that were available at Lincoln University. Although cis-3-hexenal was the compound emitted at the highest concentration, this compound was not available due to its highly transient and unstable nature. However, it has been shown to have antifungal activity as a *Botrytis* sp. was unable to grow on fruits of strawberry exposed to 200 µL of cis-3-hexenal (in a 250 mL container) (Archbold et al., 1997).

All of the pure compounds reduced the germination of conidia of *N. luteum* MM558, with germination almost completely inhibited in the presence of 100 µL trans-2-hexenal and cis-3-hexenyl acetate. Trans-2-hexenal has been reported to be important in conferring resistance of *Arabidopsis* to *B. cinerea* (Kishimoto et al., 2008). Transgenic plants having higher levels of this compound were found to be more resistant to growth of the pathogen than plants in which levels of this compound were suppressed. Trans-2-hexenal has also been shown to be inhibitory towards *P. expansum* germination (ED_{50}=10.2 µL L) (Neri et al., 2006), growth of *Escherichia coli*, *Salmonella enteritidis* and *Staphylococcus aureus* (at 6.26 µg/mL, 6.26 µg/mL and 3.13 µg/mL, respectively) (Nakamura & Hatanaka, 2002) and growth of *Aspergillus flavus* (at 10 µL per Petri dish) (Cleveland et al., 2009). Growth of *B. cinerea* was inhibited on strawberry fruits after being exposed to 60 µL of 1-hexanol and 12 µL of cis-3-hexen-1-ol (Archbold et al., 1997). Another wound-induced volatile, cis-3-hexenyl acetate, has been shown to prime plant defence signalling in leaves, which resulted in them being less susceptible to attack by herbivory (Frost et al., 2008). Although the concentrations of pure compounds used during this study would not be found in nature, they demonstrate

that volatile compounds have the potential to restrict the germination of Botryosphaeriaceae spp. conidia. In addition, using pure compounds gives an indication of which volatile compounds may be more effective at inhibiting germination and/or growth. It would be useful to perform a more comprehensive study to determine if conidia from numerous Botryosphaeriaceae species are affected, whether the effect was concentration dependent and whether the effect was permanent or temporary.

If fresh wound volatiles do effect the germination and growth of these pathogens this could be in contradiction to the reports that fresh grapevine wounds are more susceptible to infection (Úrbez-Torres & Gubler, 2011; van Niekerk et al., 2011; Amponsah et al., 2014). However the authors in these studies used artificial inoculation directly onto wounds and this may have placed an unrealistic inoculum load onto the wounds, masking the events which may occur naturally in the field. Further, assessment of pruning wound susceptibility was usually performed weeks or months after inoculation. As volatiles are transient in nature any response would likely be seen at very early stages of fungal development. If the volatile response was temporary and germination and/or growth continued after a few days the effect would go unnoticed if assessment of growth was made weeks later.

Assessment of directional growth in the current study indicated that the germ tubes of *N. luteum* isolate MM558 did not show any chemotropic growth response to the volatiles released from wounded grapevine stems. Relevant literature in this area was limited and restricted to the well-known tropic responses such as those related to sex pheromones (Brand & Gow, 2009), mycorrhizal fungi host location (Harrison, 2005) and the wood decay fungi (Mowe et al., 1983). As such, comparisons could not be made to other stem pathogens that infect via wound sites. The negative result obtained during this investigation may have been a result of the experimental system used as observations made during the investigation of infection structures appeared to show that germlings very close to the wound site on grapevine stems were demonstrating some sort of directional growth response (Appendix B.5.1, B.5.2).

During the investigation into directional growth, a 650 mL container was used with an opening at one end to try and promote a directional movement of volatiles from the wounded shoot, across the slides containing conidia towards the opening. On reflection, the container was probably too large and the volatile mix may have been quickly lost from the

container through the opening. In addition, the conidial suspension on the slides closest to the box opening (B4 and T4) were starting to dry out after 3 h, even with the smaller opening. The concern with using a Petri dish for this experiment was that the dish would get flooded with volatile mix and thus it would be difficult to establish a directional gradient. However, assays using Petri dishes have been used to successfully demonstrate chemotropic growth of *Cochliobolus sativus* towards barley roots (Jansson et al., 1988), of *Chaetomium globosum* to volatiles from wood (Carlile & Matthews, 1988) and of a number of wood decay fungi towards air-dried wood blocks (Mowe et al., 1983). It is also possible that the chemotropic responses are only activated very close to the volatile source, in which case using a Petri dish would have been more appropriate for this study. Chemotropic responses of germ tubes of *Co. sativus* were activated 2 mm or less from barley roots but were not seen at distances greater than this (Jansson et al., 1988). Sbrana and Giovannetti (2005) estimated that the distance of perception of chemotropic signals for *Glomus mossae* towards host roots was about 900 µm while Koske (1982) reported germ tubes of *Gigaspora gigantea* could perceive volatile signals from host roots up to a distance of 11 mm away. It is possible that the requirement for close proximity in these studies reflects a need for high concentrations of signal molecules. In this study, the drops of conidial suspension closest to the nearest wounded shoot piece were about 2.5 cm away which may have been too far to activate a chemotropic response. It would be advantageous for Botryosphaeriaceae spp. germ tubes to be able to find wound sites by responding to a signal emitted from wounds instead of relying on the random encounter of a wound site. As this signal may be related to volatile emission, the directional growth of germ tubes of Botryosphaeriaceae spp. towards volatiles should be further investigated using Petri dish assays. Experiments should be performed with a range of species and conidia should be placed at closer distances to the shoot piece to determine the range of any potential response. Conidia should also be placed in the vicinity of the wounded shoot at various time periods after wounding has occurred to determine if there is a time limit on volatile responses.

In conclusion, this study has shown that disruption of grapevine tissues causes an increase in the emission of a number of well-known wound associated compounds. Although the trapped, eluted and stored volatiles had little effect, exposure to volatiles released directly from freshly wounded stems reduced the germ tube growth of some Botryosphaeriaceae spp. by about 20% while exposure to volatiles from freshly wounded stems+leaves reduced

germ tube growth of *N. luteum* MM558 by about 50%. Exposure to 100 µL of some pure volatile compounds reduced germination of isolate *N. luteum* MM558 and *N. parvum* isolate G652 with almost complete inhibition in the presence of trans-2-hexenal and cis-3-hexenyl acetate. Germlings were not found to demonstrate directional growth towards wounds however observations made during microscopy studies indicate a chemotropic response may be occurring.

Chapter 5

Concluding discussion

The large number of species of Botryosphaeriaceae associated with grapevine trunk diseases throughout the world, which has led to significant economic losses, has led to these fungi being considered serious pathogens of grapevines. Effective control strategies for these diseases are proving elusive and are further complicated by the number of different species that can infect grapevines. Management of trunk diseases has traditionally been achieved by using an integrated strategy consisting of a combination of vineyard sanitation practices, pruning wound protectants, remedial surgery or replanting. As the main point of entry of these diseases into the vine is thought to be via pruning wounds, liquid or paint fungicides are often used to protect newly cut areas. However, this has not been completely effective because many wounds may be made on a single vine throughout the year, and it is not possible to time all pruning and trimming events to coincide with periods of low spore dispersal, especially with respect to the many vineyard blocks being managed in a single vineyard.

Development of new control strategies requires a more detailed knowledge of the disease cycle in order to target specific infection stages, preferably before penetration into the plant occurs. Although understanding of the factors that contribute towards disease development has increased dramatically, the complete disease cycle of Botryosphaeriaceae species has yet to be elucidated. With this in mind, the study aimed to investigate an aspect of the disease cycle which has not been investigated, the properties of Botryosphaeriaceae spp. spores and processes that occur before penetration into the plant, information which could prove useful in preventing subsequent infection.

5.1 Adhesion

The conidia of the *N. luteum*, *N parvum* and *B. dothidea* isolates used during this study all adhered well and quickly to a range of surfaces differing in their wettability. Since the Botryosphaeriaceae spp. pathogens need to infect through wounds or other openings, it is advantageous for the spores to adhere quickly to prevent being displaced from the host, thereby remaining attached long enough to germinate and then grow towards/into an

opening. As Botryosphaeriaceae species conidia are dispersed in water droplets, and many plant surfaces are hydrophobic, they risk being displaced when water droplets roll off such surfaces. Adhering quickly would lower the chances of this happening. This research indicates that conidia would be able to adhere to a range of grapevine plant tissues differing in their wettability, such as petals, leaves, flowers, young shoots and older wood, suggesting that all parts of the vine could potentially harbour the inoculum. Given that the chances of landing directly onto a fresh wound site may be low if the source of inoculum is at a distance from the wound, it would be advantageous for these pathogens to adhere to a range of tissues, particularly as buds, flowers and fruit have been shown to be susceptible to infection (Amponsah et al., 2011; Wunderlich et al., 2011). Since fresh wounds are less hydrophobic than the plant cuticle, the ability to adhere to tissues of different wettabilities provides for greater chance of finding potential infection sites.

As conidia adhere well to artificial surfaces, it appears as if the attachment process is not specific to the plant host, indicating early adhesion of conidia is not reliant on host signals. Further evidence for an infection process that is not reliant on host specific signals has been provided by Amponsah et al. (2011) who found non-grapevine isolates of Botryosphaeriaceae spp. from hosts such as cherry, apple, oak, broom and willow were equally pathogenic on grapevines. In addition, during this study a non-grapevine isolate of *N. luteum* from hardy Kiwifruit was used to produce conidia by infecting grapevine shoots. For a non-specific host pathogen it would be advantageous to avoid relying on specific host signals therefore non-specific adhesion may reflect the ability of these pathogens to adhere to a wide range of species and tissue types.

The short time required to reach maximum adhesion indicates that conidia of Botryosphaeriaceae spp. are likely to travel over relatively short distances during a dispersal event, even with wind assisted dispersal of water droplets. Therefore it is likely that most new infections come from within the vineyard rather than from other hosts or other vineyards in the surrounding area. This indicates that for prevention of stem disease, emphasis should be placed on removing inoculum sources from the vineyard prior to pruning and, where possible, only planting disease-free vines. The quick attachment process also has implications for grapevine nurseries. During nursery propagation of grapevines,

cuttings taken from mother vine blocks are washed and hydrated at various stages. An investigation into sources of inoculum in New Zealand grapevine nurseries showed that incidence of infection was high on bark of cuttings but in wash tanks inoculum was at very low levels or absent (Billones-Baaijens, 2011). While this may indicate effective sanitation practices, it could also be because conidia that are adhering to plant surfaces are not dislodged during the washing/rehydration steps. Evidence that this may be the case was demonstrated by Billones-Baaijens (2011) who investigated the numbers of conidia washed from surfaces of grapevine cuttings after various incubation times. It was shown that when dormant cuttings were inoculated with approximately 10,000 conidia and then immediately washed (after 2-3 min processing) significantly fewer conidia were recovered in the wash water (mean 862.5). After 1 h incubation, the number of conidia recovered in the wash water was even fewer (mean 287.5). It is possible that changes in the washing procedure to enable conidia to be washed off could have a large impact so should be investigated further. Washing regimes could incorporate the use of different surfactants or detergents that could help to 'wash' conidia from cutting surfaces. Alternatively, washing/hydration solutions could be amended with appropriate fungicides or bleach to reduce the viability of any attached conidia.

While this study demonstrated the quick attachment of conidia to surfaces, the histochemical labelling of conidia and germlings in the current study indicated that the adhesion of Botryosphaeriaceae conidia occurs in two phases. The first phase occurs quickly after making surface contact and the second coincides with germination. This suggests that the adhesion of both non-germinated and germinated conidia may contribute to disease development. Indeed this study has demonstrated that conidia do not require surface contact for germination, so it is possible that conidia can germinate within water droplets and then adhere with the aid of the mucilage released at this stage. The mean adhesion rates recorded during this study reached only about 50% and it is unclear whether this reflects the qualities of the conidia used, the short duration (1 h) of the experiment, or the use of artificial surfaces. In order to establish whether a temporal relationship exists between the secretion of matrix materials and adhesion, a further study should be conducted over a longer time period that encompasses the early stages of germination and uses a more natural surface such as reconstituted cuticle (Podila et al., 1993). In addition, the strength of adhesion should be ascertained during these two adhesion phases by

increasing the volume or number of washes or by using a method where the water can be agitated. If conidia can be easily dislodged from surfaces during the initial adhesion phase then it may be possible to devise some method of targeting this stage as a control strategy.

This study was unable to determine whether the adhesion process was mediated by active metabolic processes as results were inconclusive. However, heat treatment of conidia did not completely prevent the attachment of conidia to surfaces and as no growth on PDA indicated conidia had been killed, this indicates that active metabolism is not required during the early stages of the adhesion process. The complete inhibition of adhesion shown by conidia treated with a protease demonstrated that proteins in the spore coat play a major role in the attachment process and this should be investigated in more detail. Further studies should be conducted with different incubation regimes to determine if conidia exposed to pronase and then washed can regain their ability to adhere and also whether treatment of attached conidia with pronase can cause them to be removed.

5.2 Germination

Germination rates were also examined on the same surfaces as those used during the adhesion study. For all isolates, germination rates were higher on cellulose than on glass, parafilm or polystyrene. In comparison to glass, the mean increases in percent germination were 24.0% for *N. luteum* isolate CC445, 6.3% for *N. luteum* isolate MM558, 4.9% for *N. parvum* G652 and 10.7% for *B. dothidea* 007. As the other three surfaces tested during the study did not have an effect on the germination rate of these isolates it was felt that surface hydrophobicity did not play a major role in germination of these pathogens, which could germinate on a range of surfaces differing in their wettabilities. It is possible that some other property of cellulose influenced germination rate but this was not determined during this study. Conidia germinated on all artificial surfaces which suggests that specific host plant signals are not required for initiation of germination. A non-specific germination mechanism would allow conidia to germinate wherever they landed and if conditions were favourable, growth may continue until a wound or other opening was encountered.

In contrast to the highest germination rates occurring on cellulose, germ tube growth on this surface was significantly less than on the other surfaces and this pattern was observed across all species. Plate assays performed with cellulose agar indicated that cellulose may

have been utilised as a food source thus negating the requirement for exploratory growth of the mycelium. If a certain species can degrade cellulose then it may be able to begin growth on exposed plant tissues very rapidly and so have greater potential for swiftly invading the stems. Further, since the cuticles of green tissues are often composed of discontinuous granules, these fungi may be able to penetrate the unprotected cellulose on green stems directly, rather than requiring an opening. This would have an impact on the methods used to control them. Pruning wound protectants may not be the most effective method of controlling these pathogens if they were capable of penetrating the host via degradation of the cell wall over time. Alternatively, the rate of cellulose degradation may not be sufficient to penetrate the host but to allow survival on the plant surface until an opening on the plant surface is encountered. This strategy seemed to be operating in Austrian and Scots pine trees, where *Diplodia pinea* was isolated from symptomless shoots and was mainly associated with bark and phloem tissues (Flowers et al., 2001). In addition, inoculations on non-wounded stems of peach trees with *B. dothidea*, *B. rhodina* and *B. obtusa* that were evaluated 18 months later showed that isolation frequency of these species from outer bark was 35-100% whereas the frequency of isolation from inner bark or xylem was much lower (0-13%) (Pusey, 1993). Further, even though pathogens were isolated the stems displayed no obvious signs of disease, further indicating that Botryosphaeriaceae spp. pathogens have the ability to survive for periods of time on the bark and this may be related to their ability to be able to degrade cellulose as a food source.

As the ability of Botryosphaeriaceae spp. pathogens to degrade cellulose may have a major impact on their ability to infect tissues directly or to survive for long periods on the plant surface, there is a need to further investigate this aspect of their nutrition. Development of a test that has the ability to quantitatively measure the rate of cellulose breakdown would be useful and would eliminate the use of the less reliable agar plate assays. In addition, inoculation of non-wounded canes with a marker isolate of Botryosphaeriaceae would allow monitoring of pathogen development on the outer tissues and demonstrate whether they were able to colonise internal tissues over time without wounds.

Further evidence for non-specific germination cues was provided by the lack of any discernible germination patterns relating to surface hardness and the ability of the conidia to germinate without surface contact. The ability to germinate equally well on soft or hard

surfaces confers increased flexibility to the pathogen and may be one reason for the reported ability of *N. luteum* to infect types of tissues that differ in their hardness such as buds, leaves, mature berries as well as woody and green stems (Amponsah et al., 2012b). The lack of requirement for a hard surface or indeed for any surface contact at all, may reflect the lack of infection structures reported for these pathogens. Surface contact, often with a hard surface, is conducive for differentiation of germ tubes into appressoria for *M. grisea* (Jelitto et al., 1994), *C. trifoli* (Warwar & Dickman, 1996), *C. lindemuthianum* (Rawlings et al., 2007) and *C. truncatum* (Egley, 1994). To date, no infection structures have been reported for Botryosphaeriaceae species on grapevines and the investigation of infection structures undertaken during this study was unsuccessful. The ability to form infection structures influences the way in which host penetration can occur and this in turn has an impact on the effectiveness of control strategies. Further investigations into the ability of Botryosphaeriaceae spp. to form infection structures should be performed using microscopy, with a range of hosts and a range of species.

The apparent non-specific adhesion and lack of germination cues highlight the potential of Botryosphaeriaceae spp. pathogens to infect many different hosts. These non-specific mechanisms found during the early infection process may in some part explain the lack of resistance seen in grapevine varieties and other woody hosts to-date. Host defences are often triggered by recognising specific signals from the pathogen and a lack of such recognisable signals may prevent resistance from developing. This is extremely important as it prevents resistant varieties being used as part of an integrated control strategy.

5.3 Volatiles

Investigations into the volatiles produced upon the disruption of grapevine tissues showed an increase in many compounds known to be produced from wounded tissues and referred to as the green leaf volatiles. The green leaf volatile (GLV) compounds can be quickly emitted upon mechanical wounding, herbivory and pathogen infection and have been reported to be involved in eliciting plant defence responses (Scala et al., 2013). It would be advantageous to grapevines to be able to mount a defence response upon wounding to decrease infection rates from both fungi and bacteria. This study has demonstrated that GLV compounds are released at increased emission rates upon wounding and indications are that volatiles from freshly wounded plant tissue can affect the germination and growth of Botryosphaeriaceae

pathogens and as such this area warrants more investigation. Although confirmation of the effects of volatiles on germination and growth should be performed in vitro, further experiments should be conducted on living, mature plants to assess the effect of fresh wounds on germination of Botryosphaeriaceae spp. conidia in the natural environment.

As Botryosphaeriaceae spp. pathogens infect mainly through wounds (Úrbez-Torres, 2011) the effect of wound age on infection has been investigated by inoculating the wound sites with conidia and then assessing disease development some weeks/months later (van Niekerk et al., 2007; Serra et al., 2009; Úrbez-Torres & Gubler, 2011). These studies did not investigate the interactions that may occur immediately after wounding occurs when volatiles are released from grapevine tissues, nor the potential for growth towards the wound, perhaps guided by some kind of chemotropic signal. When Amponsah at al. (2014) investigated the effect of wound age on *N. luteum* infection, they reported no infection on a 30 day old wound from conidia but a 40% incidence of infection from mycelium. These findings suggest that mycelium is less affected by wound age than conidia and has the potential to infect wounded tissue for extended periods of time. The differential response of conidia and established mycelia to wound age reported by these authors may have been associated with the effects of different concentrations of chemotropic signals, which could affect germination and mycelia growth differently. It is also possible that initiation of a response may only require a very brief exposure to volatile compounds. Evidence that this may be the case has been provided by French et al. (1977). These authors examined the minimum exposure time required for maximum germination of uredospores of some rust fungi to volatile compounds. Both *Puccinia coronata* and *P. graminis* var *tritici* required only 20 s exposure to nonanal in order to stimulate germination, with the stimulation effect lasting for up to 90 min. The percent germination of the control uredospores was less than 5% after 90 min. In the current study, only volatiles emitted within 20 to 60 min after wounding were collected and assessed. However, the period of volatile emission from a new wound is unknown. In a study by Brilli et al. (2012) the volatile emissions from mountain grassland were monitored before cutting, upon cutting and for 5 days thereafter. They reported that volatile emissions increased due to cutting and that it took 3-4 days before levels of emissions returned to those of pre-cut levels. Clearly, it would be worthwhile for a similar study to be conducted with grapevine wound signals. Prolonged periods of post-

wounding volatile emissions would increase the chances that a volatile signal wound be received by mycelium growing on the vine or surviving within the outer tissues.

This research has provided new information about the pre-infection behaviours of Botryosphaeriaceae spp. and indicated the potential for new research directions which may result in new innovative control methods. Application of volatile compounds to grapevine tissues may have the potential to reduce incidence of infection, either by its direct action or through priming of defence responses. Since the volatile profiles of plants of tomato (Lewinsohn et al., 2001), tobacco (Kessler et al., 2004) and potato (Vancanneyt et al., 2001) have been reported to be modified through genetic engineering, it is possible that grapevine green tissues could be induced to emit increased levels of certain volatile compounds, possibly resulting in lower infection rates by Botryosphaeriaceae species. Clearly, further research is warranted into the role of wound volatiles and ways to exploit such knowledge for development of control strategies; they have the potential to improve management of Botryosphaeria dieback, which has not been achieved to date.

Appendix A

Supplementary material for Chapter 2

A.1 Dye and media recipes

Lactoglycerol aniline blue

20 mL Lactic acid
40 mL Glycerol
20 mL Distilled water
0.075g Aniline blue

Skimmed milk agar (SMA)

The SMA was prepared by combining 20 g water agar (Davies) with 2.8 g nutrient agar (Oxoid) to 500 mL SROW in a 1 L Schott bottle (Dore, 2009). Into a 500 mL bottle, 50 g skimmed milk powder (Anchor) was dissolved in 500 mL SROW. These bottles were both autoclaved (121^{o}C for 15 min), then cooled in a water bath to ~50^{o}C. Once cooled, the skimmed milk was slowly poured into the agar suspension under aseptic conditions. These were mixed by slowly rolling the bottle on its side before being poured into standard Petri dishes and left to set.

Dyes for histochemical characterisation of mucilage

Table A.1.1 Recipes for dyes used in the histochemical labelling of spore mucilage

Dye	Recipe
Alcian blue (Sigma Aldrich)	1% in 5% acetic acid
Coomassie brilliant blue (BDH, Global Science)	0.2g Coomassie brilliant blue 40 mL methanol 10 mL acetic acid 50 mL SROW
Congo red (Coomak Chemicals Ltd)	0.1 g Congo red 0.05 mL ethanol (95%) 100 mL SROW
Acid fuchsin (BDH stains)	1% in SROW

A.2 Raw data for testing the consistency of the spore mixing procedure

Table A.2.1 Counts of conidia present on a glass slide within a 10 uL drop of *N. luteum* isolate MM558 conidial suspension (~$1.5x10^4$ conidia/mL) that had been vortexed briefly for a few seconds before the start of each test then swirling by hand between each pipette for that test.

Number of conidia present					
Test1	**Test2**	**Test3**	**Test4**	**Test5**	**Test6**
148	151	155	148	148	162
135	131	130	155	179	164
130	144	141	152	151	158
158	146	149	125	148	168
170	165	125	155	157	145

A.3 Analysis of variance (ANOVA) tables for adhesion experiments

Table A.3.1 ANOVA for adhesion (log_{10}) of conidia of *N. luteum* isolate CC445 to different surfaces at different time points

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	0.2827	0.0566	1.50	
Surface	3	4.4915	1.4972	39.73	<.001
Time	4	25.6686	6.4172	170.27	<.001
Surface.Time	12	2.4458	0.2038	5.41	<.001
Residual	95	3.58040	0.0377		
Total	119	36.4690			

Table A.3.2 ANOVA for adhesion (log_{10}) of conidia of *N. luteum* isolate MM558 to different surfaces at different time points.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	0.6200	0.1240	3.12	
Surface	3	1.0572	0.3524	8.86	<.001
Time	4	21.8922	5.4730	137.64	<.001
Surface.Time	12	3.2461	0.2705	6.80	<.001
Residual	95	3.7776	0.0398		
Total	119	30.5931			

Table A.3.3 ANOVA for adhesion (log_{10}) of conidia of *N. parvum* isolate G652 to different surfaces at different time points.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	6.3490	1.2698	28.08	
Surface	3	0.8483	0.2828	5.59	0.001
Time	4	16.1873	4.0468	79.94	<.001
Surface.Time	12	4.4227	0.3686	7.28	<.001
Residual	95	4.8092	0.0506		
Total	119	32.6165			

Table A.3.4 ANOVA for adhesion (log_{10}) of conidia of *B. dothidea* isolate 007 to different surfaces at different time points.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	1.3432	0.2686	5.56	
Surface	3	1.0977	0.3659	7.57	<.001
Time	4	18.6074	4.6519	96.30	<.001
Surface.Time	12	1.7121	0.1427	2.95	0.002
Residual	95	4.5890	0.0483		
Total	119	27.3494			

Table A.3.5 ANOVA for adhesion of treated conidia of isolate *N luteum* MM558 to glass after incubation for 20 min with various treatments – Experiment 1.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	192.20	38.44	0.73	
Treatment	6	14668.55	2444.76	46.13	<0.001
Residual	30	1589.83	52.99		
Total	41	16450.58			

Table A.3.6 ANOVA for adhesion of treated conidia of isolate N. luteum MM558 to glass after incubation for 20 min with various treatments – Experiment 2.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	3960.7	792.1	2.67	
Treatment	6	24086.0	4014.3	13.51	<0.001
Residual	30	8915.8	297.2		
Total	41	36962.5			

Table A.3.7 ANOVA for adhesion of conidia of isolate *N. luteum* MM558 to glass after being heat treated (95^{o}C and 110^{o}C) for various time points (10, 15, 20 and 30 min).

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	85.77	17.15	0.49	
Treatment	8	39684.87	4960.61	141.50	<0.001
Residual	40	1402.25	35.06		
Total	53	41172.89			

Table A.3.8 ANOVA for adhesion of conidia of isolate *N. luteum* MM558 to glass after being washed (0, 2 or 4 times).

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	524.3	104.9	0.60	
Treatment	2	982.1	491.0	2.83	0.106
Residual	10	1737.7	173.8		
Total	17	3244.0			

A.4 Supplementary photographs of colonies growing on PDA from droplets of conidial suspensions which had been treated with various inhibitors.

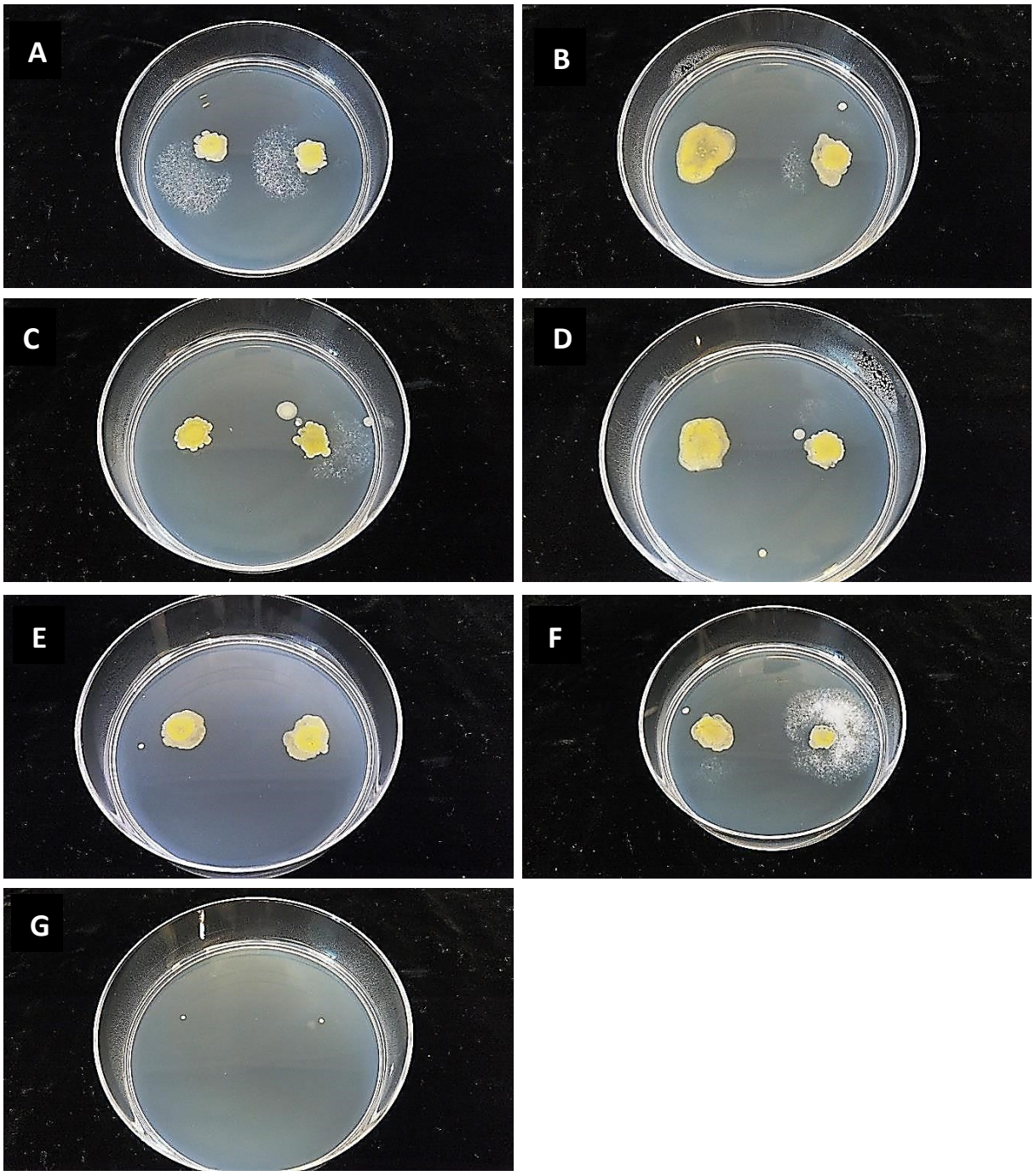

Figure A.4.1 Three day old colonies growing from droplets of conidial suspensions of *N. luteum* isolate MM558 after being subjected to various treatments. A) No treatment (control); B) buffer (Tris-HCl); C) pronase E; D) denatured protease; E) cycloheximide; F) sodium azide; G) heat.

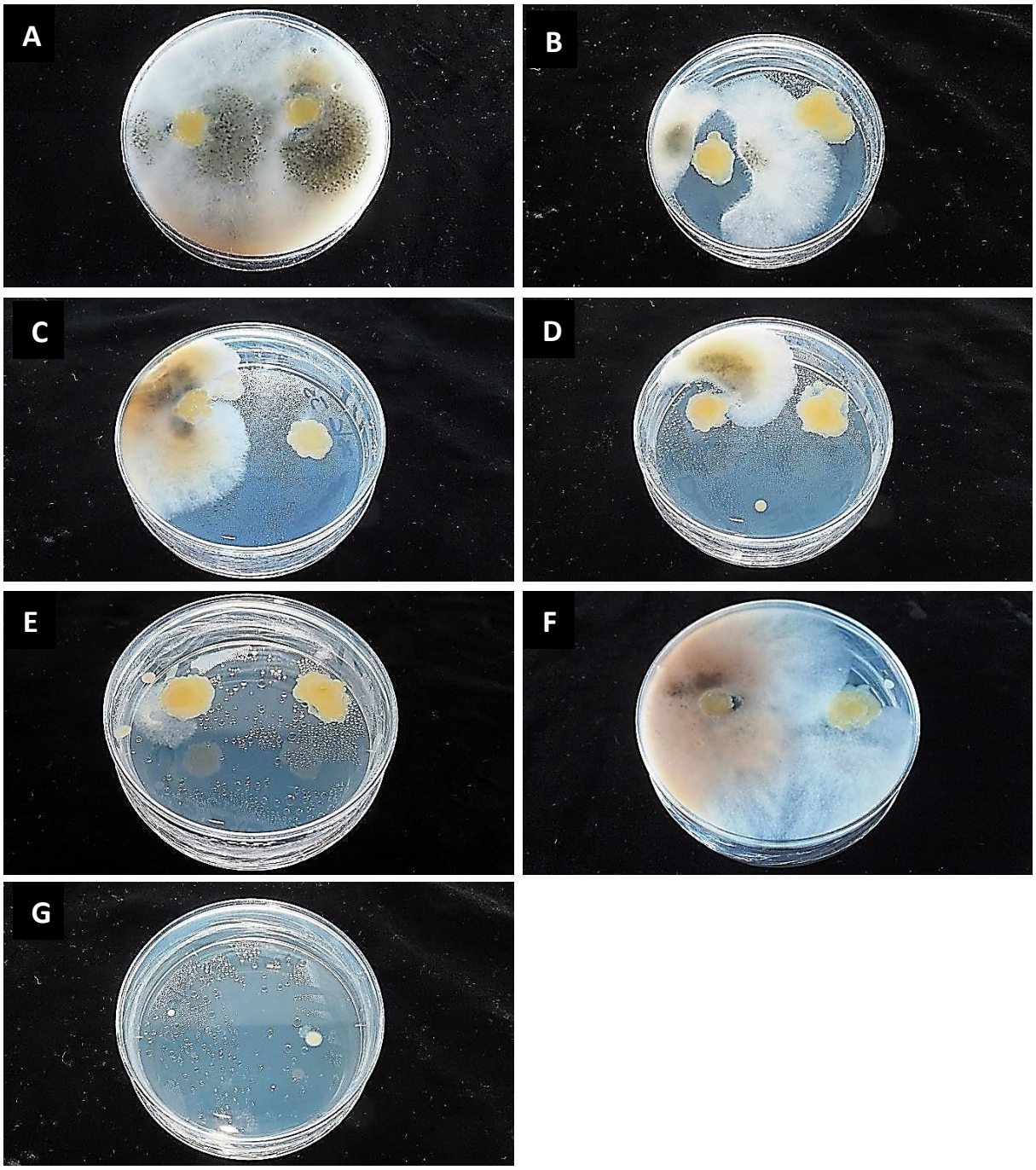

Figure A.4.2 Five day old colonies growing from droplets of conidial suspensions of *N. luteum* isolate MM558 after being subjected to various treatments. A) No treatment (control); B) buffer (Tris-HCl); C) pronase E; D) denatured protease; E) cycloheximide; F) sodium azide; G) heat.

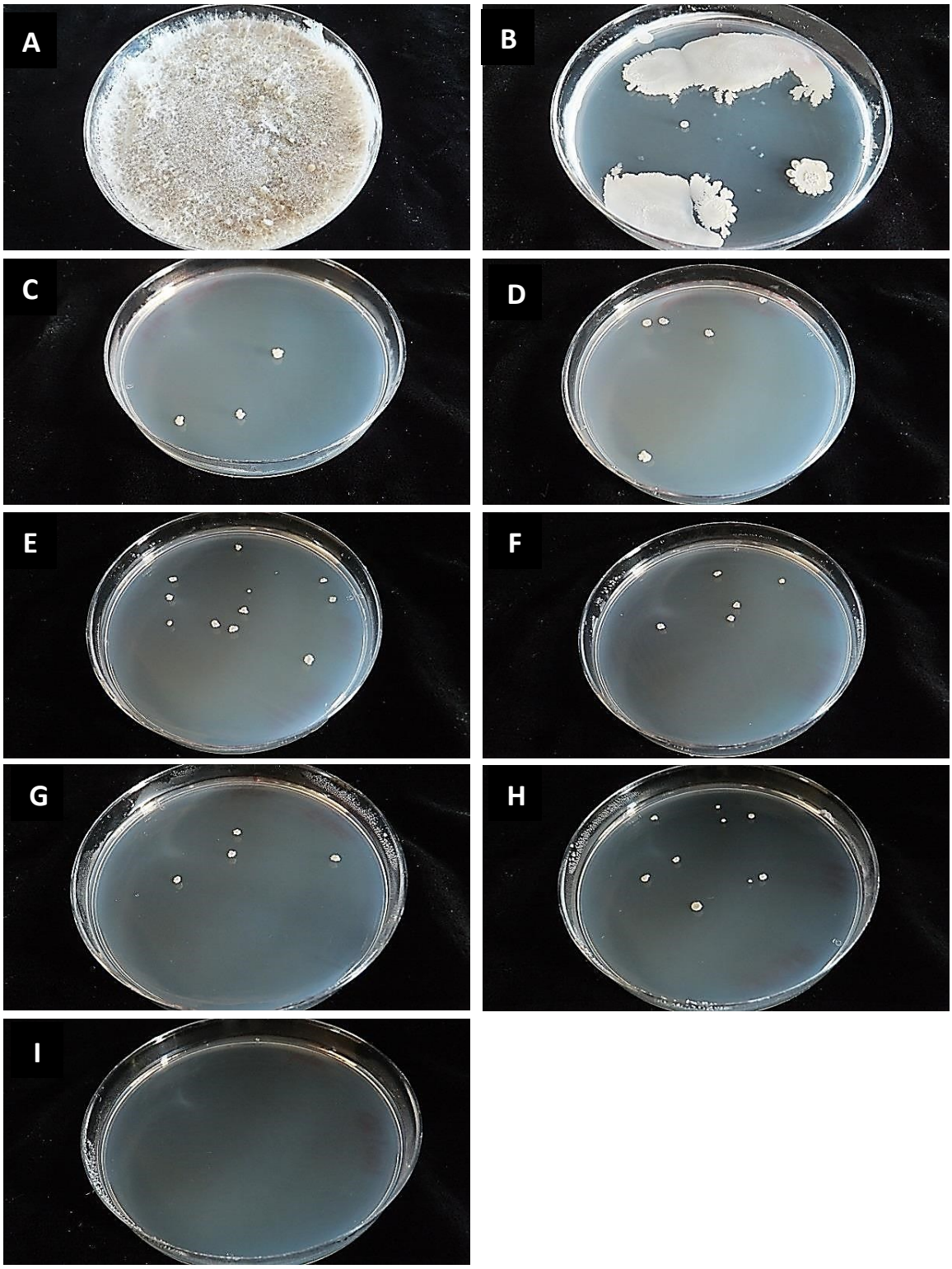

Figure A.4.3 Conidia of *N. luteum* isolate MM558 subjected to various heat treatment regimens and then incubated for ten days at 25°C 12/12 (light/dark) on PDA to check viability. A) control (no heat); B) 95°C 10 min; C) 95°C 15 min; D) 95°C 20 min; E) 95°C 30 min; F) 110°C 10 min; G) 110°C 15 min; H) 110°C 20 min; I) 110°C 30 min.

A.5 Supplementary photographs of conidia stained with a range of dyes

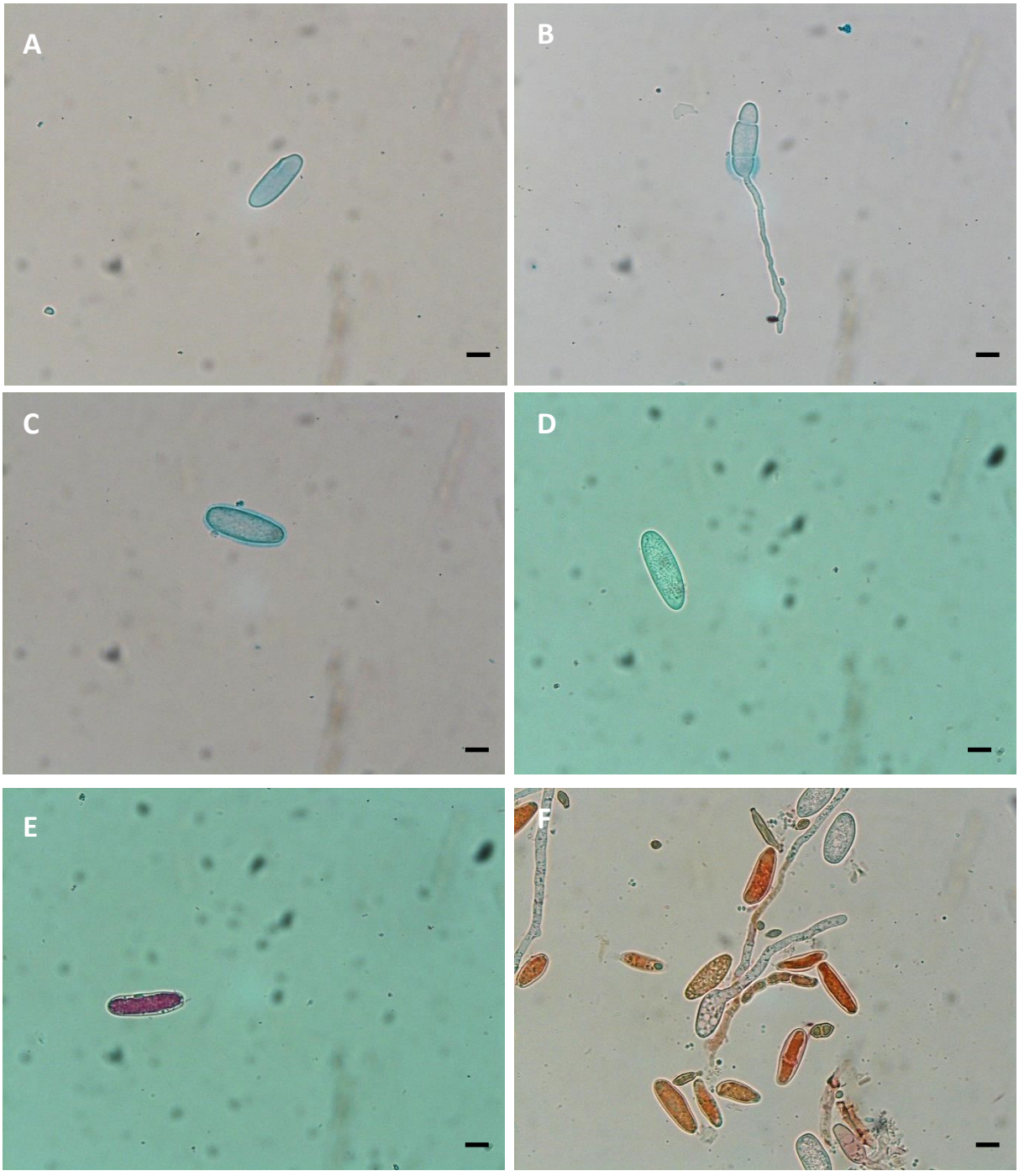

Figure A.5.1 Conidia of *N. luteum* isolate MM558 incubated for 150 min and then stained with a range of dyes. A) Empty conidium stained with alcian blue (-ve result); B) germling stained with alcian blue showing a blue halo at the germ tube emergence point; C) conidium stained with alcian blue with surrounding blue halo; D) conidium stained with acid fuchsin with no visible halo (-ve result); E) Shrivelled conidium stained with acid fuchsin (-ve result); F) conidia and germlings stained with Congo red, only shrivelled and empty conidia stained pink/red so were not included in results, normal viable conidia and germlings did not stain red. Scale bars = 8 μm.

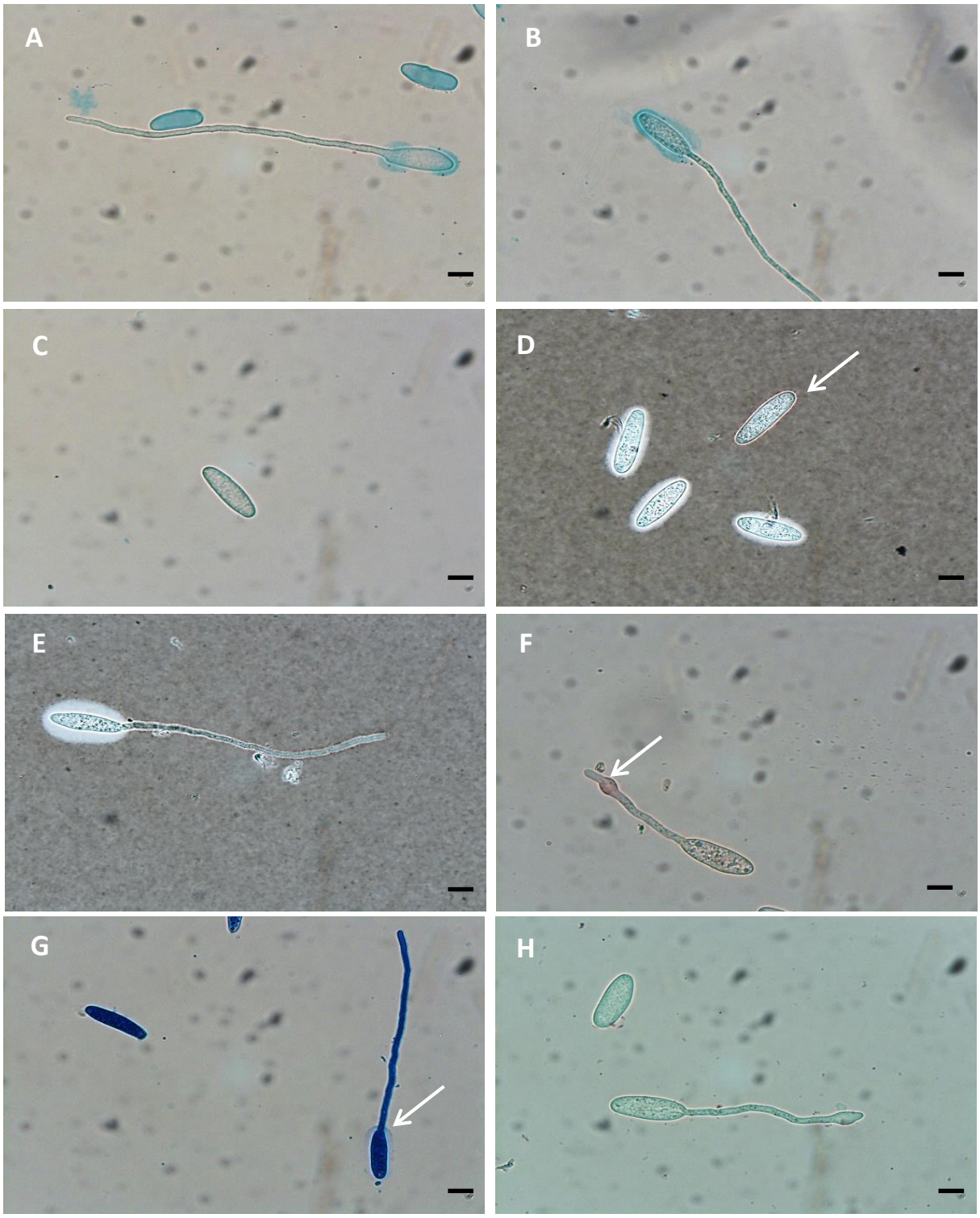

Figure A.5.2 Conidia of *N. luteum* isolate CC445 incubated for 210 min and then stained with a range of dyes. A) Empty non-viable conidia stained with alcian blue (-ve result) and germling with blue halo; B) germling stained with alcian blue showing a halo surrounding the conidium; C) conidium stained with alcian blue (-ve result); D) conidia stained with ink with bright halo and no halo (arrow); E) germling stained with ink showing bright halo around conidium; F) germling stained with Congo red showing red point near the tip of the germ tube (arrow); G) germling stained with CBB showing a blue halo at the germ tube emergence point (arrow); H) conidium and germling stained with acid fuchsin (-ve result). Scale bars = 8 μm.

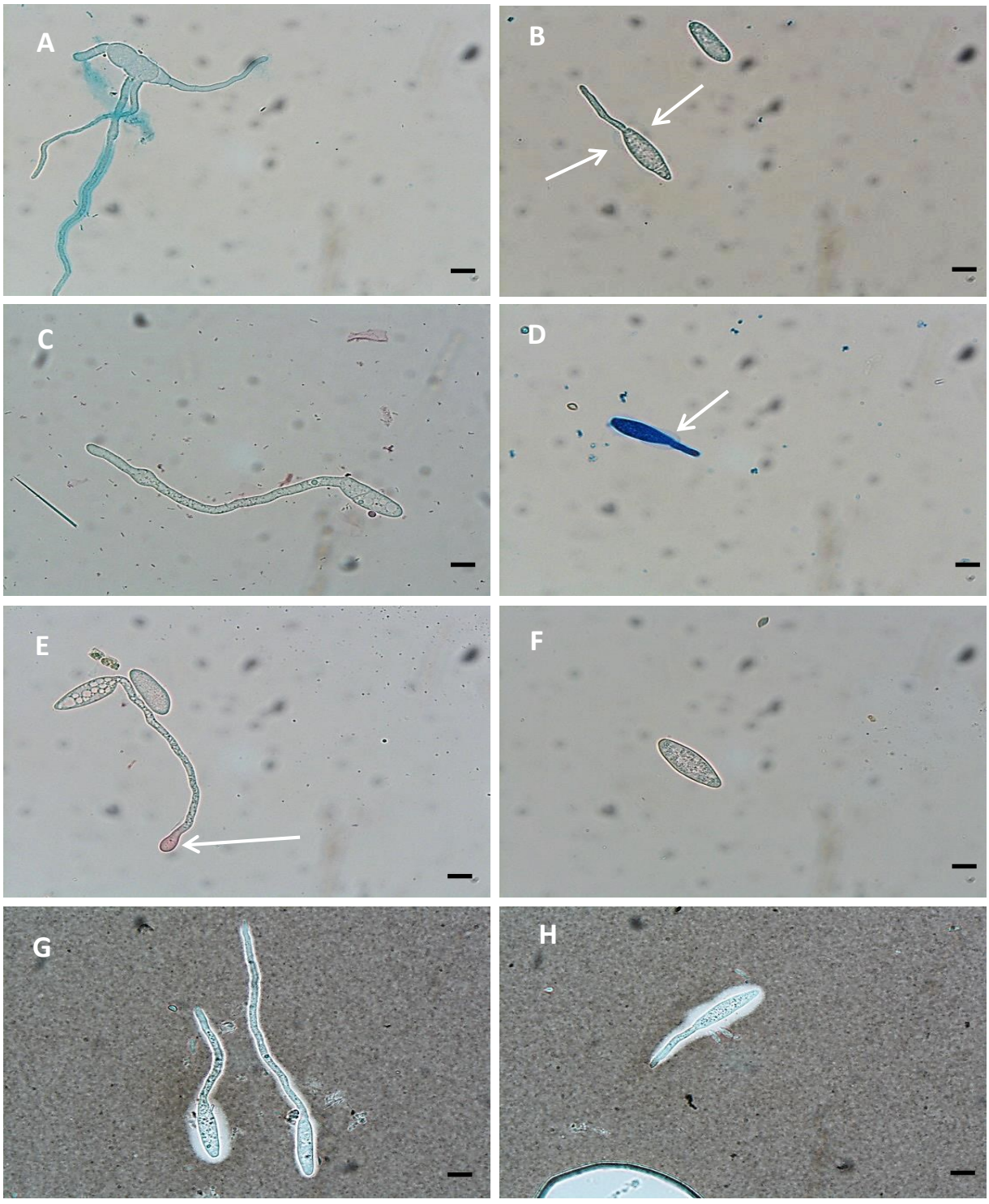

Figure A.5.3 Conidia of *B. dothidea* isolate 007 incubated for 210 min and then stained with a range of dyes. A) germling stained with alcian blue with a blue halo around the germ tube; B) germling stained with alcian blue showing a halo at the germ tube emergence point (arrows); C) germling stained with acid fuchsin (-ve result); D) germling stained with CBB showing blue halo around the germ tube emergence point (arrow); E) germling stained with Congo red showing red stain at germ tube tip (arrow); F) conidium stained with Congo red (-ve result); G) and H) germlings stained with ink showing bright halo around conidia and germ tube. Scale bars = 8 μm.

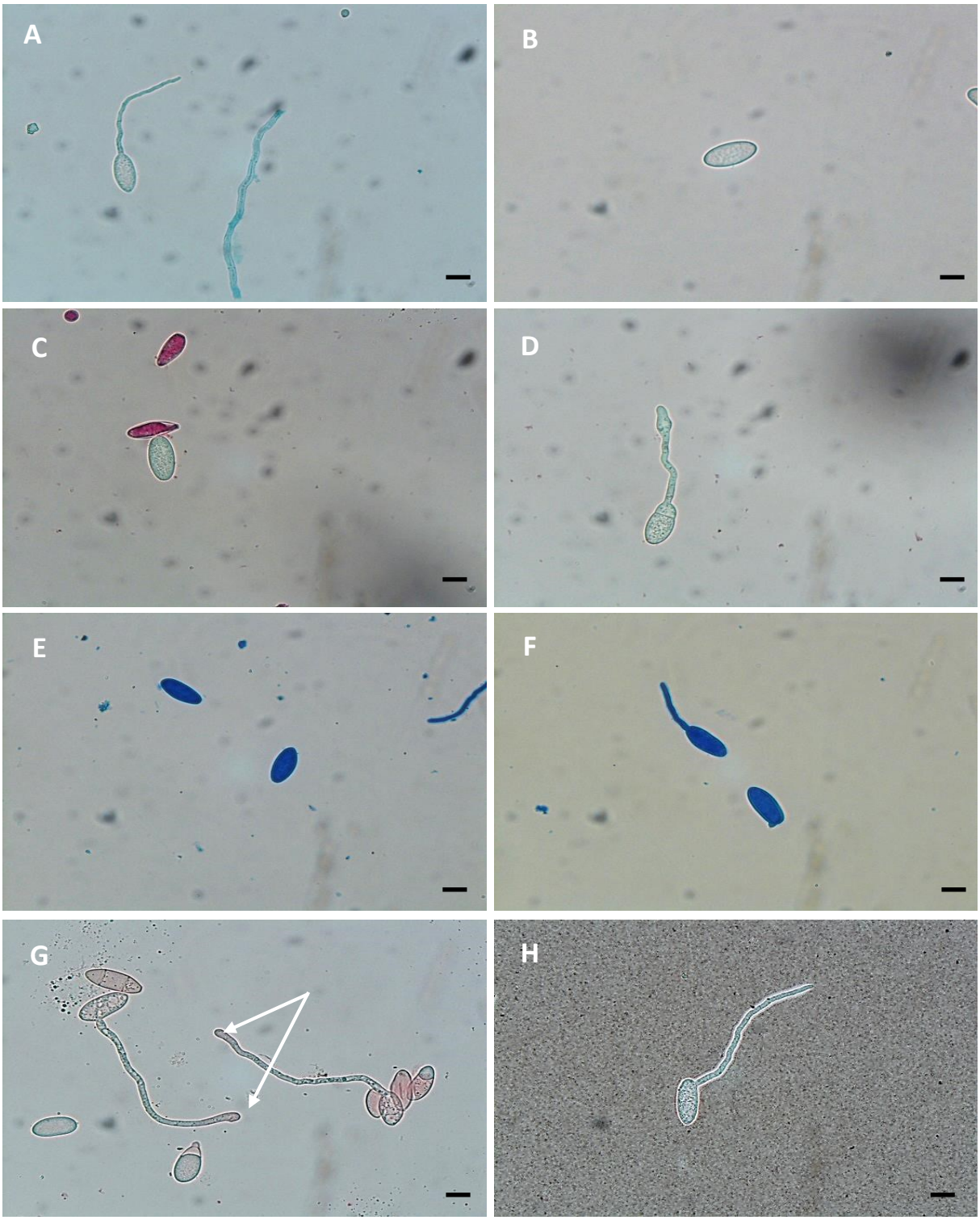

Figure A.5.4 Conidia of *N. parvum* isolate G652 incubated for 210 min and then stained with a range of dyes. A) germling stained with alcian blue with no visible halo (-ve result); B) conidium stained with alcian blue (-ve result); C) viable (clear) and shrivelled (red) conidia stained with acid fuchsin (-ve result); D) germling stained with acid fuchsin (-ve result); E) conidia stained blue with CBB; F) conidium and germling stained with CBB with no visible halo; G) Congo red stained germlings with red tip (arrows), empty conidia stained red and no stain for viable conidia; H) germling stained with ink showing narrow halo around conidium and germ tube. Scale bars = 8 μm.

Appendix B

Supplementary material for Chapter 3

B.1 Media recipes

CMC agar (g L^{-1})

17.5 Water agar (Oxoid),
0.3 yeast extract,
10.0 CM-cellulose, preswollen (Sigma-Aldrich),
6.5 $NaNO_3$,
6.5 K_2HPO_4,
6.5 KCl ,
3.0 $MgSO4.7H_2O$.

B.2 Analysis of variance (ANOVA) tables for germination experiments

Table B.2.1 ANOVA for germination of conidia of *N. luteum* isolate MM558 on different surfaces at two different time points (3 h and 6 h).

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	2112.89	422.58	26.20	
Surface	3	673.97	224.66	13.93	<.001
Time	1	10.70	10.70	0.66	0.421
Surface.Time	3	5.54	1.85	0.11	0.951
Residual	35	564.59	16.13		
Total	47	3367.69			

Table B.2.2 ANOVA for germination of conidia of *N. luteum* isolate CC445 on different surfaces at two different time points (3 h and 6 h).

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	9575.02	1915.00	21.82	
Surface	3	6673.41	2224.47	25.35	<.001
Time	1	288.61	288.61	3.29	0.078
Surface.Time	3	64.69	21.56	0.25	0.864
Residual	35	3071.06	87.74		
Total	47	19672.79			

Table B.2.3 ANOVA for germination of conidia of *N. parvum* isolate G652 on different surfaces at two different time points (3 h and 6 h).

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	10748.34	2149.67	72.33	
Surface	3	285.01	95.00	3.20	0.035
Time	1	522.28	522.28	17.57	<.001
Surface.Time	3	32.06	10.69	0.36	0.783
Residual	35	1040.19	29.72		
Total	47	12627.89			

Table B.2.4 ANOVA for germination of conidia of *B. dothidea* isolate 007 on different surfaces at two different time points (3 h and 6 h).

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	10892.9	2178.6	17.02	
Surface	3	1360.7	453.6	3.54	0.024
Time	1	1230.2	1230.2	9.61	0.004
Surface.Time	3	53.5	17.8	0.14	0.936
Residual	35	4481.1	128.0		
Total	47	18018.4			

Table B.2.5 ANOVA for germ tube length of *N. luteum* isolate MM558 conidia incubated for 6 hours on different surfaces.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	4258.5	851.7	4.49	
Surface	3	16058.1	5352.7	28.23	<.001
Residual	15	2844.3	189.6		
Total	23	23161.0			

Table B.2.6 ANOVA for germ tube length of *N. luteum* isolate CC445 conidia incubated for 6 hours on different surfaces.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	8333.4	1666.7	9.73	
Surface	3	7319.6	2439.9	14.24	<.001
Residual	15	2570.2	171.3		
Total	23	18223.2			

Table B.2.7 ANOVA for germ tube length of *N. parvum* isolate G652 conidia incubated for 6 hours on different surfaces.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	2901.86	580.37	9.94	
Surface	3	950.25	316.75	5.43	0.010
Residual	15	875.74	58.38		
Total	23	4727.86			

Table B.2.8 ANOVA for germ tube length of *B. dothidea* isolate 007 conidia incubated for 6 hours on different surfaces.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	3206.2	641.2	3.16	
Surface	3	2420.4	806.8	3.98	0.029
Residual	15	3041.2	202.7		
Total	23	8667.8			

Table B.2.9 ANOVA for percent germination of conidia of *N. luteum* isolate MM558 on surfaces of different hardness after 3 h incubation.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	205.88	41.18	2.15	
Hardness	4	484.56	121.14	6.33	0.002
Residual	20	382.89	19.14		
Total	29	1073.32			

Table B.2.10 ANOVA for percent germination of conidia of *N. luteum* isolate CC445 on surfaces of different hardness after 3 h incubation.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	164.45	32.89	1.64	
Hardness	4	211.72	52.93	2.64	0.064
Residual	20	401.31	20.07		
Total	29	777.48			

Table B.2.11 ANOVA for percent germination of conidia of *N. parvum* isolate G652 on surfaces of different hardness after 3 h incubation.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	420.29	140.10	6.14	
Hardness	4	1715.06	428.77	18.81	<.001
Residual	12	273.58	22.80		
Total	19	2408.94			

Table B.2.12 ANOVA for percent germination of conidia of *B. dothidea* isolate 007 on surfaces of different hardness after 3 h incubation.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	135.46	27.09	0.80	
Hardness	4	587.36	146.84	4.33	0.011
Residual	20	677.61	33.88		
Total	29	1400.42			

Table B.2.13 ANOVA for germ tube length of *N. luteum* isolate MM558 conidia incubated for 3 hours on surfaces of different hardness.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	54.64	10.93	2.62	
Hardness	4	57.06	14.26	3.43	0.027
Residual	20	83.27	4.16		
Total	29	194.96			

Table B.2.14 ANOVA for germ tube length of *N. luteum* isolate CC445 conidia incubated for 3 hours on surfaces of different hardness.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	26.56	5.31	1.18	
Hardness	4	71.82	17.96	3.97	0.016
Residual	20	90.35	4.52		
Total	29	188.74			

Table B.2.15 ANOVA for germ tube length of *N. parvum* isolate G652 conidia incubated for 3 hours on surfaces of different hardness.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	3.26	1.09	0.62	
Hardness	4	12.46	3.11	1.79	0.196
Residual	12	20.92	1.74		
Total	19	36.63			

Table B.2.16 ANOVA for germ tube length of *B. dothidea* isolate 007 conidia incubated for 3 hours on surfaces of different hardness.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	8.23	1.65	0.59	
Hardness	4	283.41	70.85	25.60	<.001
Residual	20	55.36	2.77		
Total	29	347.00			

Table B.2.17 ANOVA for percent germination of conidia of isolate *N. luteum* MM558 either continuously shaken or left to settle for three hours.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	2	220.05	110.03	4.35	
Treatment	2	73.48	36.74	1.45	0.244
Residual	49	1238.37	25.57		
Total	53	1531.91			

B.3 Supplementary photographs for determination of infection structures

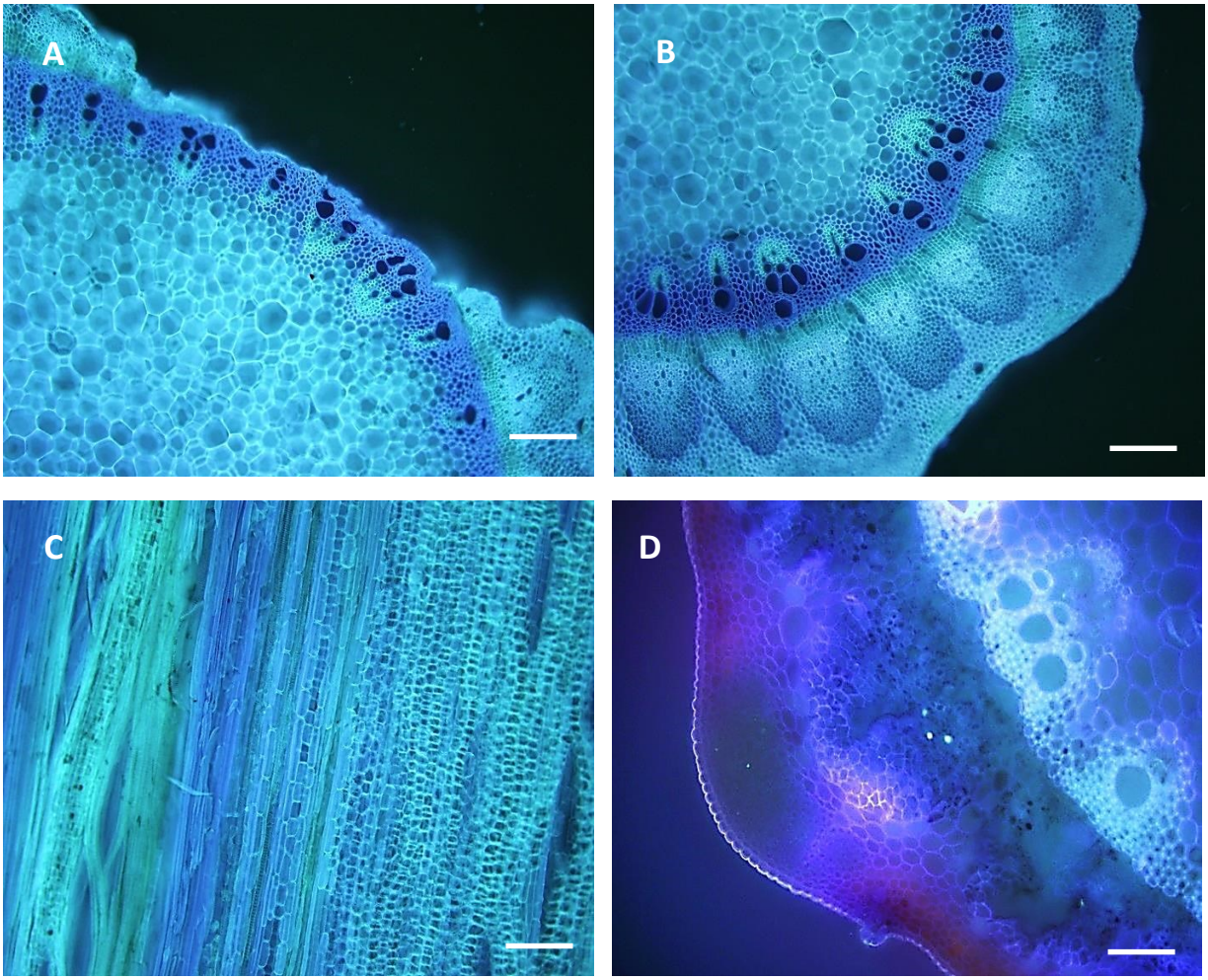

Figure B.3.1 Pinot noir shoots hand sectioned, autoclaved in 1M KOH and then stained. A) transverse tissue section of a wounded shoot stained with Calcofluor; B) transverse section of a non-wounded shoot stained with Calcofluor; C) longitudinal section of a non-wounded shoot stained with Calcofluor; D) transverse section of a non-wounded shoot stained with 0.05% aniline blue in 0.067 M K_2HPO_4. Scale bars = 100 μm.

B.4 Photographs of mycelial growth of Botryosphaeriaceae spp. germlings after 6 h incubation at 20°C on cellulose and glass.

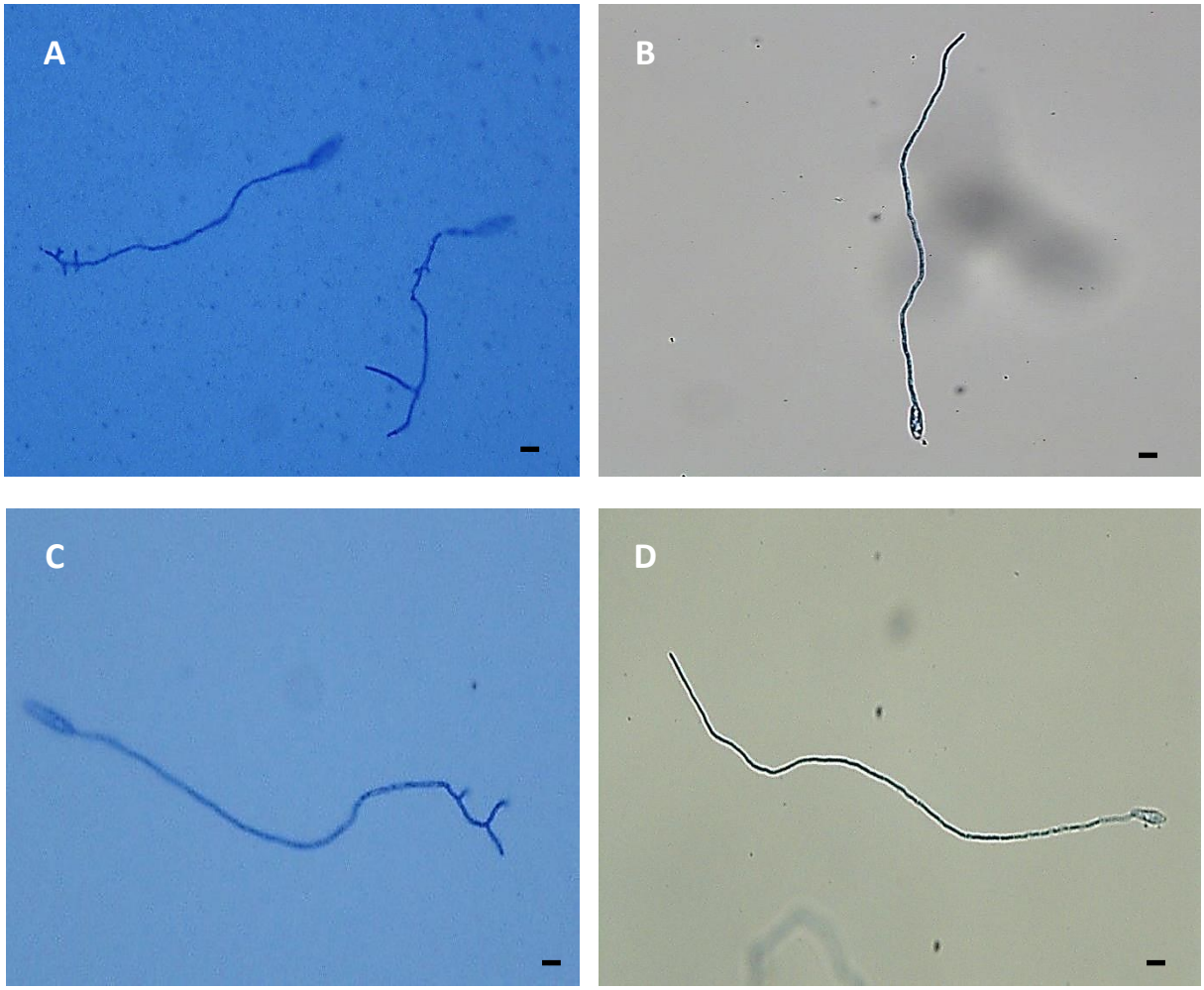

Figure B.4.1 Botryosphaeriaceae spp. germlings incubated on cellulose and glass for 6 h showing different branching patterns. A) *N. luteum* isolate CC445 on cellulose showing initiation of branching; B) *N. luteum* isolate CC445 on glass with no branching; C) *N. luteum* isolate MM558 on cellulose showing initiation of branching; D) *N. luteum* isolate MM558 on glass with no branching. Scale bars = 8 μm.

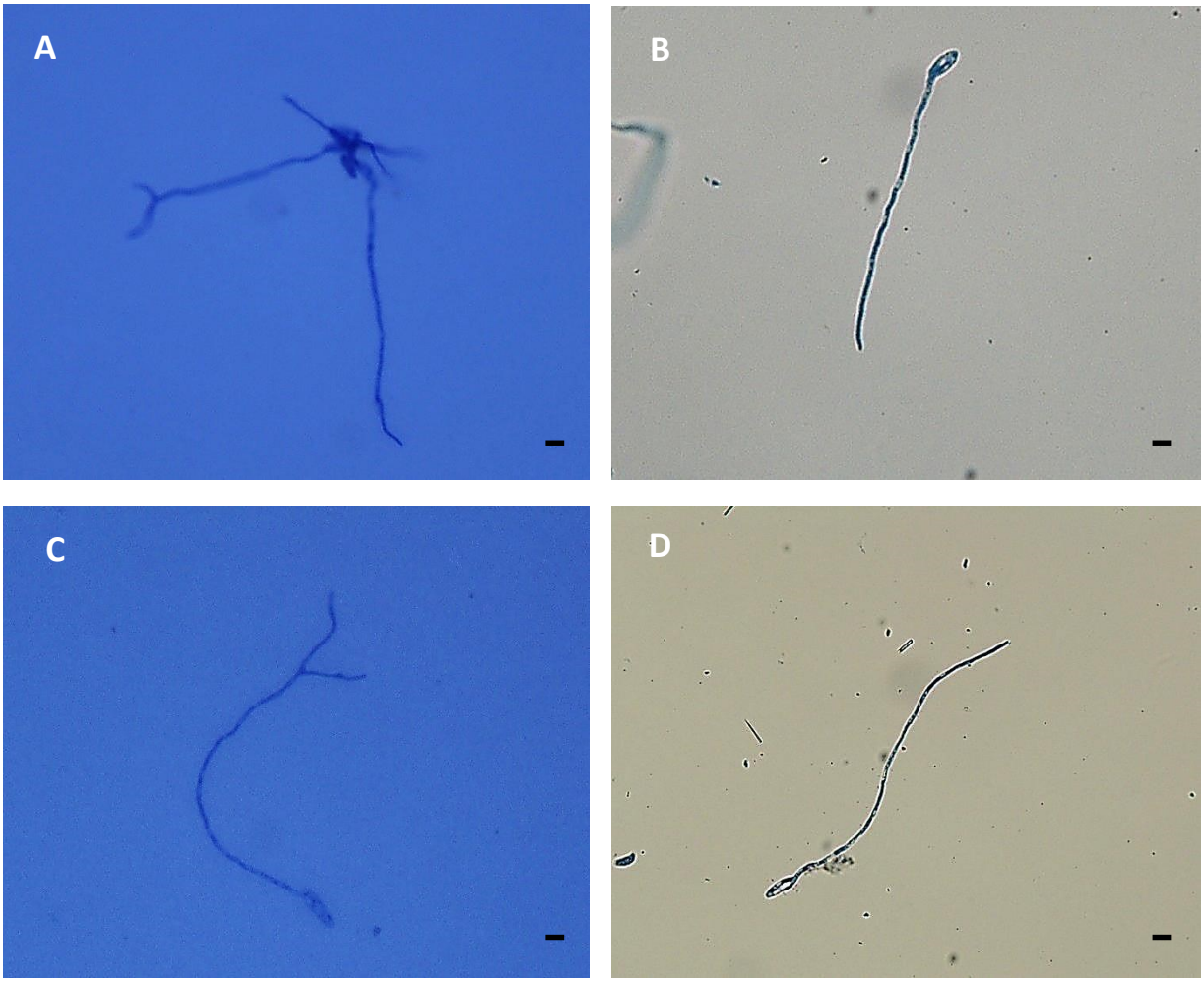

Figure B.4.2 Botryosphaeriaceae spp. germlings incubated on cellulose and glass for 6 h showing different branching patterns. A) *N. parvum* isolate G652 on cellulose showing initiation of branching; B) *N. parvum* isolate G652 on glass with no branching; C) *B. dothidea* isolate 007 on cellulose showing initiation of branching; D) *B. dothidea* isolate 007 on glass with no branching. Scale bars = 8 µm.

B.5 Photographs of germinating conidia of *N. luteum* MM558 on Pinot noir grapevine shoots showing possible directional growth towards a wound site.

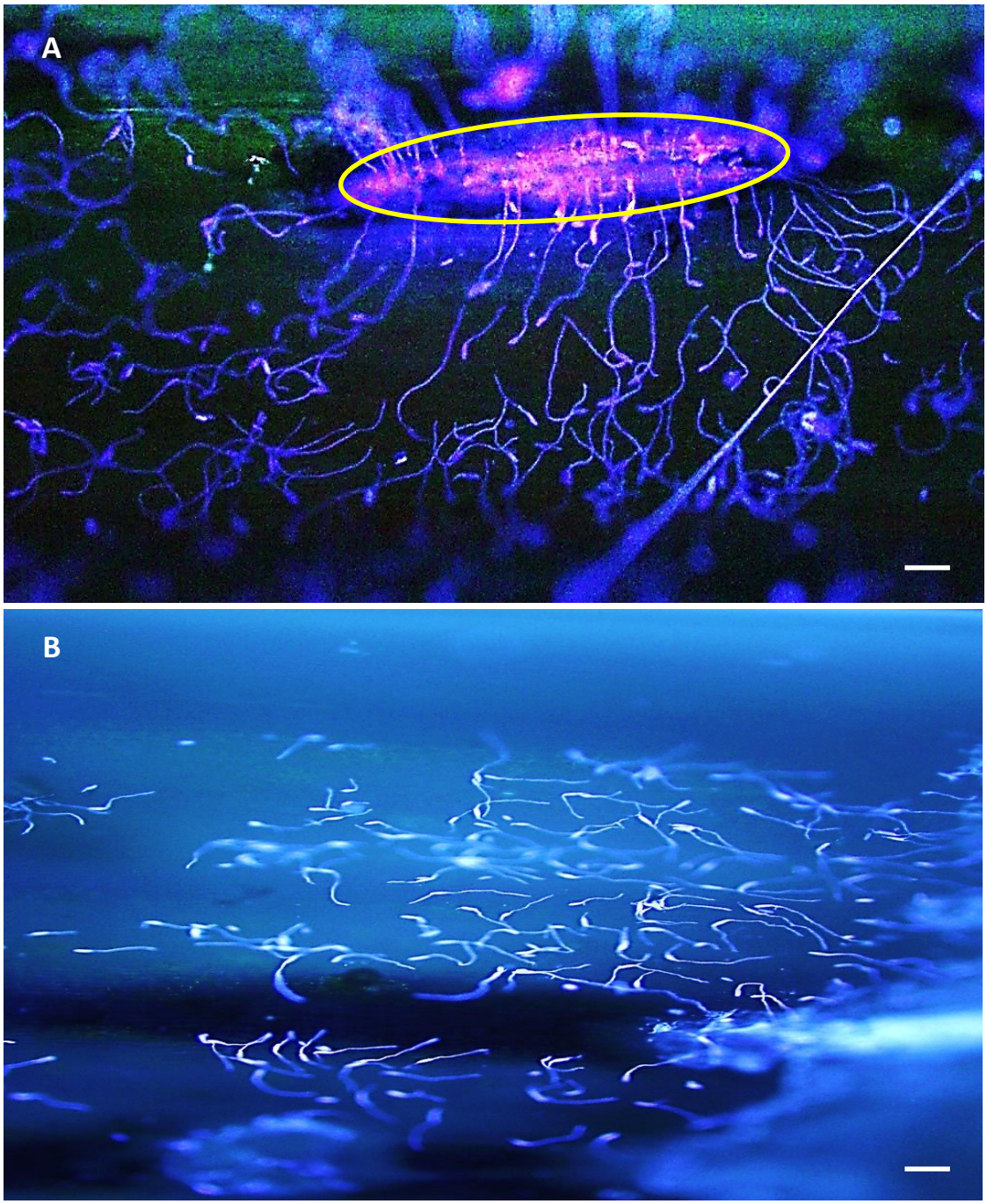

Figure B.5.1 Conidia of *N. luteum* isolate MM558 inoculated onto Pinot noir stem tissue and incubated in a humid environment at 22-25°C for 6 h. Tissue was hand sectioned and stained fresh without clearing or fixation. Germlings were stained with Calcofluor White. A) Germlings showing directional growth towards a wound site (yellow circle); B) germlings on unwounded tissue showing apparent random directional growth. Scale bars = 80 µm.

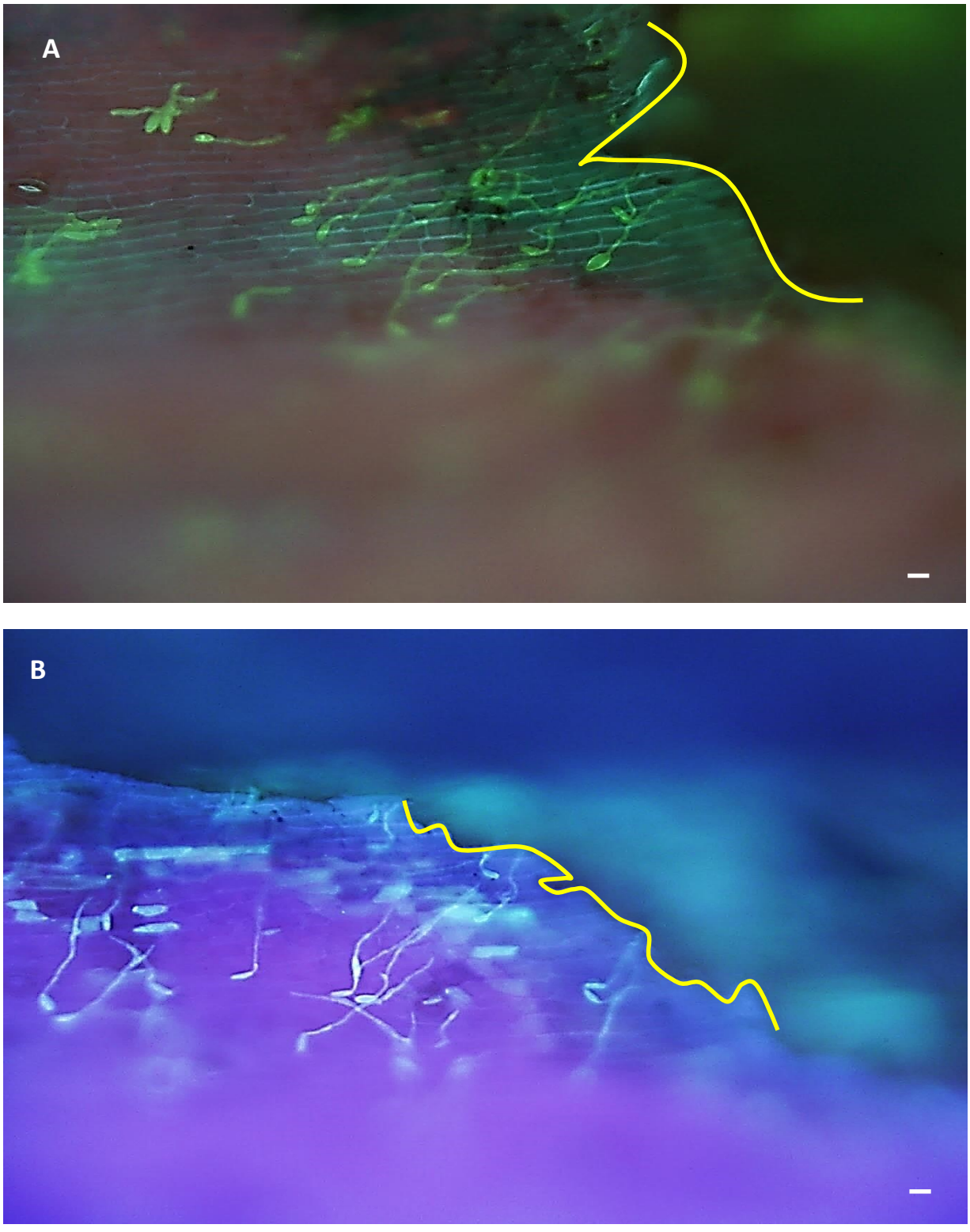

Figure B.5.2 Conidia of *N. luteum* isolate MM558 inoculated onto Pinot noir stem tissue and incubated in a humid environment at 22-25°C for 6 h. Tissue was hand sectioned and stained fresh without clearing or fixation. Germlings were stained with 0.05% aniline blue in 0.067 M K_2HPO_4. A) and B) Germlings showing directional growth towards a wound site (edged in yellow). Scale bars = 20 µm.

Appendix C

Supplementary material for Chapter 4

C.1 Raw data from GCMS analysis

Table C.1.1 Data from GCMS analysis of volatiles produced from stems+leaves (W1-W6) wounded three times over a period of 1 hr. Plant weights were 13.08 g, 13.53 g, 13.3 g, 11.5 g, 14.21 g and 16.65 g, respectively. The internal standard was octane.

	W1	W2	W3	W4	W5	W6	Mean	± SEM
Compound	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**
cis 3-Hexenal	1590.38	236.64	550.89	1247.28	3973.69	1673.55	**1545.41**	**538.70**
Octane	100.00	100.00	100.00	100.00	100.00	100.00	**100.00**	**0.00**
2-Hexenal	419.40	31.13	131.50	202.89	1040.22	389.95	**369.18**	**147.39**
cis-3-Hexen-1-ol	2483.13	200.04	394.09	1240.15	2234.65	1341.29	**1315.56**	**378.94**
trans-2-Hexen-1-ol	116.20	6.55	26.93	45.52	187.44	72.68	**75.89**	**27.21**
1-Hexanol	224.62	15.30	31.88	82.29	241.50	125.31	**120.15**	**39.11**
trans-4-Oxohex-2-enal **	6.58	0.84	1.06	4.47	9.87	3.06	**4.31**	**1.42**
6methyl-5-Hepten-2-one **	8.78	0.96	0.92	2.28	6.21	1.84	**3.50**	**1.32**
cis-3-Hexenyl acetate	1373.28	223.08	107.90	451.70	1100.21	503.67	**626.64**	**204.89**
Hexyl acetate	31.74	4.35	2.41	8.53	36.43	16.47	**16.66**	**5.88**
cis-3-Hexenyl butyrate	166.22	9.06	18.14	43.01	124.10	42.50	**67.17**	**25.84**
trans-2-hexenyl butyrate	7.35	0.28	1.22	1.44	9.72	2.05	**3.68**	**1.58**
cis-3-Hexenyl 2-methylbutanoate	3.96	0.32	0.32	0.90	1.66	0.95	**1.35**	**0.56**
Geraniol	6.97	0.00	0.00	7.19	5.19	6.73	**4.35**	**1.40**
cis-3-hexenyl hexanoate	10.51	0.30	1.13	3.75	12.42	3.10	**5.20**	**2.06**
Decyl acetate	0.20	0.02	0.15	0.96	0.74	4.10	**1.03**	**0.63**
cis-Geranyl acetone **	2.51	0.26	0.10	0.74	1.88	0.64	**1.02**	**0.39**
Lauryl acetate **	0.00	0.00	0.00	0.00	0.00	2.29	**0.38**	**0.38**
Heneicosane **	21.87	4.80	3.93	7.72	15.06	6.48	**9.98**	**2.88**
4,9,13,17-Tetramethyl-4,8,12,16-oc	1.73	0.50	0.00	14.76	34.46	1.90	**8.89**	**5.59**

** On advice from the GCMS technician (J. Breitmeyer, personal communication, 2014) rows highlighted in yellow were considered likely contaminants, showed inconsistent results or showed little difference to unwounded plants so were omitted from further analysis and discussion

Table C.1.2 Data from GCMS analysis of volatiles produced from non-wounded stems+leaves (C1-C6) over a period of 1 hr. Plant weights were 13.08 g, 13.53 g, 13.3 g, 11.5 g, 14.21 g and 16.65 g, respectively. The internal standard was octane.

	C1	C2	C3	C4	C5	C6	Mean	± SEM	-ve control 1	-ve control 2	Mean	± SEM
Compound	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**
cis 3-Hexenal	156.01	41.75	43.30	46.81	47.31	34.52	**61.62**	**18.97**	14.92	8.56	**11.74**	**3.18**
Octane	100.00	100.00	100.00	100.00	100.00	100.00	**100.00**	**0.00**	100.00	100.00	**100.00**	**0.00**
2-Hexenal	2.28	1.81	2.11	0.72	1.49	1.11	**1.59**	**0.24**	2.26	1.13	**1.70**	**0.57**
cis-3-Hexen-1-ol	17.79	11.06	5.29	6.93	10.27	3.81	**9.19**	**2.07**	6.75	4.80	**5.78**	**0.97**
trans-2-Hexen-1-ol	1.65	0.58	1.08	0.37	1.67	0.43	**0.96**	**0.24**	0.00	0.00	**0.00**	**0.00**
1-Hexanol	4.21	3.29	1.32	1.44	7.17	2.25	**3.28**	**0.90**	0.00	0.47	**0.24**	**0.24**
trans-4-Oxohex-2-enal **	0.00	0.69	1.36	0.92	3.03	2.27	**1.38**	**0.45**	3.26	1.64	**2.45**	**0.81**
6methyl-5-Hepten-2-one **	8.41	1.27	4.37	1.35	2.60	1.58	**3.26**	**1.13**	0.00	0.00	**0.00**	**0.00**
cis-3-Hexenyl acetate	1.18	3.16	0.23	0.15	11.94	1.32	**2.99**	**1.84**	0.83	0.15	**0.49**	**0.34**
Hexyl acetate	0.00	1.22	0.00	0.00	1.46	0.00	**0.45**	**0.28**	0.00	0.00	**0.00**	**0.00**
cis-3-Hexenyl butyrate	0.00	0.07	0.03	0.08	0.03	0.00	**0.04**	**0.01**	0.00	0.00	**0.00**	**0.00**
trans-2-hexenyl butyrate	0.04	0.05	0.11	0.05	0.04	0.01	**0.05**	**0.01**	0.06	0.04	**0.05**	**0.01**
cis-3-Hexenyl 2-methylbutanoate	0.00	0.30	0.14	0.09	0.22	0.10	**0.14**	**0.04**	0.00	0.00	**0.00**	**0.00**
Geraniol	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**	**0.00**	0.00	0.00	**0.00**	**0.00**
cis-3-hexenyl hexanoate	0.00	0.00	0.00	0.55	0.54	0.00	**0.18**	**0.11**	0.00	0.00	**0.00**	**0.00**
Decyl acetate	0.00	-0.01	0.00	0.01	0.00	-0.01	**0.00**	**0.00**	3.27	2.87	**3.07**	**0.20**
cis-Geranyl acetone **	0.00	0.18	0.27	0.06	0.93	0.25	**0.28**	**0.14**	0.00	0.00	**0.00**	**0.00**
Lauryl acetate **	0.00	0.02	0.00	0.00	0.00	0.00	**0.00**	**0.00**	0.00	0.00	**0.00**	**0.00**
Heneicosane **	10.47	2.83	6.56	1.95	9.10	5.13	**6.01**	**1.38**	30.94	23.01	**26.97**	**3.97**
4,9,13,17-Tetramethyl-4,8,12,16-oc	9.95	5.05	14.94	0.00	3.19	0.00	**5.52**	**2.42**	3.03	2.48	**2.75**	**0.27**

** On advice from the GCMS technician (J. Breitmeyer, personal communication, 2014) rows highlighted in yellow were considered likely contaminants, showed inconsistent results or showed little difference to unwounded plants so were omitted from further analysis and discussion.

Table C.1.3 Data from GCMS analysis of volatiles produced from stems (SW1-SW4) wounded three times over a period of 1 hr. The plant weights were 1.94 g, 2.47 g, 2.49 g and 3.11 g, respectively. The internal standard was octane.

	SW1	SW2	SW3	SW4	Mean	± SEM
Compound	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**
Toluene	3.15	2.78	2.54	2.86	**2.83**	**0.12**
cis-3-Hexenal	128.56	54.25	170.55	46.39	**99.94**	**29.94**
Hexanal	49.74	27.86	63.52	26.96	**42.02**	**8.89**
Octane	100.00	100.00	100.00	100.00	**100.00**	**0.00**
2-Hexenal	108.15	85.32	181.27	99.14	**118.47**	**21.45**
trans-2-Hexen-1-ol	8.64	6.62	13.16	8.08	**9.12**	**1.41**
1-Hexanol	14.68	8.42	18.25	8.25	**12.40**	**2.46**
Styrene	9.01	8.51	8.70	7.12	**8.34**	**0.42**
alpha-Pinene	5.12	5.01	5.29	3.58	**4.75**	**0.39**
1,2,4-trimethyl Benzene	3.91	3.67	3.89	3.31	**3.69**	**0.14**
D-Limonene	7.00	7.22	7.43	6.19	**6.96**	**0.27**
Citronellol	2.77	2.29	3.08	2.29	**2.61**	**0.19**
trans- Geraniol	22.10	21.15	31.23	10.66	**21.28**	**4.21**
trans-Citral	2.95	2.61	3.88	2.28	**2.93**	**0.35**

Table C.1.4 Data from GCMS analysis of volatiles produced from non-wounded stems (S1-S4) over a period of 1 hr. The plant weights were 1.94 g, 2.47 g, 2.49 g and 3.11 g, respectively. The internal standard was octane.

	S1	S2	S3	S4	Mean	± SEM	-ve control 1	-ve control 2	Mean	± SEM
Compound	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**	**(ng)**
Toluene	0.90	0.72	0.86	0.94	**0.85**	**0.05**	1.00	0.62	**0.81**	**0.19**
cis-3-Hexenal	17.31	17.82	25.52	24.39	**21.26**	**2.15**	1.96	1.61	**1.79**	**0.17**
Hexanal	17.24	17.64	25.44	24.08	**21.10**	**2.13**	10.10	2.90	**6.50**	**3.60**
Octane	100.00	100.00	100.00	100.00	**100.00**	**0.00**	100.00	100.00	**100.00**	**0.00**
2-Hexenal	15.45	13.40	16.11	4.70	**12.41**	**2.64**	14.10	13.23	**13.66**	**0.43**
trans-2-Hexen-1-ol	2.96	2.85	4.92	5.56	**4.07**	**0.69**	2.51	1.69	**2.10**	**0.41**
1-Hexanol	2.96	2.88	4.87	5.48	**4.05**	**0.66**	2.55	1.70	**2.12**	**0.42**
Styrene	1.72	1.76	1.30	1.77	**1.64**	**0.11**	1.28	1.01	**1.14**	**0.14**
alpha-Pinene	2.42	2.53	1.70	4.37	**2.76**	**0.57**	1.13	0.60	**0.86**	**0.26**
1,2,4-trimethyl Benzene	1.42	1.15	1.36	1.61	**1.38**	**0.10**	1.31	1.01	**1.16**	**0.15**
D-Limonene	2.18	2.13	2.06	2.56	**2.23**	**0.11**	1.96	1.03	**1.50**	**0.46**
Citronellol	0.67	0.74	0.30	0.52	**0.56**	**0.10**	0.37	0.06	**0.21**	**0.15**
trans- Geraniol	1.38	10.07	0.68	0.64	**3.19**	**2.30**	0.06	0.09	**0.07**	**0.01**
trans-Citral	0.36	0.76	0.27	0.28	**0.42**	**0.11**	0.47	0.06	**0.26**	**0.21**

C.2 Paired t-test statistics for comparisons of emitted volatile compounds from wounded and non-wounded grapevine stem+leaf tissue.

cis-3-Hexenal

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	111.519	91.926	37.529
Unwounded	6	0	4.624	3.635	1.484
Difference	6	0	106.895	91.723	37.446

t = 2.855 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 10.638 to 203.152
Two-tailed P-value = 0.0356
One-tailed P-value = 0.0178

2-Hexenal

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	26.420	25.146	10.266
Unwounded	6	0	0.117	0.0469	0.0191
Difference	6	0	26.303	25.152	10.268

t = 2.562 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -0.0923 to 52.699
Two-tailed P-value = 0.0505
One-tailed P-value = 0.0253

cis-3-Hexen-1-ol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	96.652	69.189	28.246
Unwounded	6	0	0.688	0.393	0.160
Difference	6	0	95.964	68.959	28.152

t = 3.409 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 23.596 to 168.332
Two-tailed P-value = 0.0191
One-tailed P-value = 0.00953

trans-2-Hexen-1-ol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	5.484	4.722	1.928
Unwounded	6	0	0.0710	0.0439	0.0179
Difference	6	0	5.413	4.690	1.915

t = 2.827 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.491 to 10.335
Two-tailed P-value = 0.0368
One-tailed P-value = 0.0184

1-Hexanol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	8.729	6.948	2.837
Unwounded	6	0	0.238	0.155	0.0634
Difference	6	0	8.491	6.832	2.789

t = 3.044 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 1.321 to 15.661
Two-tailed P-value = 0.0286
One-tailed P-value = 0.0143

cis-3-Hexenyl acetate

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	46.091	37.595	15.348
Unwounded	6	0	0.212	0.318	0.130
Difference	6	0	45.879	37.476	15.299

t = 2.999 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 6.551 to 85.207
Two-tailed P-value = 0.0301
One-tailed P-value = 0.0151

Hexyl acetate

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	1.204	1.042	0.425
Unwounded	6	0	0.0320	0.0498	0.0203
Difference	6	0	1.172	1.032	0.421

t = 2.783 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.0895 to 2.254
Two-tailed P-value = 0.0388
One-tailed P-value = 0.0194

cis-3-Hexenyl butyrate

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	4.961	4.752	1.940
Unwounded	6	0	0.00279	0.00293	0.00120
Difference	6	0	4.958	4.753	1.940

t = 2.555 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -0.0296 to 9.946
Two-tailed P-value = 0.0509
One-tailed P-value = 0.0255

trans-2-Hexenyl butyrate

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	0.268	0.280	0.114
Unwounded	6	0	0.00383	0.00249	0.00102
Difference	6	0	0.264	0.281	0.115

t = 2.301 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -0.0309 to 0.559
Two-tailed P-value = 0.0697
One-tailed P-value = 0.0348

cis-3-Hexenyl 2-methylbutanoate

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	0.101	0.105	0.0429
Unwounded	6	0	0.0104	0.00776	0.00317
Difference	6	0	0.0902	0.110	0.0451

t = 2.000 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -0.0257 to 0.206
Two-tailed P-value = 0.102
One-tailed P-value = 0.0509

Geraniol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	0.321	0.266	0.108
Unwounded	6	0	0.000	0.000	0.000
Difference	6	0	0.321	0.266	0.108

t = 2.964 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.0427 to 0.600
Two-tailed P-value = 0.0314
One-tailed P-value = 0.0157

cis-3-Hexenyl hexanoate

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	6	0	0.383	0.369	0.150
Unwounded	6	0	0.0143	0.0223	0.00912
Difference	6	0	0.369	0.360	0.147

t = 2.505 with 5 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -0.00969 to 0.747
Two-tailed P-value = 0.0542
One-tailed P-value = 0.0271

Decyl acetate

Wilcoxon Signed Rank Test

Group	N	Missing	Median	25%	75%
Col 17	6	0	0.0338	0.00879	0.124
Col 18	6	0	-0.0000601	-0.000379	0.000281

W= -21.000 T+ = 0.000 T-= -21.000
Z-Statistic (based on positive ranks) = -2.201
P(est.)= 0.036 P(exact)= 0.031

The change that occurred with the treatment is greater than would be expected by chance; there is a statistically significant difference (P = 0.031).

C.3 Paired t-test statistics for comparisons of emitted volatile compounds from wounded and non-wounded grapevine stem tissue.

Toluene

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	1.172	0.311	0.156
Unwounded	4	0	0.350	0.0792	0.0396
Difference	4	0	0.822	0.243	0.121

t = 6.770 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.435 to 1.208
Two-tailed P-value = 0.00659
One-tailed P-value = 0.00329

cis-3-Hexenal

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	42.911	28.417	14.208
Unwounded	4	0	8.558	1.331	0.666
Difference	4	0	34.353	27.251	13.626

t = 2.521 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -9.009 to 77.715
Two-tailed P-value = 0.0861
One-tailed P-value = 0.0430

Hexanal

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	17.776	9.070	4.535
Unwounded	4	0	8.498	1.356	0.678
Difference	4	0	9.278	7.921	3.960

t = 2.343 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -3.326 to 21.881
Two-tailed P-value = 0.101
One-tailed P-value = 0.0505

2-Hexenal

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	48.742	19.269	9.635
Unwounded	4	0	5.342	2.759	1.379
Difference	4	0	43.400	17.500	8.750

t = 4.960 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 15.554 to 71.247
Two-tailed P-value = 0.0157
One-tailed P-value = 0.00787

trans-2-Hexen-1-ol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	3.754	1.333	0.666
Unwounded	4	0	1.610	0.357	0.178
Difference	4	0	2.144	1.173	0.587

t = 3.655 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.277 to 4.011
Two-tailed P-value = 0.0354
One-tailed P-value = 0.0177

1-Hexanol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	5.240	2.572	1.286
Unwounded	4	0	1.602	0.340	0.170
Difference	4	0	3.637	2.470	1.235

t = 2.946 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -0.292 to 7.567
Two-tailed P-value = 0.0602
One-tailed P-value = 0.0301

Styrene

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	3.469	0.962	0.481
Unwounded	4	0	0.673	0.163	0.0817
Difference	4	0	2.796	0.841	0.421

t = 6.646 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 1.457 to 4.134
Two-tailed P-value = 0.00694
One-tailed P-value = 0.00347

alpha-pinene

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	1.985	0.618	0.309
Unwounded	4	0	1.091	0.313	0.157
Difference	4	0	0.895	0.792	0.396

t = 2.259 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: -0.366 to 2.155
Two-tailed P-value = 0.109
One-tailed P-value = 0.0545

1,2,4-trimethyl benzene

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	1.531	0.389	0.195
Unwounded	4	0	0.565	0.115	0.0576
Difference	4	0	0.967	0.308	0.154

t = 6.284 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.477 to 1.456
Two-tailed P-value = 0.00814
One-tailed P-value = 0.00407

D-Limonene

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	2.876	0.667	0.334
Unwounded	4	0	0.909	0.144	0.0718
Difference	4	0	1.967	0.563	0.282

t = 6.984 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 1.071 to 2.864
Two-tailed P-value = 0.00602
One-tailed P-value = 0.00301

Citronellol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	1.083	0.308	0.154
Unwounded	4	0	0.233	0.106	0.0530
Difference	4	0	0.850	0.289	0.145

t = 5.876 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.390 to 1.311
Two-tailed P-value = 0.00983
One-tailed P-value = 0.00492

trans-Geraniol

Treatment Name	N	Missing	Mean	Std Dev	SEM
Wounded	4	0	8.980	4.063	2.031
Unwounded	4	0	1.317	1.852	0.926
Difference	4	0	7.664	4.477	2.238

t = 3.424 with 3 degrees of freedom.
95 percent two-tailed confidence interval for difference of means: 0.540 to 14.787
Two-tailed P-value = 0.0417

C.4 Analysis of variance (ANOVA) tables for volatile experiments

Table C.4.1 ANOVA for percent germination of conidia of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	15.67	3.13	0.81	0.044
Treatment	1	27.76	27.76	7.13	
Residual	5	19.46	3.89		
Total	11	62.89			

Table C.4.2 ANOVA for percent germination of conidia of isolate *N. luteum* CC445. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	56.34	11.27	1.06	0.068
Treatment	1	57.42	57.42	5.41	
Residual	5	53.05	10.61		
Total	11	166.81			

Table C.4.3 ANOVA for percent germination of conidia of isolate *N. parvum* G652. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	16.42	3.28	0.36	0.064
Treatment	1	51.05	51.05	5.63	
Residual	5	45.30	9.06		
Total	11	112.77			

Table C.4.4 ANOVA for germ tube growth of germlings of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	132.96	26.59	6.58	<.001
Treatment	1	198.73	198.73	49.17	
Residual	5	20.21	4.04		
Total	11	351.90			

Table C.4.5 ANOVA for germ tube growth of germlings of isolate *N. luteum* CC445. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	38.58	7.77	0.49	0.025
Treatment	1	158.43	158.43	9.96	
Residual	5	79.49	15.90		
Total	11	276.77			

Table C.4.6 ANOVA for germ tube growth of germlings of isolate *N. parvum* G652. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	68.4	13.68	9.14	0.640
Treatment	1	0.37	0.37	0.25	
Residual	5	7.49	1.50		
Total	11	76.27			

Table C.4.7 ANOVA for percent germination of conidia of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots plus leaves.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	213.3	42.7	0.33	0.121
Treatment	1	450.8	450.8	3.47	
Residual	5	649.4	129.9		
Total	11	1313.5			

Table C.4.8 ANOVA for germ tube growth of germlings of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of wounded grapevine shoots plus leaves.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	5	355.30	71.06	1.63	
Treatment	1	1272.01	1272.01	29.21	
Residual	5	217.73	45.55		
Total	11	1845.04			

Table C.4.9 ANOVA for percent germination of conidia of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of volatiles collected for 60 min from non-wounded grapevine shoots by dynamic headspace sampling.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	29.70	9.90	1.54	
Treatment	1	0.33	0.33	0.05	0.835
Residual	3	19.24	6.42		
Total	7	49.28			

Table C.4.10 ANOVA for germ tube growth of germlings of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of volatiles collected for 60 min from non-wounded shoots by dynamic headspace sampling.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	29.88	9.96	0.25	
Treatment	1	1.52	1.52	0.04	0.859
Residual	3	121.40	40.46		
Total	7	152.79			

Table C.4.11 ANOVA for percent germination of conidia of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of volatiles collected for 60 min from wounded grapevine shoots by dynamic headspace sampling.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	5.80	1.93	1.84	
Treatment	1	11.81	11.81	11.21	0.044
Residual	3	3.16	1.05		
Total	7	20.78			

Table C.4.12 ANOVA for germ tube growth of germlings of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of volatiles collected from wounded shoots by dynamic headspace sampling.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	10.73	3.58	2.53	
Treatment	1	1.00	1.00	0.71	0.462
Residual	3	4.25	1.42		
Total	7	15.98			

Table C.4.13 ANOVA for percent germination of conidia of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of volatiles collected for 60 min from wounded grapevine shoots+leaves by dynamic headspace sampling.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	40.02	13.34	1.29	
Treatment	1	0.72	0.72	0.07	0.809
Residual	3	31.08	10.36		
Total	7	71.82			

Table C.4.14 ANOVA for germ tube growth of germlings of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of volatiles collected from wounded grapevine shoots+leaves by dynamic headspace sampling.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	57.83	19.28	16.69	
Treatment	1	29.37	29.37	25.43	0.015
Residual	3	3.47	1.16		
Total	7	90.67			

Table C.4.15 ANOVA for percent germination of conidia of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of 30 µL pure hexane.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	20.84	6.95	0.94	
Treatment	1	7.46	7.46	1.01	0.389
Residual	3	22.15	7.38		
Total	7	50.44			

Table C.4.16 ANOVA for germ tube growth of germlings of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of 30 µL pure hexane.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	69.62	23.21	17.86	
Treatment	1	36.70	36.71	28.24	0.013
Residual	3	3.90	1.30		
Total	7	110.22			

Table C.4.17 ANOVA for percent germination (log_{10}) of conidia of isolate *N. luteum* MM558. Conidia were incubated for 2 h at 20°C in the presence of pure volatile compounds.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	0.0478	0.0159	2.28	
Treatment	4	13.767	3.4418	493.06	<.001
Residual	12	0.0838	0.0070		
Total	19	13.899			

Table C.4.18 ANOVA for percent germination (log_{10}) of conidia of isolate *N. parvum* G652. Conidia were incubated for 2 h at 20°C in the presence of pure volatile compounds.

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Block stratum	3	0.0409	0.0136	2.10	
Treatment	4	9.321	2.330	359.39	<.001
Residual	12	0.0778	0.006		
Total	19	9.439			

C.5 Calculations for the maximum concentrations of compounds present in the liquid volatile mix (30 μL) in each Petri dish during volatile experiments.

Calculations for stem+leaf volatiles

	Mean conc (ng)	ng/100ul	ng per dish (30 μl)	ug/dish
cis 3-Hexenal	1545.406	51513.520	15454.056	15.454
(Z)-3-Hexen-1-ol	1315.559	43851.976	13155.593	13.156
(Z)-3-Hexenyl acetate	626.642	20888.055	6266.416	6.266
2-Hexenal	369.184	12306.121	3691.836	3.692
1-Hexanol	120.149	4004.962	1201.488	1.201
2-Hexen-1-ol, (E)-	75.885	2529.506	758.852	0.759
(3Z)-3-Hexenyl butyrate	67.170	2239.006	671.702	0.672
Hexyl acetate	16.655	555.179	166.554	0.167
(Z)-3-hexenyl hexanoate	5.203	173.422	52.027	0.052
Geraniol	4.348	144.941	43.482	0.043
(E)-2-hexenyl butyrate	3.677	122.577	36.773	0.037
cis-3-Hexenyl 2-methylbutanoate	1.355	45.154	13.546	0.014
Decyl acetate	1.029	34.284	10.285	0.010

Calculation for stem only volatiles

	Mean conc (ng)	ng/100ul	ng per dish (30 μl)	ug/dish
cis 3-Hexenal	61.618	2053.923	616.177	0.616
(Z)-3-Hexen-1-ol	9.191	306.369	91.911	0.092
1-Hexanol	3.281	109.357	32.807	0.033
(Z)-3-Hexenyl acetate	2.994	99.801	29.940	0.030
2-Hexenal	1.586	52.864	15.859	0.016
2-Hexen-1-ol, (E)-	0.964	32.143	9.643	0.010
Hexyl acetate	0.445	14.839	4.452	0.004
(Z)-3-hexenyl hexanoate	0.181	6.044	1.813	0.002
cis-3-Hexenyl 2-methylbutanoate	0.143	4.763	1.429	0.001
(E)-2-hexenyl butyrate	0.050	1.681	0.504	0.001
(3Z)-3-Hexenyl butyrate	0.036	1.184	0.355	0.000
Geraniol	0.000	0.000	0.000	0.000
Decyl acetate	0.000	-0.011	-0.003	0.000

C.6 Chi-square results from analysis of directional growth data of *N. luteum* isolate MM558 germlings towards wounded grapevine stem tissue

Analysis of data for slides grouped together (all slides in box with wounded shoots versus all slides in control box). Upper=towards volatiles and lower=away from volatiles.

Chi-Square Test:

```
Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

        Upper  lower  Total
Shoots     98    102    200
       105.00  95.00
        0.467  0.516

Control   112     88    200
       105.00  95.00
        0.467  0.516

Total     210    190    400

Chi-Sq = 1.965, DF = 1, P-Value = 0.161
```

Analysis of data for slides closest to volatiles (T1 and B1 from box with wounded shoots versus T1 and B1 from control box). Upper=towards volatiles and lower=away from volatiles.

Chi-Square Test:

```
Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

       Upper  Lower  Total
Shoots    24     26     50
       23.00  27.00
       0.043  0.037

Control   22     28     50
       23.00  27.00
       0.043  0.037

Total     46     54    100

Chi-Sq = 0.161, DF = 1, P-Value = 0.688
```

Analysis of data for slides furthest from volatiles (T4 and B4 from box with wounded shoots versus T4 and B4 from control box). Upper=towards volatiles and lower=away from volatiles.

Chi-Square Test:

```
Expected counts are printed below observed counts
Chi-Square contributions are printed below expected counts

        Upper  Lower  Total
Shoots     26     24     50
        30.50  19.50
        0.664  1.038

Control    35     15     50
        30.50  19.50
        0.664  1.038

Total      61     39    100

Chi-Sq = 3.405, DF = 1, P-Value = 0.065
```

C.7 Raw data from assessment of direction of growth of *N. luteum* isolate MM558 germlings exposed to volatiles from wounded grapevine stems

Slide position T=top, B=base 1=closest to shoots 4=furthest from shoots	**Upper = towards volatiles**	**Lower = away from volatiles**
Wounded shoots		
T4	14	11
T3	14	11
T2	7	18
T1	14	11
Total	49	51
B4	12	13
B3	12	13
B2	15	10
B1	10	15
Total	49	51
No shoots (control box)		
T4	17	8
T3	14	11
T2	7	18
T1	9	16
Total	47	53
B4	18	7
B3	15	10
B2	19	6
B1	13	12
Total	65	35

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