The response to stress treatments of potted grapevines inoculated with *Eutypa lata* and *Botryosphaeria lutea*, fungi associated with trunk disease

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Abstract Current management options for grapevine trunk diseases are focused on ameliorating symptoms once they have occurred. In grapevines and other crops, plant stress has been observed to increase symptom expression for different pathogens, depending on timing and type of stress. This research aimed to establish whether stress to vines at the time of wounding enhances colonisation by fungi associated with trunk disease. Potted plants were wounded and inoculated with two grapevine trunk fungi, *Eutypa lata* and *Botryosphaeria lutea*. Stress treatments (leaf removal and girdling) were applied to the vines immediately prior to inoculation. Lesion size at the wound site and infection, confirmed by re-isolation of the pathogen, were measured. In these experiments stress did not induce greater infection incidence or larger lesions at the site of wounding. Some difficulties in using potted plants to simulate responses of mature vines in the field are discussed.

Keywords trunk disease, carbohydrate stress, vine, *Eutypa lata*, *Botryosphaeria lutea*.

INTRODUCTION

Visible grapevine trunk disease symptoms often develop only 10 or more years after planting (Rolshausen et al. 2010). Investigation of grapevine trunk diseases often occurs only after symptom expression is observed. Because of the cryptic nature of these diseases, there may already be a large number of established vines infected by trunk disease pathogens in New Zealand that are not expressing symptoms. Studies in New Zealand (Whiteman et al. 2007; Graham et al. 2009) and overseas (Edwards &

Pascoe 2004; Fourie & Halleen 2004; Aroca et al. 2006) have indicated that mother vines often carry a range of endophytic fungi. However, younger vines that are infected can express young grapevine decline, which can lead to a decrease in productivity and vine death (Rolshausen et al. 2010). Currently nurseries cannot economically test grafted plants for the presence of trunk disease fungi, as the diseases are not uniformly distributed and sampling is destructive.

During a recent survey of trunk diseases in New Zealand, only three of the 41 vineyard blocks sampled across eight regions had no detectable fungal trunk pathogens (Manning & Mundy 2009; Mundy et al. 2009). However, many of the vines sampled were not expressing any symptoms of trunk diseases even when the pathogens were present. The pathogens of interest have been identified as wound invaders (Mundy & Manning 2010) and stress on the vines at the time of wounding has been reported as one possible factor that could influence vine response to pathogen invasion (Rudelle et al. 2005; Desprez-Loustau et al. 2006; Petit et al. 2006; Mundy & Manning 2011). Carbohydrates are important both to the vine's defence mechanisms against wounding and to the wound invading process of many pathogens (Mundy & Manning 2011). If carbohydrate stress can influence pathogen invasion of the wound and trigger latent endophytic infections to become pathogenic, this has implications for the management of the vineyard in terms of risk of trunk diseases. Ongoing research has demonstrated that over-cropped grapevines, which have carried more fruit than the vine can ripen with the available canopy, have a higher soluble sugar concentration and lower starch in the trunk than lower cropped vines (Trought & Bennett 2009). Leaf removal and girdling have also been reported to change the availability of sugars within the vine (Caspari et al. 1998). Decreases in carbohydrate reserves (starch) or changes in allocation to different sinks such as roots and fruit have been hypothesised to reduce the rate of wound healing and decrease fungal colonisation of wounds (Mundy & Manning 2011).

In long-term studies on older vines in Italy, stress has been shown to increase disease expression of esca, a grapevine trunk disease that is not present in New Zealand (Corino et al. 2004). Symptom expression for trunk diseases in the field can vary from year to year (Rolshausen et al. 2010), due to plant stress, environmental interactions with the pathogen and initial infection rates of pathogens. Symptoms such as lesion formation require not only the pathogen to be present but also the plant

to respond to the pathogen by accumulation of phytoalexins and other pigmented defence compounds at the site of invasion (Mundy & Manning 2011). Carbohydrates have been shown to be important in trunk disease response of vines even when symptoms are not observed (Petit et al. 2006). Carbohydrates appear to influence both the vine's defence mechanisms against wounding and the wound invading process of many pathogens (Mundy & Manning 2011).

The present research aimed to determine whether stress is an important factor in wound invasion by trunk diseases under New Zealand conditions.

METHOD

Overall design

Potted 2-year-old grapevines were used to investigate the effect of stress and pathogen interactions on vines. Experimental treatments included leaf removal (to induce carbohydrate stress), girdling (wound response and carbohydrate stress) and a control where stress was not induced. Vines subjected to these stress regimes were inoculated with isolates of *E. lata* and *B. lutea*.

Experiment 1

Potato dextrose agar (PDA) plugs without or with one of the two fungal isolates were prepared and used to inoculate potted grapevines as described above. The two stress treatments with the addition of a non-stress control created a 3×3 factorial experiment. Each of the nine treatment combinations was repeated nine times, with 81 vines in total being used. Stress treatments were applied to vines prior to wounding and inoculation on 10 February 2010 and the wood samples from the vines were harvested in September 2010.

Experiment 2

This experiment was established at the same time as Experiment 1 to determine if a time period longer than 12 months was required for latent infections to express symptoms. The same treatments were applied using 14 replicates, and the vines were harvested in October 2011.

Experiment 3

This experiment compared methods of inoculation (following poor establishment of *E. lata* in Experiments 1 and 2). *Eutypa lata* plugs (which contained agar and mycelium) and ascospore suspensions (2.5×10^4 /ml) were tested. This experiment was established in January 2012 and assessed in March 2012.

Inoculation

Inoculation was carried out with isolates of *E. lata* and *B. lutea* as mycelium/agar plugs (5 mm) cut from the edges of 8-day old cultures on PDA. Inoculation wounds were made to freshly-cut end wounds on 1-year-old wood above the point where the main shoot branched from the lignified wood. The fungal plugs or agar controls were placed onto freshly-cut end wounds and covered with Parafilm® for 3 days. The vines were incubated in a non temperature controlled, vented plastic house for different periods of time depending on the experiment (Table 1). For Experiment 3, both 10 µl ascospore suspensions (2.5×10^4 viable ascospores/ml) and plugs of *E. lata* were used, following the discovery of fruiting bodies of the pathogen on dead vines in Marlborough (D.C. Mundy, unpublished data). The ascospore inoculations, using the methods of Sosnowski et al. (2008), involved wetting the end wound before applying spores but the wound was not covered with Parafilm® following application. This method was included to allow comparisons with the plug method often used in plastic house experiments and the spore infection method used in field experiments on grapevines.

Vine preparation

Two-bud grafted Sauvignon blanc vines on Schwarzmann rootstock were chosen for uniformity from a larger pool of vines, re-potted into individual 5-litre black polythene bags and grown to break dormancy and establish a single healthy shoot in leaf (5-6 months). Plants were selected for uniformity of growth, and assigned randomly to the experimental treatments. Two

stress treatments of leaf removal and girdling were used to simulate carbohydrate stress and an untreated control. For the leaf removal treatment, half of the leaves were removed from the shoot. Girdling was applied to the vine by cutting the bark and phloem tissue around the trunk of the vine 15 cm from ground level using two scalpels taped together to produce parallel cuts 5 mm apart. Wounding was applied to the treatments immediately following inoculation.

Measurements

At the end of each experiment, each vine was assessed for the presence of disease symptoms, including vine death, lesion formation and discoloration of internal tissue. Each vine was split length-wise and the length of the symptomatic tissue within the vine measured.

Each split vine was dipped into 70% ethanol for 1 min to kill any surface contamination. Inner tissue was taken from the lesion sites with a scalpel from 10 mm below the cut surface (Experiments 1 and 2) or from the edge of the lesion (Experiment 3) and plated onto PDA. These plates were then incubated at 20°C and isolates observed over a 2-week period. For Experiments 1 and 2, isolations were made 10 mm below the cut and treated surface. For Experiment 3, the isolations were taken from the edge of the discoloration zone distal to the treated wound. Once sufficient growth had occurred, the colonies were then sub-cultured to fresh PDA to obtain a pure culture that could be further identified.

Statistical methods

The effects of the stress and fungal treatments on lesion lengths were analysed using two-way ANOVA. The lengths were log-transformed before analysis to equalise variances, and means were back-transformed for presentation of results. The proportions of vines from which the pathogen was re-isolated were analysed using a binomial generalised linear model with a logit link. The analyses presented excluded vines that died during the experiment. Contrasts were used to compare main effects means.

RESULTS

Visual assessment of internal symptoms

No significant effect of any of the treatments was detected on discoloration of internal tissues in any of the experiments (Table 1). In Experiment 1, the length of internal symptomatic tissue did not differ significantly with fungal treatment ($P=0.97$) or stress treatment ($P=0.30$) and there was no significant Stress×Fungi interaction ($P=0.30$). Similarly, in Experiment 2, the length of lesions did not differ significantly with fungal treatment ($P=0.43$) or stress treatment ($P=0.67$). There was a tendency for the combination of stress and fungal treatment to affect the lesion lengths (Stress×Fungi, $P=0.083$). For the *Botryosphaeria*-inoculated treatment, lesions tended to be longer on the vines that had leaves removed than on the girdled vines ($P<0.05$; least significant ratio = 1.63). In contrast, for the untreated control and *Eutypa*-inoculated treatments, the lesion lengths were not significantly different between the stress treatments. In Experiment 3, the length of discoloration did not differ significantly with inoculation method ($P=0.28$) or stress treatment ($P=0.90$) and there was no significant Stress×Inoculation interaction ($P=0.84$). In Experiments 1 and 3, the internal discoloured tissue was sometimes absent or indistinct.

Re-isolation of *E. lata*

The success of re-isolation was not consistent across the three experiments (Table 2). In Experiment 1, no significant differences were found, although very low numbers of re-isolations were achieved making the biological relevance of this result questionable. In Experiment 2, the percentage of vines from which *E. lata* was re-isolated differed significantly with respect to the inoculating fungal species ($P<0.001$) but not with the stress treatment ($P=0.47$), and there was no significant Stress×Fungi interaction ($P=0.32$). In Experiment 3, the percentage of vines that had *E. lata* re-isolated from them differed significantly with the Inoculation treatment ($P<0.001$) but not with the stress treatment ($P=0.99$), and there was no significant Stress×Inoculation interaction ($P=0.57$). When the data from the three stress treatments in Experiment 3 were pooled, the vines that were inoculated with plugs had more *E. lata* re-isolated from them (85%) than the vines that received the ascospores (54%), which in turn had more successful re-isolations than the vines that received no fungus (4.5%). The results also indicated that there may have been some pre-existing latent infections of *E. lata* in the test plants or that there had been some natural contamination during the periods of the experiments.

Table 1 Lengths of discoloration of tissues (mm) from the cut ends of grapevines at the end of Experiments 1, 2 and 3.

Fungal treatment	Stress treatment		
	No stress	Leaf removal	Girdling
Experiment 1¹			
None added	6.0	7.4	14.9
<i>Botryosphaeria lutea</i>	9.5	7.2	8.3
<i>Eutypa lata</i>	6.7	10.1	8.8
Experiment 2²			
None added	3.6	5.0	5.3
<i>Botryosphaeria lutea</i>	6.0	7.0	3.9
<i>Eutypa lata</i>	5.4	4.7	5.6
Experiment 3¹			
None added	7.4	4.9	7.2
<i>Eutypa</i> plugs	3.8	4.4	4.3
<i>Eutypa</i> spores	8.0	7.4	4.9

¹length of discoloration.

²length of lesion with distinct edges following lignifications.

Table 2 Percentages of successful re-isolations of *Eutypa lata* at the end of Experiments 1, 2 and 3.

Fungal treatment	Stress treatment		
	No stress	Leaf removal	Girdling
Experiment 1			
None added	0	10.0	20.0
<i>Botryosphaeria lutea</i>	0	0	0
<i>Eutypa lata</i>	10.0	10.0	10.0
Experiment 2			
None added	6.7	6.7	6.7
<i>Botryosphaeria lutea</i>	6.7	20.0	0
<i>Eutypa lata</i>	60.0	40.0	40.0
Experiment 3			
None added	14.3	0.0	0.0
<i>Eutypa</i> plugs	77.8	88.9	87.5
<i>Eutypa</i> spores	50.0	55.6	55.6

Re-isolation of *Botryosphaeria*

In Experiments 1 and 2, the percentage of vines that had *B. lutea* (identified by morphology on plates) re-isolated from them, differed significantly with the fungal treatment ($P=0.005$ and $P<0.001$, respectively) but not the stress treatment ($P=0.34$ and $P=0.94$, respectively) (Table 3). In Experiment 3 when *B. lutea* was not used as inoculum, only a single isolate was recovered indicating that vines were not contaminated with this pathogen during the experiment.

DISCUSSION

Field-grown vines have been shown to have different methods of wound healing in the xylem in winter and summer (Sun et al. 2008) when carbohydrate availability will also differ in the the wood tissue. The current experiments were

established during summer when carbohydrate stress could be induced and when a reduced availability of carbohydrates might interfere with the vines' normal defence mechanisms (Mundy & Manning 2011). It was hypothesised that the decrease in wood carbohydrate concentration may give the fungi a competitive advantage, allowing greater colonisation of the wood tissue. The aim of the experiment was to induce increased colonisation and spread of the fungi within the lignified tissue by manipulating carbohydrate levels in the vine. However, the timing of the experiment was different to field inoculations of trunk disease pathogens that are generally conducted in winter and sampled 9–12 months later (Sosnowski et al. 2008; Rolshausen et al. 2010). The difference in timing of inoculation may have reduced the rate of infection as wound

Table 3 Percentages of successful re-isolations of *Botryosphaeria lutea* at the end of Experiments 1 and 2.

Fungal treatment	Stress treatment		
	No stress	Leaf removal	Girdling
Experiment 1			
None added	10.0	0	10.0
<i>Botryosphaeria lutea</i>	30.0	20.0	40.0
<i>Eutypa lata</i>	10.0	0	0
Experiment 2			
None added	20.0	13.3	0
<i>Botryosphaeria lutea</i>	46.7	53.3	66.7
<i>Eutypa lata</i>	0	6.7	6.7

healing may have been increased due to the higher temperatures and greater plant activity during summer compared to winter when the vines are dormant. Reported susceptibility of grapevine pruning wounds to infection by *E. lata* during late pruning was lower than that of vines pruned early in the dormant season (Munkvold & Marois 1995). The pathogens used in the present experiments may not be adapted to invading wounds during the summer growth of grapes. Experiments conducted on young potted vines using plugs of *E. lata* (Sosnowski et al. 2011) were conducted in summer but inserted into a hole drilled into the stem of the vine rather than a cut end. Experiments using *B. lutea* inoculated with mixed isolate conidial suspension in spring (Amponsah et al. 2011) resulted in 92% infection rate, suggesting that this method of infection may be useful for further studies of this type. Thus, it is thought that the experimental design used in the present series of experiments may have contributed to non detection of significant differences between the treatments.

Previous research on the effects of plant stress on response to pathogen challenge or disease symptom development suggested that stress (Ferreira et al. 1999; Treutter 2005; Desprez-Loustau et al. 2006), particularly carbohydrate stress (Rudelle et al. 2005), may be important for decreasing vine defence response but also can reduce the ability of the pathogen to invade and establish depending on the timing of the stress. Desprez-Loustau et al. (2006) reported that many of the positive drought–disease interactions involved increased severity or impact of the disease rather than increased incidence of infection and so the timing of stress is important.

The present experiments have failed to show a difference in either the extent of internal symptoms or the establishment (as determined by re-isolation rate) of trunk disease fungi used in this potted vine system following summer infections. In Experiment 3, inoculations made with *E. lata* plugs, which contained some nutrients in the media, had a consistently higher rate of re-isolation than that following inoculation with the

ascospores, the preferred method of inoculation for winter infection experiments reported overseas.

In five field experiments at the Nuriootpa Research Centre in South Australia using *E. lata* ascospores, the mean percentage recovery from the inoculated controls ranged from 29 to 74% for vines inoculated within 1 day of wounding (Sosnowski et al. 2008). Other studies have also reported re-isolation rates ranging from 30% to 80% following inoculation of *E. lata* ascospores (John et al. 2005). The re-isolation rate of 50% for Experiment 3 is consistent with these reports. The relatively low rate of re-isolation in Experiment 3 for ascospores and the higher rate for plugs may indicate that the importance of nutrient availability for the fungus, and timing of the stress during the infection process should be further investigated. However, as the plug system was covered for 3 days and the spore method was not, differences in wound moisture levels may also have been a factor. The present experiments were also conducted on single isolates of *E. lata* obtained from vines in the local Marlborough region where the presence of *E. lata* in grapes has only recently been confirmed (Mundy et al. 2009). Differences have been shown in the rate of invasion and wood staining between different isolates of *E. lata* (Sosnowski et al. 2007). Studies of the virulence of isolates present in New Zealand on commonly grown grapevine rootstock and scion varieties of New Zealand isolates should be conducted before further studies of stress, vine and pathogen interactions are conducted.

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