Evaluation of single-node plantlets as a model system for grapevine trunk diseases

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Abstract Single-node plantlets were evaluated as a model system for experiments on grapevine trunk diseases. Experiments were conducted to determine how to grow the plantlets, how *Botryosphaeria* sp. and *Eutypa lata* symptoms were expressed in the plantlets compared with field-grown plants, and how the detached single-node plantlets (from living canes) responded compared with canes attached to vines under controlled conditions. Single-node plantlets could be grown in distilled water for up to 6 months. Under controlled conditions, significantly longer lesions formed on the single-node plantlets than on attached canes, suggesting that the single-node system may be more sensitive to infection than whole vines. The system shows promise for use in initial experiments on trunk diseases, before field experiments are conducted, but further work is required to validate the method.

Keywords grape, model system, trunk disease, symptom expression.

INTRODUCTION

Trunk disease refers to a disease syndrome in which fungi of various kinds colonise the trunk of grapevines (often via pruning wounds), particularly in the region of the head. These diseases include, but are not limited to, Esca, Petri disease, *Botryosphaeria* dieback and *Eutypa* dieback (Mundy & Manning 2010). Following pruning, vascular occlusions are produced in response to both wounding and pathogen invasion of the xylem (Sun et al. 2008) and these cause a loss of function of the affected vessels. Individual diseases have different combinations of symptoms generally related to poor function of the vascular system of the plant. For example, Petri disease symptoms range from general poor growth to

complete vine death (Edwards & Pascoe 2004). *Eutypa* die-back and other trunk diseases can take a number of years from infection to the expression of visible symptoms in mature vines. Even when vines are infected with a pathogen, the infected wood may not be vital to the function of a large vine, so symptoms may not express until the fungus depletes the vine's reserves, or some other stress factor triggers expression. However, once trunk fungi are established, options for successful management of the resulting diseases are limited by the systemic nature of these infections.

Conducting field experiments on vines to investigate trunk diseases can be difficult and time consuming (Mundy et al. 2009b). Mature

vines sampled from the field with symptoms of trunk disease often yield multiple pathogens from the same tissue (Edwards & Pascoe 2004; Mundy et al. 2009a). Established vines also have a long lag time from planting until they are ready for experimentation, and even after that, successful deliberate infection may not result in symptoms for some time. Substantial land area is required to conduct experiments with sufficient replicates to produce useful and significant data. Understandably, large-scale infection of productive vines, and the resulting potential of vine death and loss of production, are undesirable.

Single-node plantlets were investigated as a model system for studying fungal diseases, to be used alongside mature vines. The use of plantlets to investigate possible triggers of symptom expression of fungal grapevine trunk pathogens has four main advantages over using mature vines. First, the small size of the single-node plantlets allows each “plant” to be surface sterilised before propagation starts. Second, a small section of wood can be removed for plating to check whether a fungus is already present in the wood before inoculation, giving a baseline for comparisons to be made. Third, a large number of cuttings can be grown in a small area in a few months, rather than having to grow them in the field over a number of seasons before destructively sampling. Fourth, the plantlets can be grown out of season. This paper describes the development of this method and initial results.

METHODS

Preparation of single-node plantlets

Cane material was sourced from Sauvignon blanc vines grown both in the field and in a plastic house in Marlborough, New Zealand. Well lignified canes were selected from the current season's growth, so that there were sufficient reserves for the developing plantlet (Coombe & Dry 1992). The canes were also visually clear of damage and disease, with an even wood colour, and no staining or other apparent symptoms of fungal diseases, such as powdery mildew, Phomopsis or Botrytis.

A single-node plantlet consists of a section of cane with a dormant bud (node) with 1.5-

2 cm above and 4-5 cm of bare cane below the dormant bud. A shoot and leaves grow from the bud and adventitious roots develop from the cambium of the lower section of the cane. Each potential plantlet was surface sterilised by dipping the entire plantlet into 70% ethanol, then 3% (w/v) sodium hypochlorite (Janola® bleach) followed by a repeat of the 70% ethanol, with 2 min per submersion (Fourie & Halleen 2006). Cross sections were cut from the top end of the sterilised single-node plantlet and internal slivers were plated on Difco potato dextrose agar (PDA) plates to ascertain if any fungi were already present in that particular plantlet.

The plantlets were suspended with the dormant bud above a sheet of Parafilm® stretched across a 120-ml pot filled with distilled water so that the lower part of the bare cane was covered with water. The pots were then placed in 1-litre square plastic containers, which acted as individual growth chambers (Figure 1), and these were placed in an incubator at 20°C with a 12-h light/dark cycle.

Figure 1 Single-node grapevine plantlet in individual 1-litre plant growth chamber (left) and small section of tissue from the top of the plantlet plated on to potato dextrose agar (PDA) (right) to determine if the fungal pathogens were already present in the plantlet before the experiment.

Experiment 1 – Establishment of single-node plantlets

It was anticipated that the single-node cuttings would form root systems in the water and the upper section would develop leaf and/or shoot growth. Addition of ethanol and sugar to solutions had been recommended for bioassays to improve survival of tissues and to reduce microbial contamination (Wurms et al. 2003). Therefore this protocol was implemented for comparison with plain distilled water. Twenty cuttings in total were set up in individual chambers and grown with either distilled water only or distilled water with 5% sugar and 5% ethanol in the rooting area of the plantlet. Plants were incubated for up to 6 months under the growth chamber conditions previously described. When multiple leaves developed and senesced, these were removed to stop shading of new leaves.

Experiment 2 – Comparison of single-node plantlets and field-grown vines

This experiment tested the hypothesis that the single-node plantlet system would respond to treatment with anti-fungal pruning wound pastes and fungal inoculation in a similar way to field-grown vines. There were 7 treatments. Treatments 1 to 3 consisted of no pruning paste on the wound and inoculation with (1) no pathogen, (2) *Neofusicoccium australis* or (3) *Eutypa lata*. Treatments 4 to 7 consisted of (4, 5) Vinevax™ or (6, 7) Greenseal™ pruning paste treatments inoculated with (4, 6) *Neofusicoccium australis* or (5, 7) *Eutypa lata* (Table 1).

Ninety single-node plantlets were surface-sterilised and internal slivers plated as described above. Once the plantlets were 2 months old, seventy of them were selected for experimentation on the basis of which fungi had been isolated from the initial platings and specifically for the absence of *Eutypa lata* and *Neofusicoccium australis*. The experiment ran from December 2008 until May 2009, at which time plants were harvested and split open, to measure the length of any lesions.

The field experiment was set up in a vineyard in Rapaura Road, Marlborough. Seven replicates

of selected single vines were allocated the same seven treatments (n=49) as used on the plantlets in the laboratory. The vines were at least 15 years old and required removal of the heads (section of the trunk retained during pruning) to lower the position of the fruiting wood within the canopy system. Lowering the head is a standard vineyard maintenance practice with older cane pruned grapevines on a 10- to 15-year cycle. The old trunks were left as high as possible to allow experimentation on living plant tissue without threatening the long-term viability of the vine with fungal infection. The experimental trunk section was harvested (Table 1) before the visible lesion (wood staining) advanced past the point of abscission, 20-30 cm down the trunk from the initial wound.

A vertical hole was drilled into the centre of the trunk, i.e. in the centre of the flat surface that was left by the removal of the top of the vine, to provide a place to lodge an agar plug, which was either plain (sterile) or sourced from the edge of 1-week-old plates of actively growing selected fungal pathogens. Once the hole was drilled, a cross section of the top of the trunk was removed so that an internal sliver from the centre could be taken and plated to ascertain which pathogens were already present in each vine. The new top horizontal wound on the vine created by the sample removal and the remaining section of the drill hole were then either left untreated or treated with a wound dressing. The agar plug was then placed in the drilled hole. The wound was covered with Parafilm® for five days, after which the film was removed.

The trunk heads were removed from all vines 112 days after plugs had been inserted (established July 2009, harvested November 2009) and split open with a sterile hand axe (surface sterilised with 70% ethanol between samples) in the laboratory to establish the length of the lesions from the cut surface to the longest visible point. Isolations from the lesions were also made onto PDA amended with 100 µg/ml streptomycin sulphate and 100 µg/ml penicillin G potassium salts, to confirm by colony morphology that the lesions resulted from the

fungi applied (or from a natural infection of the same fungus).

One way analysis of variance was conducted using Genstat 10.2 for Windows, after checking for normality. LSDs were calculated at $P = 0.05$.

Experiment 3 – Comparison of single-node plantlets and canes still attached to the parent vine

This experiment tested the hypothesis that fungal growth (expressed by lesion length) would occur at the same rate in attached canes and detached single-node plantlets from the same parent vines.

This experiment was set up on vines in a plastic house. Long lignified canes that had not been pruned were selected and tagged. They were cut back to approximately 60 cm in length. A section from the cut end was taken and plated in the laboratory to ascertain which fungal pathogens were already present. Plain or colonised agar plugs (actively growing mycelium of *Eutypa lata*) were placed on the cut ends and wrapped in Parafilm for 5 days, after which the Parafilm was removed. The canes were harvested after 2 months, then split lengthways in the laboratory for measurement of lesion length (internal lesion length). Lesion formation limited to the bark was also observed and measured on both treatments (external lesion length).

Concurrently with the above lignified cane inoculations, a batch of twenty single-node plantlets that had been established for 2 months were also inoculated. The plantlets were taken in their growth chambers to the plastic house and set up on a table beside the vines being used in the experiment, thus placing them in a matching light and temperature environment. Isolations from the lesions were also made onto PDA with antibiotic, to confirm that the lesions resulted from the *Eutypa lata* applied. One way analysis of variance was conducted using Genstat 10.2 for Windows after checking for normality.

RESULTS

Experiment 1

In all but three of the plantlets, at least one fungal species (including saprophytic fungi) was isolated from the internal slivers taken after surface sterilisation. Over a 2-month period, all the plantlets treated with 5% ethanol and 5% sugar died, without any sign of growth. Eight of the 10 plantlets in distilled water produced between one and five leaves; half of these also developed roots. The two plantlets in distilled water that did not grow were heavily covered in fungal mycelium. The surviving plantlets in distilled water only were still growing 6 months after the start of incubation. Some leaf management (removal)

Table 1 Length of the longest lesion (mm) for single-node plantlets and field-grown grapevines that had been inoculated with *Neofusicoccium australis* or *Eutypa lata* with or without two types of pruning paste (Vinevax™ or Greenseal™) or left uninoculated.

	Treatment	Fungal inoculum	Field	Single-node
1	No pruning paste	No fungus	11.3 ab	13.1 cd
2	No pruning paste	<i>N. australis</i>	12.7 abc	17.3 d
3	No pruning paste	<i>E. lata</i>	21.3 cd	11.7 bcd
4	Vinevax™ ¹	<i>N. australis</i>	18.9 cd	6.3 ab
5	Vinevax™	<i>E. lata</i>	9.0 a	8.6 abc
6	Greenseal™ ²	<i>N. australis</i>	23.6 d	3.8 a
7	Greenseal™	<i>E. lata</i>	11.6 abc	5.6 ab
		P-value	0.025	0.001
		LSD at $P=0.05^3$	9.8	6.9

¹Commercial preparation of *Trichoderma* sp.

²Commercial chemical fungicide contained in a sealant paste.

³Means within a column with the same letter are not significantly different.

was required to keep the plantlets growing for this period of time.

Experiment 2

Plantlets showed clear differences in lesion length when treated with pruning pastes (Table 1). In the field part of the experiment lesion length of vines inoculated with *Eutypa lata* was highest with no pruning paste (Treatment 3) and significantly lower with no inoculum, and Vinevax™. However, in the same experiment the longest lesions for vines inoculated with *Neofusicoccium australis* were observed for treatments using Greenseal™ and Vinevax™. In the single-node part of the experiment plantlets when *Neofusicoccium australis* was the inoculum, no pruning paste (Treatment 2) and with Greenseal™ (Treatment 6) were observed to have the longest and the shortest mean lesions respectively. The two parts of the experiment cannot be directly compared as they were conducted at different times and the plantlet trial was carried out in a more controlled environment and with higher plant numbers. However, mean lesion lengths observed were of a similar range of values in both parts of the experiment.

Experiment 3

Plantlets showed significantly greater lesion length following inoculation than did long spurs ($P < 0.001$, Table 2). In addition to longer mean length of staining, all the plantlets' leaves abscised and died within 2 months of the inoculation. None of the long spurs lost their leaves or died within that time. Under the plastic house conditions, external staining became apparent on both the long spurs and plantlets, which was not observed in the other experiments. Recovery of *E. lata* isolates from the dead plantlet material

was at a lower rate (65%) than from the live long spur material (95%, Table 2).

DISCUSSION

The method of growing plantlets for experimentation is still under development, but it appears to be a suitable substitute for initial experiments on trunk disease fungi. It should be emphasised that careful selection of cane material is necessary to ensure that the plantlets do not have high levels of background fungal infection. Within 2 months it is possible to have sufficient numbers of healthy plantlets for use in experimentation. Screening by plating of stem tissue ensures that these plants should have little or no background infection.

Others have proposed that the use of conidia on green shoots may be a more effective method to inoculate grapevines artificially than the use of agar plugs (Amponsah et al. 2008). However, the risk of cross contamination when using conidia under field conditions made the use of plugs preferable for initial comparative trials, especially as a commercial vineyard was being used that had little apparent trunk infection present. The use of conidia, possibly in suspension, may be considered for further investigations, as a form of inoculation that more closely mimics field conditions.

Despite previous studies that used sugar and ethanol in plant tissue bioassays (Wurms et al. 2003), the results from Experiment 1 led to the development of a protocol using distilled water only as the solution for root growth for the plantlets. In the kiwifruit leaf bioassays of Wurms et al. (2003), the ethanol would have some preservative effect slowing micro-organism growth in the solution, and the sugars would provide energy reserves for the leaf during the short duration of

Table 2 Length of the longest lesion (mm) in long grapevine spurs and single-node plantlets that had been inoculated with *Eutypa lata* fungal disease. Values are for lesions observed within the split open tissue (internal lesions) and bark limited lesions (external lesions).

	plantlets	long spurs	P-value
Internal lesions (mm)	63	2	<0.001
External lesions (mm)	72	41	<0.001
Re-isolation rate of <i>E. lata</i> (%)	65	95	

the experiment. However, with the plantlets in the current experiment, ethanol appears to have inhibited adventitious root development from the cambium of the lower section of the cane during the longer duration of the experiment and may have killed the actively growing cells that, in the distilled water medium, resulted in plantlet root and leaf growth. The plantlets in the distilled water also had reserves of carbohydrate sufficient to allow 5 months or more of growth. Therefore, the addition of ethanol or sugars to the solution was not further investigated.

Experiment 2, over approximately 4 months, indicated different treatment effects, with the pattern differing between the plantlets and the field trial. The plantlets showed significantly shorter lesion lengths when the proprietary pruning pastes were used under controlled conditions. The results are consistent with findings of experiments reported for pruning wound dressings and *Eutypa lata* on grapevines in South Australia, with lower rates of recovery of the fungus when wound dressings were used than from the controls, for both glasshouse and field experiments (John et al. 2005). While the lower rates of recovery indicate that the wound dressing reduced the risk of disease symptoms in the vine, only percentage recovery of the pathogen was reported in these South Australian experiments, not lesion length. However, the present experiments did not consistently measure shorter lesion length in field-grown vines with the same treatments. A possible explanation for this could be that the plantlets were protected from external factors, such as weather or mechanical breakdown of the physical seal over the wound; another explanation could lie in background infection pressure, which may reduce the effectiveness of the pruning pastes in the field. John et al. (2005) discuss the variation in natural infection rates of *E. lata* in field experiments; these can be as high as 24% on small wounds, and perhaps higher on large pruning cuts. Additional investigation of ascospore numbers would be required to determine if the field vines were under high background infection pressure. The difficulties in controlling external factors in field experiments provide support for the use

of plantlets as a model system. The proprietary pastes both appeared to be effective in suppressing lesions from the *Eutypa lata* inoculation in the field, but treatments with *Neofusicoccium australis* showed less clear-cut results. This may be due to the high initial fungal population of the field-grown vines before the experiment (resulting in high lesion lengths in the control, Treatment 1) or may be a result of poor establishment of the *Neofusicoccium australis* in the field (low lesion length for the positive control, Treatment 2).

Ideally, the plantlet experiment would have been conducted at the same time as the in-field experiment, but the in-field vines were only available during a limited window for experimentation. Sun et al. (2008) observed seasonal differences in types of vascular occlusions (tyloses in summer and gels in winter) as wound responses in *Vitis vinifera*. Different wound responses by the vascular tissue due to differing physiological states of the plant in response to temperature or other environmental stimuli may be important in determining how or if vascular pathogens invade the tissue. Wound response may explain the observed differences in the rate of recovery of *Eutypa lata* reported in early and late winter in South Australia (John et al. 2005). The influence of temperature on possible differences in vine wound responses during in-field and plantlet experiments needs to be further investigated.

Experiment 3 indicated that the plantlets had a greater measurable response than mature vines to the inocula that were used. Some of the observed differences may result from lower levels of reserves and/or a higher percentage of damaged cells in the plantlets, resulting from the propagation method. Fungi that colonise wood interact with the wood tissue as part of the infection process. In pine lumber, inhibitory compounds produced by living cells in wood as they senesce are important in influencing which fungi colonise the wood and how successfully (Strong et al. 2005). Toxin production by *E. lata* can result in foliar symptoms even on mature vines (Pitt et al. 2010) and may have a proportionally larger effect on the plantlets, which would have a much more limited ability

to produce a plant-resistance response to the disease; this would explain the observed larger lesions in the plantlets.

It was possible to re-isolate the pathogen from a higher percentage of long spurs despite the fact that they displayed an apparently lower severity of infection (represented by lesion length). All the single-node plantlets died from their infections, and it could be for this reason that the re-isolation percentage was lower. This could be an advantage of using plantlets as a model system, as they would show a “worst case scenario” more quickly than would field trials.

Further planned research will test how vine stress affects disease symptom expression, to gain a better understanding of how the plants and fungi interact. Ultimately mature vines are needed for testing, but initial use of a model system should reduce the number of commercially viable vines that have to be used in experiments, and increase the speed with which initial results can be obtained.

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REFERENCES

- Amponsah NT, Jones EE, Ridgway HJ, Jaspers MV 2008. Production of *Botryosphaeria* species conidia using grapevine green shoots. New Zealand Plant Protection 61: 301-305.
- Coombe BG, Dry PR 1992. Viticulture. Volume 2. Practices. Winetitles, Adelaide. 376 p.
- Edwards J, Pascoe IG 2004. Occurrence of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* associated with Petri disease and esca in Australian grapevines. Australasian Plant Pathology 33 (2): 273-279.
- Fourie P, Halleen F 2006. Chemical and biological protection of grapevine propagation material from trunk disease pathogens. European Journal of Plant Pathology 116 (4): 255-265.
- John S, Wicks TJ, Hunt JS, Lorimer MF, Oakey H, Scott ES 2005. Protection of grapevine pruning wounds from infection by *Eutypa lata* using *Trichoderma harzianum* and *Fusarium lateritium*. Australasian Plant Pathology 34 (4): 569-575.
- Mundy DC, Casonato SG, Manning MA 2009a. Incidence of fungi isolated from grape trunks in New Zealand vineyards. The Australasian Plant Pathology Society 2009, Newcastle City, NSW, Australia. p. 86 (Abstract).
- Mundy DC, Casonato SG, Manning MA 2009b. Sampling techniques for isolating trunk disease fungi from a Nelson vineyard. New Zealand Plant Protection 62: 406 (Abstract).
- Mundy DC, Manning MA 2010. Ecology and management of grapevine trunk diseases in New Zealand: A review. New Zealand Plant Protection 63: 160-166.
- Pitt WM, Huang R, Trouillas FP, Steel CC, Savocchia S 2010. Evidence that *Eutypa lata* and other diatrypaceous species occur in New South Wales vineyards. Australasian Plant Pathology 39 (1): 97-106.
- Sun Q, Rost TL, Matthews MA 2008. Wound-induced vascular occlusions in *Vitis vinifera* (Vitaceae): tyloses in summer and gels in winter. American Journal of Botany 95 (12): 1498-1505.
- Strong N, Webber J, Eaton R 2005. Factors affecting the fungal colonization of pine lumber. Forest Pathology 35 (3): 195-203.
- Wurms KV, George MP, Lauren DR 2003. Involvement of phenolic compounds in host resistance against *Botrytis cinerea* in leaves of the two commercially important kiwifruit (*Actinidia chinensis* and *A. deliciosa*) cultivars. New Zealand Journal of Crop and Horticultural Science 31: 221-233.