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A method using leafed single-node cuttings to evaluate downy mildew resistance in grapevine

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Summary

A method using leafed single-node cuttings, incubated at 25 ± 2 °C and 100 % humidity, was examined to screen grapevine genotypes for resistance to downy mildew (*Plasmopara viticola* (Bert. & Curt.) Berl. & de Toni). Cuttings were taken at the 4th, 5th and 6th nodes back from apices of actively growing shoots. Disease symptoms, namely chlorosis, sporulation and necrosis, were observed on the leaves of cuttings within 6 days of incubation after inoculation. Based on chlorosis and sporulation, genotypes were ranked from highly susceptible to resistant in the order of Sultana, M46-32 (Bicane x Villard blanc), Joannes Seyve 23.416, Marroo Seedless and Chambourcin. The expressions of both symptoms increased with the concentration of inocula applied to leaves (1×10^5 and 5×10^4 sporangia per ml), but the overall genotypic ranking was unaltered. The third symptom of leaf necrosis occurred on infected leaves either as progressively enlarging dead areas or as smaller localised necrotic spots. The latter has been called the 'necrotic response' symptom and its expression depended on genotype, although its value for separating or ranking genotypes was unreliable. Leaf chlorosis was associated with leaf sporulation (r^2=0.41-0.47). Genotypes with necrotic response exhibited reduced leaf chlorosis and sporulation. A comparison between this new leafed single-node cutting method and a previously published leaf disc method indicated it was more reliable for separating genotypes for downy mildew resistance.

K e y w o r d s : *Plasmopara viticola*, disease resistance, laboratory protocol, leafed single-node cutting, *Vitis* spp.

Introduction

Downy mildew, caused by *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is a destructive disease of grapevines in warm and humid climatic regions (Lafon and Clerjeau 1988). The disease infects all green shoot tissues including leaves, tendrils, shoots, inflorescences and fruit bunches, and significantly depresses productivity and quality (Lafon and Clerjeau 1988). In Australia, downy mildew occurs sporadically in all grape growing regions and annual costs due to the disease and its control have been estimated at over $13 million ($230 per ha) during low rainfall seasons, and more than $47 million ($835 per ha) during wet seasons (Magarey *et al*. 1991). Although fungicides provide control against downy mildew, fungicide tolerant variants of pathogens can develop reducing their effectiveness (Cohen and Coffey 1986; Lafon and Clerjeau 1988). Economical and sustainable viticulture demands the use of disease resistant varieties to manage downy mildew.

Effective and accurate screening methods are required to select and breed for downy mildew resistance and a number of techniques have been developed for this purpose. Field observations (*e.g.* Alleweldt 1980; Becker and Zimmermann 1980; Eibach *et al.* 1989; Borgo *et al.* 1990; Brown *et al.* 1999 b, c; Kozma 2000), and glasshouse-based screening methods (*e.g.* Becker and Zimmermann 1978; Doazan 1980; Denzer *et al.* 1995) have been used along with laboratory-based techniques. Laboratory-based techniques, for example leaf disc (Stein *et al.* 1985; Denzer *et al.* 1995; Staudt and Kassemeyer 1995), detached leaf (Song *et al.* 1998) and *in vitro* dual culture methods (Barlass *et al.* 1986), are capable of screening large numbers of breeding progenies quickly (Brown *et al.* 1999 a), and are particularly valuable for resistance screening where natural vineyard infection occurs sporadically and infrequently. Of the methods reported, the wide use of the leaf disc method (Stein *et al.* 1985; Denzer *et al.* 1995; Staudt and Kassemeyer 1995) suggests it is the most reliable technique used to assess downy mildew resistance in the laboratory.

Under vineyard conditions, downy mildew infected leaves develop a series of disease symptoms. These are leaf chlorosis, sporulation due to the development of white downy sporangiophores and sporangia on the abaxial surface and, as infection progresses, leaf necrosis (Emmett *et al.* 1992). Leaf maturity and the prevailing climate critically affect symptom expression and development (Emmett *et al.* 1992). In general, genotypic variation in downy mildew resistance has been described based on disease severity and estimated by visually scoring symptom expression (Stein *et al.* 1985; Staudt and Kassemeyer 1995; Brown *et al.* 1999 a, b, c). Coutinho (1964) and Dai *et al.* (1995) described downy mildew resistance under vineyard conditions in various ways including the necrosis response (*i.e.* hypersensitive reaction), diffuse necrosis with limited sporulation, and as sporulation without necrosis. Brown *et al.* (1999 c) showed that the hypersensitive reaction was associated with reduced leaf chlorosis and sporulation. From these reports it appears that the expressions of various symptoms need to

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be assessed simultaneously to identify different types of downy mildew resistance of grapevines. Apparently this is also important for laboratory-based screening methods. The leaf disc method relies on scoring sporulation symptom to identify resistant genotypes. Neither chlorosis nor necrosis symptoms were shown to be reliable with this method (Brown *et al.* 1999 a), which suggests a more sophisticated laboratory-based technique needs to be developed.

This paper reports a method of using leafed single-node cuttings to screen for downy mildew resistance. This technique quantifies the intensity of chlorosis and sporulation and records the necrotic response of infected leaves under laboratory conditions. In optimizing the technique, the effects of leaf maturity and sporangia suspension concentrations on infection and symptom expression were examined. The reliability of the method relative to a leaf disc method was demonstrated by assaying a range of commercial varieties and a number of hybrids from CSIRO's breeding program.

Material and Methods

Leafed single-node cutting (LSNC) method: The experimental unit was a leafed single-node cutting (LSNC), which consisted of a node, a fully expanded leaf and part of the internode below the leaf (Fig. 1 a). LSNCs were collected from healthy, developing grapevine shoots at the 4-6 node position below the apex during early spring from the vineyard at CSIRO Plant Industry, Merbein, N. W. Victoria. Collected shoots were immersed in soapy water for 1 min, rinsed first with tap water and then distilled water before being separated into LSNCs.

LSNCs were laid abaxial surface up on a double layer of wet paper towel that overlaid a compressed bed of perlite, which had been saturated with distilled water, held in a plastic tray (47 x 37 x 10.5 cm). Leaf abaxial surfaces were inoculated by spraying with a sporangia suspension (see below) until covered completely with fine droplets. The concentration of sporangia in the suspension was adjusted using a hemacytometer. After inoculation, the tray was covered by a layer of wet paper towel and enclosed using a tight fitting layer of cling wrap to maintain relative humidity close to 100 % during incubation. The tray was illuminated using cool-white fluorescent lights (310 µmol m^{-2} s^{-1}) and incubated for 16 h in a culture room at 25 ± 2 °C. The tray was then uncovered, the paper towel removed and the LSNCs inverted and planted by inserting the internode stem into the perlite bed (Fig. 1 b). The tray was again enclosed by a tight fitting layer of cling wrap and sealed into a plastic bag and returned to the culture room with a 16 h photoperiod.

Disease severity was rated 6 d after incubation when more than 80 % of Sultana leaves became chlorotic and were covered by white sporangiophores. Ratings were given for chlorosis, sporulation and necrosis independently on a scale of 1-9, where 1 = no symptoms, 2 > 0 to 2.5 % leaf area affected, 3 > 2.5 % to 10 %, 4-5 > 10 % to 25 %, 6-7 > 25 % to 50 %, 8 > 50 % to 80 % and 9 > 80 %. From rating scales used in previous reports (Patil *et al.* 1989; Brown *et al.* 1999 a, b), genotypes tested were classified as: 1= immune; 2 = highly resistant (HR); 3 = resistant (R); 4-5 = moderate resistant (MR); 6-7 = moderate susceptible (MS); 8 = susceptible (S) and 9 = highly susceptible (HS). These incubation conditions and rating methods were used in the experiments described below.

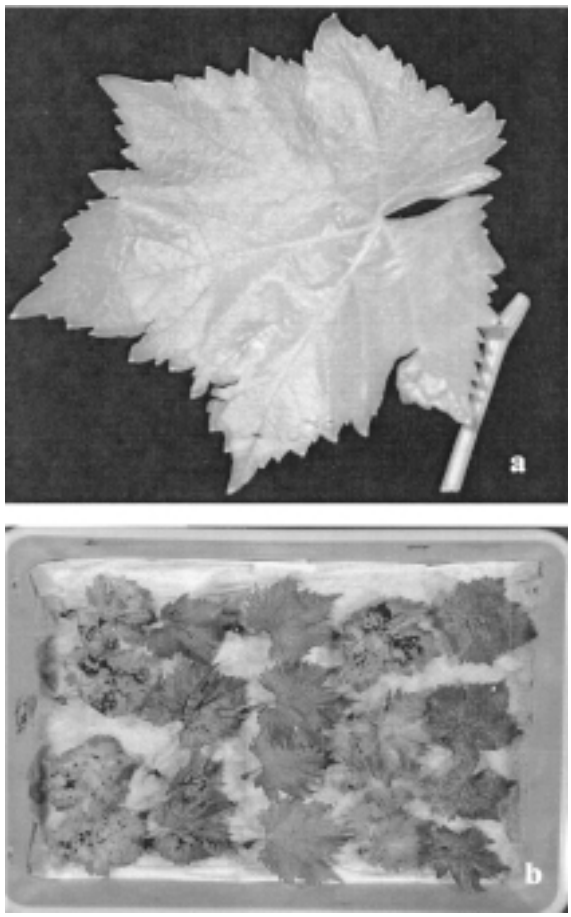

Fig. 1: (**a**) A leafed single-node cutting (LSNC); (**b**) LSNCs planted in a compressed bed of perlite saturated with distilled water contained within a plastic tray. The photo was taken 6 d after incubation and shows genotypic differences in response to downy mildew inoculation. Genotypes from left to right were Sultana, Joannes Seyve 23.416, Chambourcin, M46-32 and Marroo Seedless.

Source and multiplication of inoculum: The initial source of inoculum was collected from diseased leaves of an unsprayed Sultana vine grown at Koorlong in N. W. Victoria during May 2001. The disease was subsequently propagated and maintained on container-grown Sultana vines through winter 2001 using a host-pathogen dual culture system under glasshouse conditions.

Downy mildew infected leaves were harvested from dual cultured vines in mid September, 2001, washed in soapy water for 1 min and then rinsed once with tap and then 3 times with distilled water. Leaves were blotted dry with paper towel, placed into a moistened plastic bag, sealed and incubated at room temperature overnight. White sporangiophores and sporangia were visible on abaxial surfaces of leaves by the following morning. Sporangia were harvested by washing sporangiophores into a beaker with distilled water at 4 °C applied using a wetted camel hair brush. The collected suspension was filtered through two layers of cheesecloth to remove sporangiophores and other particles.

Inoculum was multiplied further by inoculating LSNCs of Sultana. When dense sporangiophores and sporangia appeared on abaxial surfaces of inoculated leaves, sporangia were harvested and used as the inoculum for the following experiments.

Effect of leaf maturity on disease infection: Young shoots of Sultana, M46-32 (a hybrid of Bicane x Villard blanc), Joannes Seyve 23.416 (GALET 1979) and Chambourcin were divided into LSNCs as described, that is into single-node cuttings collected from nodes 4-6. Leaf maturity was defined based on the nodal position with a fully matured leaf at the 6th node. Sultana, Joannes Seyve 23.416 and Chambourcin were chosen as they react differently to the disease (BARLASS *et al.* 1986).

LSNCs were inoculated using a 1 x 10^5 sporangia per ml suspension. The experiment was laid out as a split-plot design with genotypes as main plots and leaf maturity as subplots. Four LSNCs were tested for each genotype/leaf maturity combination with each cutting as a replicate.

Effect of inoculum levels on disease development: LSNCs of Sultana, M46-32, Joannes Seyve 23.416, Marroo Seedless and Chambourcin at the 4th node from shoot apices were used. Four concentrations of sporangia suspensions, *i.e.* 5 x 10^3, 1 x 10^4, 5 x 10^4 and 1 x 10^5, were used as inocula. The experiment was laid out as a split-plot design with inocula as main plots and genotypes as subplots (Fig. 1 b). Four LSNCs from each genotype were tested and arranged in a row with each cutting as a replicate.

Comparison of LSNC and leaf disc methods: Thirty-two genotypes were evaluated for downy mildew resistance using LSNC and leaf disc methods during October 2001. Sultana and Chambourcin were chosen as susceptible and resistant controls respectively. The other vines were three hybrid varieties, Villard Noir, Cascade and Muscat Hamburg and 27 hybrids from CSIRO's grape breeding program.

These experiments were conducted using healthy, fully expanded leaves collected from the 4th node back from shoot apices. Leaves were washed and rinsed as already described. The LSNC experiment was laid out as a randomised complete block design with three replicates. The leaf disc experiment was conducted as described by BROWN *et al.* (1999 a). One 16-mm-diameter disc was cut with a cork borer from each of 4 leaves for each genotype, which were placed at random abaxial surface up on a bed in a plastic tray as prepared for LSNC method. Each leaf disc was considered as a replicate.

The inoculum was a 1 x 10^5 sporangia per ml suspension and trays were incubated as already described. LSNCs and leaf discs were observed and scored for chlorosis and sporulation independently using the 1-9 scale 6 d after incubation. The presence of the necrosis response was also recorded.

Statistical analysis: Data were subjected to analysis of variance according to experimental design. Where F tests were significant, means were separated using least significant difference (LSD) independently for leaf chlorosis, sporulation and necrosis. T-tests were used to compare the mean differences between hybrids with and without the necrosis response under individual methods. Spearman rank coefficients were used to rank and compare data for chlorosis and sporulation within and between methods.

Results

Symptom expression: Chlorotic patches appeared on leaves of susceptible genotypes 3 or 4 d after incubation. Large genotypic differences for disease symptom expression were evident 6 d after incubation (Fig. 1 b and Fig. 2). With Sultana, the majority of the leaf lamina became chlorotic and white sporangiophores covered almost the entire abaxial surface at a high density. Necrotic patches expanded progressively during the later stages of incubation as chlorosis and sporulation coverage increased. Sultana petioles were also heavily infected and became fragile leading to lamina abscission. A similar response to the disease was observed for M46-32 (Fig. 1 b). For Joannes Seyve 23.416, chlorosis was visible in only a limited area and coverage was less extensive. White sporangiophores were observed at low density on only part of the abaxial surface and localised necrotic patches appeared as irregular shapes. Most parts of Marroo Seedless laminae became brownish

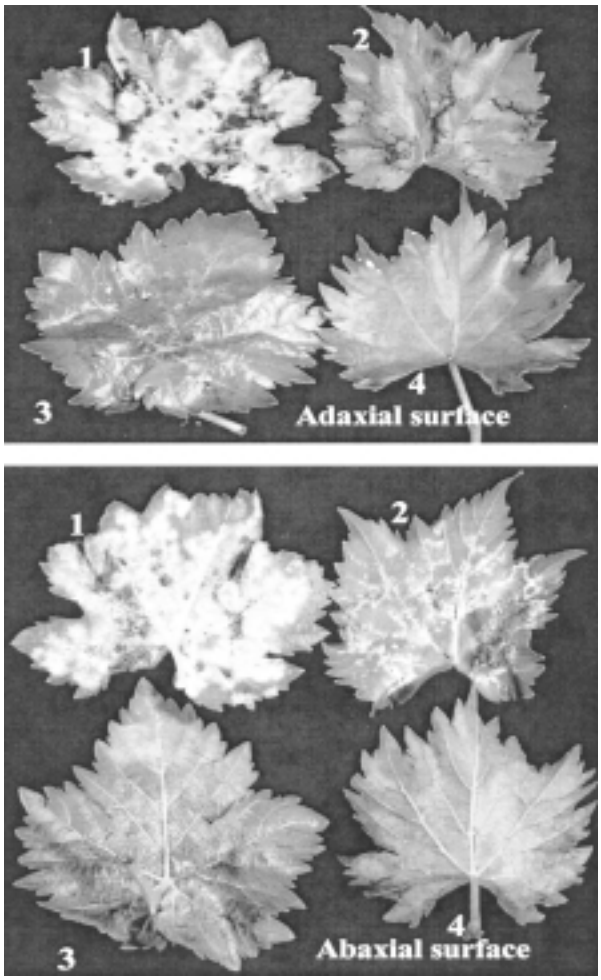

Fig. 2: Leaf chlorosis, sporulation and necrosis symptoms on leaves from single-node cuttings of Sultana (**1**), Joannes Seyve 23.416 (**2**), Marroo Seedless (**3**) and Chambourcin (**4**) following incubation for 6 d after inocula were applied following the LSNC method.

and sporangiophores were evident, although these were at a low density within the discoloured margin on abaxial surfaces. Small chlorotic patches and localised fine necrotic spots were visible on leaves of Chambourcin. The necrotic spots appeared between 3 and 4 d after incubation on Chambourcin laminae, although the majority remained green and there was no evidence of petiole abscission either at lamina or axil. Only a few sporangiophores were visible on necrotic spots where moisture condensation occurred on Chambourcin laminae.

Genotypic differences in disease symptom expression were observed using the LSNC method. The ranking of genotypes from susceptible to resistant was Sultana, M46-32, Joannes Seyve 23.416, Marroo Seedless and Chambourcin.

Effect of leaf maturity on the expression of disease symptoms: There were significant differences between genotypes for the expression of leaf chlorosis ($P < 0.01$), sporulation ($P < 0.01$) and necrosis ($P < 0.05$) symptoms of downy mildew (Tab. 1 a). There were also significant differences ($P < 0.01$) between different aged leaves for chlorosis and sporulation symptoms and significant ($P < 0.01$) genotype x leaf maturity interactions for all three symptoms.

Based on chlorosis and sporulation symptoms of leaves from the 4th and 5th nodes, Sultana and M46-32 had similar reactions to the disease. These genotypes had the highest disease severity ratings (> 8.0), followed by Joannes Seyve 23.416 and Chambourcin (< 3.0), which had the lowest rating (Fig. 3). Chlorosis and sporulation symptom expressions were less intense for leaves from the 6th node for Sultana and M46-32. The expression of leaf necrosis symptoms was

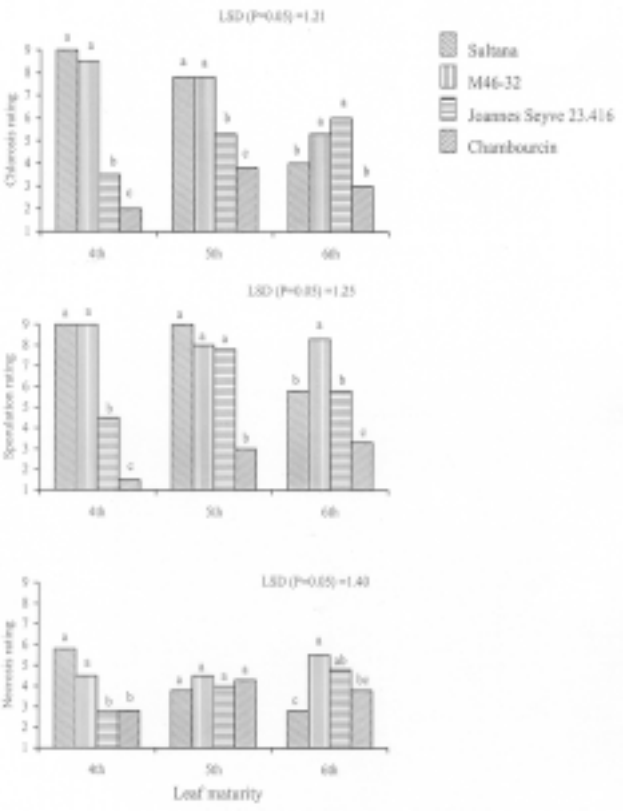

Fig. 3: Effects of leaf maturity, measured as node position back from the shoot apex, on symptoms of leaf chlorosis, sporulation and necrosis on leaves borne on single-node cuttings of four genotypes incubated for 6 d after being sprayed with downy mildew spores. Bars with different letters for each leaf maturity were significantly different at $P \leq 0.05$.

Table 1

Analysis of variance tables highlighting the effects of genotype (G), leaf maturity (LM), inoculum concentration (IC) and interactions on leaf chlorosis, sporulation and necrosis in separate split-plot designed experiments using LSNC

Source	df	F value		
		Leaf chlorosis	Leaf sporulation	Leaf necrosis
a) Genotype x leaf maturity				
Main plots:				
Block	3	2.66	3.71	0.61
G	3	50.84 **	105.07 **	3.86 *
Error	9			
Sub-plots:				
LM	2	18.25 **	8.52 **	0.28
G x LM	6	21.54 **	10.52 **	5.81 **
Error	24			
b) Inoculum concentration x genotype				
Main plots:				
Block	3	0.60	0.78	0.49
IC	3	8.07 **	27.18 **	2.82
Error	9			
Sub-plots:				
G	4	252.62 **	381.54 **	10.88 **
G x IC	12	2.77 **	2.15 *	6.45 **
Error	48			

*, ** significant at $P < 0.05$ or 0.01, respectively.

considerably less compared to those for other symptoms with Sultana, M46-32 and Joannes Seyve 23.416 irrespective of leaf maturity (Fig. 3). In contrast, and in comparison with the other two symptoms, expression of the necrosis symptom was greater with Chambourcin.

Effect of inoculum levels on disease ratings: The effect of inoculum concentration was significant ($P > 0.01$) on the expression of leaf chlorosis and sporulation but not necrosis symptoms (Tab. 1 b). There were significant genotype ($P < 0.01$) and genotype x inoculum concentration ($P < 0.05$) effects for all three symptoms.

Expressions of chlorosis and sporulation symptoms on Sultana and M46-32 leaves were significantly greater when they were inoculated with higher (1×10^5 and 5×10^4 sporangia per ml) than lower concentrations of inocula (Fig. 4). Inoculum concentration did not alter chlorosis expression for Joannes Seyve 23.416 and Marroo Seedless, but high concentrations increased sporulation. A few sporangiophores and sporangia grew spontaneously on necrotic spots on the abaxial surfaces of Chambourcin leaves after they were treated with high concentrations of inocula. High inoculum concentrations also increased the expression of necrosis on leaves of Sultana, M46-32 and Joannes Seyve 23.416, but not on those of Marroo Seedless and Chambourcin (Fig. 4).

Regardless of inoculum concentrations, genotypic ranking for resistance was consistent when based on either leaf chlorosis or sporulation, although data for node 4 LSNCs of Joannes Seyve 23.416 varied between experiments (Figs 3 and 4). Genotypic ranking for resistance was not consistent when based on necrosis (Fig. 4). When ratings for leaf chlorosis and sporulation were considered in combina-

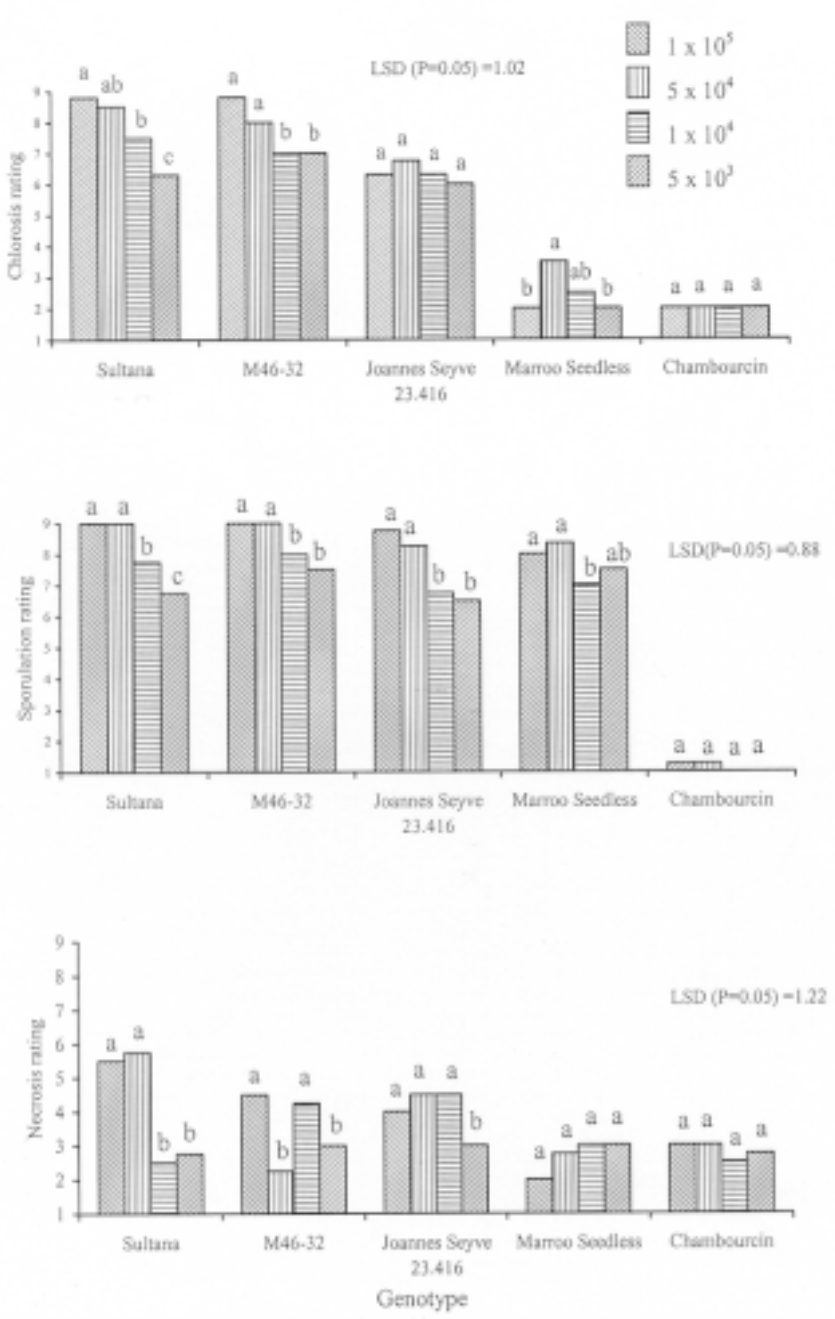

Fig. 4: Effects of downy mildew inoculum concentrations on expression of leaf chlorosis, sporulation and necrosis symptoms for five genotypes 6 d after incubation. Bars with different letters for each genotype were significantly different at $P < 0.05$.

tion, genotypes were ranked from susceptible to resistant in the order of Sultana, M46-32, Joannes Seyve 23.416, Marroo Seedless and Chambourcin (Fig. 4).

Comparing LSNC and leaf disc methods: None of the genotypes used to compare the two methods were immune to downy mildew, but their susceptibility varied greatly (Tabs 2 and 3). Genotypic means for leaf chlorosis and sporulation were significantly higher using the LSNC method (Tab. 2). In addition, coefficients of variation for both symptoms using the LSNC method were two thirds of those observed using leaf disc method, even though the leaf disc experiment had an extra replicate (Tab. 2). This indicated that the LSNC method produced less variable results.

Table 2

Means, ranges and coefficients of variance (CV) for leaf chlorosis[a] and sporulation[a] of 32 genotypes evaluated for downy mildew resistance/susceptibility using LSNC and leaf disc methods

	Leaf chlorosis		Leaf sporulation	
	LSNC	Leaf disc	LSNC	Leaf disc
Mean	6.6**	5.1	8.1**	6.4
Range	3.0-9.0	2.5-8.0	4.0-9.0	2.8-9.0
CV (%)	17.3	26.8	13.1	20.8

** significant at $P < 0.01$.

[a] leaf chlorosis and sporulation were scored using a hedonic scale of 1-9 where 1 = no symptoms, 2 >0 to 2.5 % leaf area affected, 3 > 2.5 % to 10 %, 4-5 > 10 % to 25 %, 6-7 > 25 % to 50 %, 8 > 50 % to 80 % and 9 > 80 %.

The susceptibility rating of a genotype depended on which disease symptom was used to describe response (Tab. 3). For example, with the LSNC method, there were 2 and 9 resistant and moderately resistant genotypes respectively based on leaf chlorosis. In contrast, only one genotype was identified as moderately resistant and none resistant based on leaf sporulation. With the leaf disc method, 22 genotypes were rated as moderately or highly resistant based on leaf chlorosis, but only 10 based on leaf sporulation.

Agreement in rating genotypes as resistant or susceptible was poor between methods (Tab. 3). Only 11 and 9 genotypes were assigned the same ratings based on leaf chlorosis and sporulation, respectively, using the two methods. Based on leaf chlorosis, 11 out of 22 genotypes ranked moderately to highly resistant using the leaf disc method, were rated moderately susceptible using the LSNC method. Similarly, based on leaf sporulation, 10 out of 11 genotypes rated moderately to highly resistant with the leaf disc method were moderately or highly susceptible using the LSNC method. In contrast, all genotypes rated as resistant using the LSNC method were assigned the same phenotype using the leaf disc method.

Ratings for leaf chlorosis and sporulation were correlated for the 32 genotypes evaluated using the LSNC method ($r^2 = 0.41$, $P < 0.05$) and the leaf disc method ($r^2 = 0.47$, $P < 0.05$). Similarly, ratings were correlated between the two methods for leaf chlorosis ($r^2 = 0.50$, $P < 0.05$) and sporulation ($r^2 = 0.14$, $P < 0.05$). Though all significant, these associations were not strong.

Table 3

Number of genotypes ranked as resistant-to-susceptible based on symptoms of leaf chlorosis and sporulation respectively using LSNC and leaf disc methods to screen for downy mildew resistance

Leaf disc	LSNC						Total
	I	HR	R	MR	MS	S+HS	
Leaf chlorosis							
I	0	0	0	0	0	0	**0**
HR	0	0	0	1	0	0	**1**
R	0	0	2	4	2	0	**7**
MR	0	0	0	4	9	0	**14**
MS	0	0	0	0	3	5	**8**
S+HS	0	0	0	0	0	2	**2**
Total	**0**	**0**	**2**	**9**	**14**	**7**	
Leaf sporulation							
I	0	0	0	0	0	0	**0**
HR	0	0	0	0	1	0	**1**
R	0	0	0	0	2	2	**4**
MR	0	0	0	1	1	4	**6**
MS	0	0	0	0	3	11	**14**
S+HS	0	0	0	0	2	5	**7**
Total	**0**	**0**	**0**	**1**	**9**	**22**	

I = immune; HR = highly resistant; R = resistant; MR = moderately resistant; MS = moderately resistant; S = Susceptible; HS = highly susceptible.
Data within diagonal lines represent hybrids that exhibited identical responses to the disease using both methods.

Localised necrotic spots of various sizes were observed for 16 of the 32 genotypes tested and 14 of these were scored positively for this regardless of the method. When tested using the LSNC method, genotypes with the leaf necrosis response had a mean rating of 5.9 for leaf chlorosis, which was significantly less than the mean value of 7.7 observed for genotypes without necrosis response ($t = 2.82$, $P < 0.01$). There was no significant difference, however, for ratings of leaf sporulation between genotypes with and without necrosis response (7.7 versus 8.4). Genotypes that had necrosis response in the test using the leaf disc method had a significantly lower mean rating of 4.3 for leaf chlorosis compared to a mean of 5.9 assigned to those that did not show necrosis response ($t = 3.68$, $P < 0.01$). A similar difference was not apparent with regard to leaf sporulation.

Discussion

Three symptoms attributable to downy mildew infection were used to rate grapevine genotypes for resistance to the disease using a leafed single-node cutting (LSNC) method. The ranking obtained for Sultana, Joannes Seyve 23.416 and Chambourcin using this method agreed with that reported by Barlass *et al.* (1986) based on vineyard obser-

vations and results using an *in vitro* dual culture method. The resistance assigned to Marroo Seedless reported here supports its description as a resistant variety (CLINGELEFFER and POSSINGHAM 1988).

Marroo Seedless and Joannes Seyve 23.416 exhibited a response to the infection that was intermediate and different to other genotypes (Figs 3 and 4). Genotypes with this intermediate type of resistance, however, could only be separated from others when both leaf chlorosis and sporulation were assessed together (Fig. 4). The reasons for this were that genotypic response to the disease, as either resistance or susceptibility, relied on the symptoms used to score disease severity (Tab. 3), and the expression of these symptoms was not highly correlated across the genotypes assessed ($r^2 < 0.50$). These suggest that factors limiting the development of leaf chlorosis and sporulation may be different, even though both symptoms occurred sequentially after infection. Further investigation to understand these factors may improve our knowledge of resistance expression as infection progresses, which would assist downy mildew resistant breeding programs.

From the objective of improving the reliability of resistance screening techniques, the results reported here indicate that it is essential to assess both leaf chlorosis and sporulation in determining genotypic resistance or susceptibility to the disease. This, however, contradicts BROWN *et al.* (1999 b) who reported that measurement of any one disease symptom would give an accurate assessment of downy mildew resistance under vineyard conditions.

As a symptom of the disease, leaf necrosis was expressed in various forms according to genotype. For example, with Sultana and M46-32 leaf necrosis occurred as progressively enlarging patches during the incubation period after leaf chlorosis and sporulation occurred. If left to progress after the experimental period, leaves became completely necrotic and rotted (data not shown). This complete necrosis and rot may have been due to death of diseased tissue followed by secondary infection by necrotrophic parasites. In contrast, leaf necrosis in Chambourcin was expressed as localised fine necrotic spots that appeared 3-4 d after incubation as a typical hypersensitive response (COUTINHO 1964; LANGCAKE and LOVELL 1980; BORGO *et al.* 1990; DAI *et al.* 1995). The hypersensitive response has been shown to be a form of programmed localised cell death at infection points and is commonly observed in other plant species such as lettuce and hop, for example (MATTHEWS 1981; KAMOUN *et al.* 1999). The presence of localised necrotic spots was associated with a reduction in other symptoms, especially leaf chlorosis, which agreed with BROWN *et al.* (1999 c) and it appeared that the necrotic response was a good indicator of resistance to the disease in grapevines. However, there may be a difficulty associated with the accuracy of visually estimating the coverage of localised fine necrotic spots across a leaf. Even though genotypic differences based on leaf necrosis were comparatively small, this symptom appeared to be less useful than chlorosis and sporulation rankings for evaluating genotypic variation for resistance (Fig. 3).

Leaf maturity, defined by node position back from the shoot apex, was a crucial factor affecting disease symptom expression and the reliability of the LSNC screening method. The expression of leaf chlorosis and sporulation was higher on leaves at the 4th and 5th nodes compared to those at the 6th node, particularly for susceptible genotypes. SRINIVASAN and JEYARAJAN (1976) obtained similar results when different aged leaves of a susceptible genotype were tested. Our findings also support COUTINHO (1964), who observed that mature leaves were less susceptible. The severity of chlorosis and sporulation on leaves at the 4th and 5th node was better for distinguishing genotypes on the basis of resistance and susceptibility, and ratings of four genotypes, Sultana, Joannes Seyve 23.416, Marroo Seedless and Chambourcin, agreed with reported field data (BARLASS *et al.* 1986; CLINGELEFFER and POSSINGHAM 1988). Thus, leafed cuttings collected at the 4th and 5th nodes provided the most suitable material for screening for downy mildew resistance using the LSNC method.

Disease pressure for the LSNC method was manipulated by concentration of inocula. The results demonstrated that higher concentrations of inocula led to increased disease severity and ensured optimal expression of disease symptoms within the reported incubation period. Thus, higher concentrations of inocula assisted in identifying true resistance and improved the efficiency of the LSNC screening technique.

The LSNC method proved to be more reliable than the leaf disc method because genotypes tested received a higher and more uniform infection. With the LSNC method, leaf chlorosis and sporulation symptoms were highly expressed and could be visually quantified using a disease severity key. The leaf necrosis response, as a component of resistance, could be surveyed as effectively as that of the published leaf disc method. Furthermore, the LSNC method is as simple to use as the leaf disc method, especially since there are no pre-conditioning requirements to be conducted under laboratory conditions. Thus, the LSNC method is an improved alternative method for screening grapevines for resistance to downy mildew.

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