Effectiveness of sodium hypochlorite as a disinfestation treatment against genetically diverse strains of grape phylloxera *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae)

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Abstract

Background and Aims: Grape phylloxera, *Daktulosphaira vitifoliae* Fitch, causes damage to ungrafted European grapevines, *Vitis vinifera* L. Where distribution is limited such as in Australia and China, phylloxera is managed primarily through quarantine protocols. In Australia, there are 83 known phylloxera genetic strains, and studies have shown differences in susceptibility to some disinfestation treatments. First instar nymphs are the most dispersive stage and can be transferred from infested to healthy vineyards on footwear and hand held tools. The current disinfestation protocol recommends a footbath treatment for 30 s with 2% sodium hypochlorite (NaOCl) followed by a water rinse.

Methods and Results: Survival of six endemic phylloxera genetic strains, G1, G4, G7, G19, G20 and G30, was determined by immersing first instars in 0, 2, 3 and 4% NaOCl for 30, 40 and 60 s followed by a 30 s water rinse. Although phylloxera survival was significantly reduced by increasing NaOCl concentration and treatment duration, none of the treatment combinations were 100% effective. By excluding the post-treatment water rinse, 100% mortality was achieved across all the six genetic strains using a treatment of 2% NaOCl for 60 s.

Conclusions: Results from this study recommend a revised disinfestation treatment for footwear and hand held tools.

Significance of the Study: It is important to consider differences in susceptibility to disinfestation treatments by phylloxera genetic strains.

Keywords: *disinfestant, genetic strains, grape phylloxera, quarantine, sodium hypochlorite*

Introduction

Grape phylloxera (herein referred to as phylloxera), *Daktulosphaira vitifoliae* (Fitch) (Hemiptera: Phylloxeridae), is a sap-sucking insect pest that causes substantial damage through feeding on commercial grapevines *Vitis vinifera* L. The pest is found in most viticultural regions worldwide (European and Mediterranean Plant Protection Organization 1990). Phylloxera originated from eastern North America in the 1850s and spread throughout Europe, North and South America, South Africa and Australasia impacting on viticultural industries by the end of the 19th century (Granett et al. 2001a, Powell 2008). In most grape-producing countries, the pest is controlled through the use of phylloxera-resistant rootstocks (Granett et al. 2001b, Powell 2012). In some countries with limited distribution, however, quarantine is the preferred option in combination with rootstocks. Phylloxera was first detected in Australia near Geelong, Victoria, in 1877 and later spread to the states of New South Wales (NSW) and Queensland (Buchanan 1990). Currently, Victoria and NSW are the only Australian states where phylloxera is known to be present. Management is primarily through the use of controlled designated phylloxera quarantine zones (Vinehealth Australia 2016) and associated National Phylloxera Management protocols (Powell 2008, National Vine Health Steering Commitee 2009).

Phylloxera exists as two forms, those that feed on the roots (radicicolae) and on foliage (gallicolae), inducing galls in both instances (Kellow et al. 2004). Radicicolae forms predominate on ungrafted European *V. vinifera* and can also be found on tolerant American *Vitis* rootstocks whilst gallicoliae forms are more common on leaves of *Vitis* species. In Australia, radicicoliae phylloxera reproduce asexually, and depending on genetic strain, an adult female apterous lays up to 200 eggs (Granett et al. 2001b). Eggs hatch into first instars and undergo five nymphal instars before developing to the adult stage. Peak phylloxera populations in the field are dependent on abiotic and biotic factors, such as soil characteristics (Powell et al. 2003), age and genetic composition of the host plant (Omer et al. 2002), phylloxera genetic strain (Corrie et al. 2002, Herbert et al. 2010) and climatic conditions (Forneck et al. 2001, Herbert et al. 2010). In Australia, peak populations of all phylloxera life-stages are recorded in the summer months. Winter survival is in the form of first instars (Powell et al. 2013).

Phylloxera reduces grapevine vigour and yield as a result of root feeding damage (Powell et al. 2013). Secondary damage can also be a consequence of soil-borne pathogens entering phylloxera feeding sites and causing root necrosis, although this association of secondary damage is variable (Omer and Granett 2000, Powell et al. 2013). The first visible signs of a phylloxera infestation in vineyards are usually yellowing of vines and stunted canopy growth due to reduced root function (Granett et al. 2001b). Phylloxera is not usually diagnosed until several years after its introduction into a vineyard and when populations have built up over time causing significant root damage. Infested vines may ultimately die depending on the

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relative virulence or aggressiveness of the phylloxera strain attacking it (Kellow et al. 2004, Kingston et al. 2007b, Powell et al. 2013). As well as ungrafted *V. vinifera*, phylloxera survives on tolerant *Vitis* hybrid rootstocks, where there may be no visible above-ground symptoms of the insect, and these can be a source of new infestations. The only long term management strategy once a vineyard is infested with phylloxera is to remove susceptible vines and replant with tolerant or where possible resistant rootstocks, which is a costly and time-consuming operation (Powell 2009, Powell and Krstic 2015). The implementation of, and adherence to, quarantine procedures to restrict the spread of phylloxera reduces the associated costs and, subsequently, the need for extensive replanting with rootstocks.

Grape phylloxera usually spreads from vineyard to vineyard via human pathways, including footwear, clothing, grape-picking bins, machinery and equipment, soil, grapes, grape foliage and planting material (Deretic et al. 2003). The most dispersive life stage is the first instar nymph as this can be present both below-ground (throughout the season) and above-ground (spring to autumn) and is relatively abundant and more active compared with other life stages (King and Buchanan 1986, Herbert et al. 2006, Powell et al. 2013). Strict quarantine procedures restricting movement of people, equipment, soil and other potentially infested materials between vineyards were originally introduced in Australia in 1917 (De Castella and Brittlebank 1917). Thus, phylloxera has primarily been contained within a few geographic regions in Victoria and NSW (Umina et al. 2007). Quarantine protocols involve thoroughly disinfesting and cleaning all items that come into contact with infested vineyards to minimise the risk of spread to non-infested vineyards. Under the Plant Health and Plant Products Act 1995 (National Vine Health Steering Commitee 2009), the Australian government has declared specific areas as Phylloxera Infested Zones (PIZ) where phylloxera is present (Buchanan 1988, Vinehealth Australia 2016).

National Phylloxera Management Protocols are designed to restrict the movement and spread of phylloxera out of a PIZ if phylloxera status is determined and prevents entry of potentially infested material into a Phylloxera Exclusion Zones where phylloxera is absent (National Vine Health Steering Commitee 2009). The risk that vineyard personnel and visitors inadvertently transfer phylloxera on their footwear is particularly high between September and May in Australia (Powell 2008), when first instars are likely to be present both on the soil surface and in the grapevine canopy. For footwear, a disinfestation protocol of dipping footwear in a freshly prepared household bleach and water solution containing a.i 2% sodium hypochlorite (NaOCl) for a minimum contact time of 30 s is currently recommended (National Vine Health Steering Commitee 2009). The protocol recommends a thorough rinse in clean water post treatment so as to avoid damage of footwear and eliminate the bleach odour (National Vine Health Steering Commitee 2009). Hand held tools are also disinfested in the same manner.

The efficacy of NaOCl, the active ingredient of household bleach, against insects and nematodes has previously been demonstrated in laboratory and field studies (Stanton and O'Donnell 1994). Household bleach has been used as an ovicide for mosquitoes (Domenico et al. 2006, Jacups et al. 2013, Mackay et al. 2015), and a 5–10% concentration of NaOCl is recommended by the Australian Quarantine Inspection Service to eradicate *Aedes* spp in water-holding containers (Lamache and Whelan 2002). Grzegorczyk and Walker (1997) prevented microbial contamination in in vitro cultures of grape phylloxera eggs on roots of *Vitis* species using a.i 5.25% NaOCl. The aforementioned study, however, showed that bleach treatments required rinsing with sterile water to encourage hatching of eggs. Studies on phylloxera disinfestation by Dunstone et al. (2003) demonstrated a dose response to NaOCl in the mortality of first instars using a phylloxera population of undetermined genetic status sourced from Victoria. In that study, it was not clear if a post treatment water rinse treatment was applied, and 2% NaOCl treatment achieved 100% first instar mortality after immersion for 30 s.

In the past decade, there has been an increase in the number of infested vineyards and an expansion of PIZs in Victoria, Australia (Dr Kevin Powell, pers. comm., 2016). Opportunely, there has also been an improved characterisation of the genetic diversity of endemic phylloxera strains (Corrie et al. 2002, Umina et al. 2007). Eighty-three genetic strains are known to be present in Australia (Umina et al. 2007). Thus, a re-evaluation of disinfestation protocols for endemic phylloxera strains is considered a high priority. In this study, we focused on the disinfestation protocol currently recommended for footwear and hand held tools (National Vine Health Steering Commitee 2009). We investigated the survival of first instars of six phylloxera strains, G1, G4, G7, G19, G20 and G30, following immersion in four concentration values of NaOCl for 30, 40 and 60 s. Our specific objectives were as follows: (i) to identify the optimal NaOCl concentration and duration required to achieve 100% first instar mortality for all six genetic phylloxera strains; (ii) to investigate the effect of NaOCl treatments on first instars ability to establish feeding sites, develop into adults and reproduce; and (iii) to establish the interaction between first instar survival and a water rinse treatment following NaOCl treatments.

Materials and methods

Collection, characterisation and maintenance of phylloxera strains

Six phylloxera strains, G1, G4, G7, G19, G20 and G30, were selected for this study. The phylloxera were collected from ungrafted *V. vinifera* in commercial vineyards located in central and north-east Victoria, Australia. The strain G1, which is found in most PIZs, was collected from the Yarra Valley, Central Victoria, in the Maroondah PIZ. All other genetic strains were sourced from the north-east Victoria PIZ with G4 collected from the King Valley, G7, G19 and G30 from Rutherglen and G20 from the Buckland Valley. The insects were genotyped using six nuclear DNA microsatellite markers (Umina et al. 2007).

All six phylloxera strains were mass-reared in vitro at Agriculture Victoria, Rutherglen, Vic., Australia, in a dark controlled environment growth room (25 ± 2°C) under quarantine conditions. Insect stock cultures were maintained on excised *V. vinifera* cv. Chardonnay roots in 90 × 25 mm Petri dishes (Kingston et al. 2007a). The end of the roots were wrapped in cotton wool and moistened once every week with sterile water to keep them viable for the insects nutritional requirements. Where fungal growth was evident, the roots were cleaned by dabbing the contaminated root section with a sable-haired paintbrush slightly moistened in 80% ethanol. Eggs aged 1–4 days old were collected using an artist's paintbrush and gently placed on moistened filter paper in a 35 mm Petri dish. The Petri dishes were sealed with cling film (Rapfast PVC food packaging, Integrated Packaging, Reservoir, Vic., Australia) to create a hatching chamber and incubated in the dark at a constant temperature of 22°C. Eggs were

monitored daily until hatching. Newly emerged active first instars were collected and used for all experiments.

Sodium hypochlorite as a disinfestation treatment for six phylloxera genetic strains

The effectiveness of household bleach [White King, Pental, Melbourne, Vic., Australia; active ingredient: NaOCl 42 g/L (available chlorine 4.0% m/v); sodium hydroxide 9 g/L] was investigated against six phylloxera genetic strains. Bleach was diluted in tap water to obtain the NaOCl concentration required for the different treatments.

The current recommended disinfestation protocol for footwear and handheld tools requires immersing in 2% NaOCl solution for 30 s followed by a thorough rinse in water (National Vine Health Steering Commitee 2009). Thus, the effectiveness of this disinfestation protocol was validated by immersing first instars phylloxera from six genetic strains, G1, G4, G7, G19, G20 and G30, in treatments containing 2% NaOCl for 30 s followed by a 30 s water rinse to reflect the current recommended disinfestation protocol (National Vine Health Steering Commitee 2009).

To examine the effect of time of immersion on survival, first instars were also treated in 2% NaOCl for 40 and 60 s followed by a 30 s water rinse. To investigate the effect of a water rinse following NaOCl treatment on first instars survival, insects were subjected to 2% NaOCl for 30, 40 and 60 s, but a post-treatment water rinse was omitted. The effect of increasing NaOCl concentration and time of immersion was in addition investigated by subjecting first instars to 3 and 4% NaOCl for 30, 40 and 60 s followed by a 30 s rinse in water. First instars from the six genetic strains were also subjected to a water Control (0% NaOCl) treatment for 30, 40 and 60 s.

The experiments were conducted by placing newly emerged first instars in a plastic vial (5.5 cm high, 2.5 cm in diameter) that was sealed with a screw top lid with a metal mesh (53 µm aperture) at the top and bottom ends (Korosi et al. 2012). The vial was immersed in the respective NaOCl solutions prepared to a volume of 500 mL in a beaker. Each treatment was conducted in separate trials with five replications of ten insects per replicate from each of the six genetic strains. After treatment, first instars were removed from the vials using a sable-haired paintbrush that was moistened in water, placed onto a filter paper in a Petri dish and examined under a low-power microscope. Where there was observable movement or if the antennae and legs moved when stimulated with the tip of the paintbrush, the first instars were classified as surviving the treatment. Where there was no apparent movement 2 h after treatment, first instars were scored dead. The total number of first instars recovered from the vials and those that were missing was recorded.

Effect of NaOCl treatment on feeding, development and reproduction on phylloxera

To examine the effect of NaOCl on feeding, development to adults and reproduction, first instars that survived treatments were placed on excised *V. vinifera* roots. First instars from the three immersion durations (30, 40 and 60 s) were pooled and placed on a single separate root piece in a 90 × 25 mm Petri dish for each NaOCl concentration (i.e. 0, 2, 3 and 4%) due to low numbers of insects (<30) surviving the three time treatments. Root pieces in the bioassay plates were checked once every day following treatments, and first instars that died on the roots and those that established feeding sites were counted and recorded. Dead first instars were characterised by a dark brown colour and lack of movement within 3 days post treatment. Root pieces were thereafter cleaned and checked weekly, and first instars that developed to adulthood and laid eggs were counted.

Experimental design

All experiments were conducted under controlled laboratory conditions at a set temperature of 22°C. Experiments consisted of two treatment factors: (i) NaOCl at five concentration values, that is, 0% NaOCl water Control, 2% NaOCl rinsed in water for 30 s after immersion (R), 2% NaOCl not-rinsed (NR) in water after immersion, 3% NaOCl (R) and 4% NaOCl (R); and (ii) time at three levels; 30, 40 and 60 s. Each experiment implemented a randomised complete block design with full factorial combination of NaOCl and time with five replications each. The blocking factor was replication.

Statistical analysis

Percentage first instar phylloxera survival was calculated as the number of insects surviving treatments out of the total recovered from vials multiplied by 100. Percentage survival was subjected to ANOVA. To compare survival in different NaOCl concentration values (2, 3 and 4%) and to compare with the Control treatments, liquid (NaOCl and water) were treated in a factorial combination with a genetic strain by time interaction. This treatment structure was coded in GenStat as NaOCl*Time*Genetic strain with replication as the blocking factor.

The effect of post-treatment water rinse on first instar survival was assessed only for the 2% NaOCl treatments using the treatment structure 'rinse' (Yes and No) with genetic strain and time as factors. A contrast analysis, where contrasts were constructed using the treatment factors (NaOCl, time and genetic strain), was used to compare and validate the effectiveness of the recommended disinfestation protocol for footwear and handheld tools between the six genetic strains for 2% NaOCl treatments.

The proportion of first instars that survived treatments, established feeding sites, developed to adulthood and subsequently laid eggs was analysed using a general ANOVA with NaOCl concentration as the treatment factor and proportion of dead crawlers, those that established feeding sites and reproduced as the response variables.

Residual values were examined graphically to ensure normality and homogeneity of variances. Observations with standardised residuals greater than 3.0 were excluded from analyses. Fisher's protected least significant difference (LSD) test ($P < 0.05$) was used to separate means where F-tests were significant. All data were analysed using GenStat 17 software (GenStat Release 16., VSN International, Hemel Hempstead, England).

Results

Effectiveness of 2% NaOCl disinfestation treatment on six phylloxera genetic strains

When subjected to the recommended treatment of 2% NaOCl for 30 s followed by a water rinse, a mean survival of 71% (±1.6) was observed for first instars across all the six genetic strains (Table 1). Compared with the water Control treatments (0% NaOCl) for 30 s, an average of 95% first instars survived treatment (Table 1).

First instars from G1 and G4 genetic strains were most susceptible to the 2% NaOCl treatment for 30 s compared with G7, G19, G20 and G30 (Table 2). Increasing the time of immersion in 2% NaOCl from 30 to 40 s followed by a water

Table 1. Effect of treatment at four levels of sodium hypochlorite concentration and three times on percentage mean survival of six phylloxera genetic strains.

Treatments		Mean survival (%) Phylloxera genetic strains					
Time (s)	**NaOCl [C]**	**G1**	**G4**	**G7**	**G19**	**G20**	**G30**
30	0	98	95	95	97	95	95
	2NR	2	2	5	10	14	4
	2R	65	62	75	73	76	78
	3R	61	27	40	72	68	67
	4R	17	15	32	69	64	62
40	0	96	87	93	98	98	98
	2NR	0	0	2	4	7	0
	2R	57	35	64	58	64	58
	3R	31	19	32	49	53	59
	4R	4	14	28	33	48	50
60	0	92	88	92	93	98	95
	2NR	0	0	0	0	0	0
	2R	28	26	51	36	63	38
	3R	25	14	29	30	44	35
	4R	2	0	24	30	35	23
Standard error				4.8			
F test probabilities							
Genetic strains (G)				<0.001			
NaOCl [C]				<0.001			
Time (T)				<0.001			
Interaction (G × [C] × T)				<0.001			

rinse impacted on survival of first instars (Table 1) with a significant decrease in survival for G4, G7, G19, G20 and G30 first instars ($P < 0.05$) but not for G1 ($P = 0.156$) (Table 2). When immersion time in 2% NaOCl solution followed by a water rinse was increased from 30 to 60 s, first instar survival decreased significantly ($P < 0.01$) across all six genetic strains (Table 1) with the treatment impacting significantly on G1 and G4 first instars, which had the least mean survival (Table 2). First instars from G7 and G20 strains were most resistant to the 2% treatment at the higher immersion time while G19 and G30 strains were moderately susceptible (Table 2).

Effect of a water rinsing following 2% NaOCl treatment on survival of first instars from six phylloxera genetic strains

Omitting a water rinse after immersion in 2% NaOCl significantly impacted on the survival of phylloxera first instars with a significant genetic strain and time interaction ($P < 0.001$) (Table 1).

When immersed in 2% NaOCl for 30 s without a water rinse afterwards, at least 2% (range 2–5%) first instars from G1, G4, G7 and G30 genetic strains survived treatment compared with 10 and 14% of G19 and G20, respectively (Table 1). In contrast, an average of 68% (range 61–78%) of first instars treated in 2% NaOCl for 30 s followed by a water rinse survived treatments (Table 2).

When immersion time in 2% NaOCl without a water rinse afterwards was increased from 30 to 40 s, none of the first instars from G1, G4 and G30 genetic strains survived the

Table 2. A summary of six main contrasts showing mean differences in the survival of first instars from six phylloxera strains when subjected to 2% NaOCl solution followed by a water rinse and without a water rinse.

Contrasts	Phylloxera genetic strains					
	G1	**G4**	**G7**	**G19**	**G20**	**G30**
2R30 vs 2R40						
Estimate	7	27	11	14	12	20
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	0.156	<0.001	0.043	0.006	0.015	<0.001
2R30 vs 2R60						
Estimate	36	36	24	37	13	40
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	<0.001	<0.001	<0.001	0.009	<0.001
2R40 vs 2R60						
Estimate	29	9	14	22	1	20
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	0.024	0.011	<0.001	0.850	<0.001
2R30 vs 2NR30						
Estimate	62	60	70	62	61	74
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2R30 vs 2NR40						
Estimate	65	62	73	68	68	78
SE	5.1	4.0	5.1	5.1	4.7	4.8
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
2R30 vs 2NR60						
Estimate	50	41	63	56	67	58
SE	4.2	3.3	4.2	4.1	3.8	3.9
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Estimate value is the difference between the respective treatments, 2% NaOCl solution and time of immersion 30, 40 and 60 s, of first instars across the six genetic strains. R, water rinse for 30 s after immersion; NR, treatments where a water rinse after immersion in NaOCl solutions was omitted. SE, standard error.

treatment while 2, 4 and 7% of first instars from G7, G19 and G20, respectively, survived the treatment (Table 1). In contrast, 56% (range 35–64%) of the first instars immersed in 2% NaOCl for 40 s followed by a water rinse survived the treatment (Table 1).

With treatment in 2% NaOCl for 60 s without a water rinse afterwards, none of the first instars across all the six genetic strains survived (Table 1). In contrast, an average of 40% (range 28–63%) first instars survived in 2% NaOCl when immersed for 60 s with a water rinse afterwards (Table 1).

Survival of phylloxera first instars in 3 and 4% NaOCl

Survival of first instars immersed in 3 and 4% NaOCl solutions followed by a water rinse varied with a significant interaction between the six phylloxera genetic strains; NaOCl concentration and the time insects were exposed to treatments ($P < 0.001$) (Table 1).

When immersed for 30 s in 3% NaOCl, survival of first instars from G4 and G7 genetic strains was 27 and 40%, respectively, and significantly lower than that of first instars from G1, G19, G20 and G30 strains where survival was greater than 61% (range 61–72%) (Table 1). When immersed for 30 s in 4% NaOCl, first instar survival for G1 and G4 genetic strains was 17 and 15%, respectively, and was significantly lower than that of first instars from G7, G19, G20 and G30 where survival was greater than 32% (range 32–69%) (Table 1).

When immersed for 40 s in 3% NaOCl, survival of G1, G4 and G7 first instars ranged between 19 and 32% and was significantly lower than that of first instars from G19, G20 and G30 genetic strains where survival ranged between 49 and 59% (Table 1). When immersed for 40 s in 4% NaOCl, G1 and G4 first instars were most susceptible with a significantly low survival of 4 and 14%, respectively. First instars from G20 and G30 genetic strains were least susceptible with 48 and 50%, respectively, surviving the treatment (Table 1). First instars from G7 and G19 were moderately susceptible to 4% NaOCl when immersed for 40 s with 28 and 33%, respectively, surviving the treatment (Table 1).

When immersed for 60 s in 3% NaOCl, G4 first instars were most susceptible with 14% surviving treatments compared with G1, G7, G19, G20 and G30 where survival ranged from 25 to 44% (Table 1). With immersion time of 60 s in 4% NaOCl, only G4 first instars did not survive the treatment (Table 1). First instars from G1 genetic strain were highly susceptible when immersed for 60 s in 4% NaOCl with only 2% surviving the treatment (Table 1). First instars from G20 were moderately susceptible to the treatment in 4% NaOCl for 60 s with 35% survival while first instars from G7, G19 and G30 genetic strains were least susceptible to the treatment with at least 23–30% survival observed (Table 2).

Compared with the water Control (0% NaOCl) where at least 87% of first instars survived treatments, survival of first instars in 3 and 4% NaOCl was much lower with an average of 36% insects across the six genetic strains and time of immersion surviving the latter treatments (Table 1).

Effect of immersion time in NaOCl treatments on survival of grape phylloxera

Survival of first instars in NaOCl treatments that were followed by a water rinse was significantly reduced the longer the insects were exposed to treatments (for the four NaOCl concentration values were combined) ($P < 0.001$) (Figure 1a). Across all genetic strains, a mean of 45% (±2.9) first instars survived

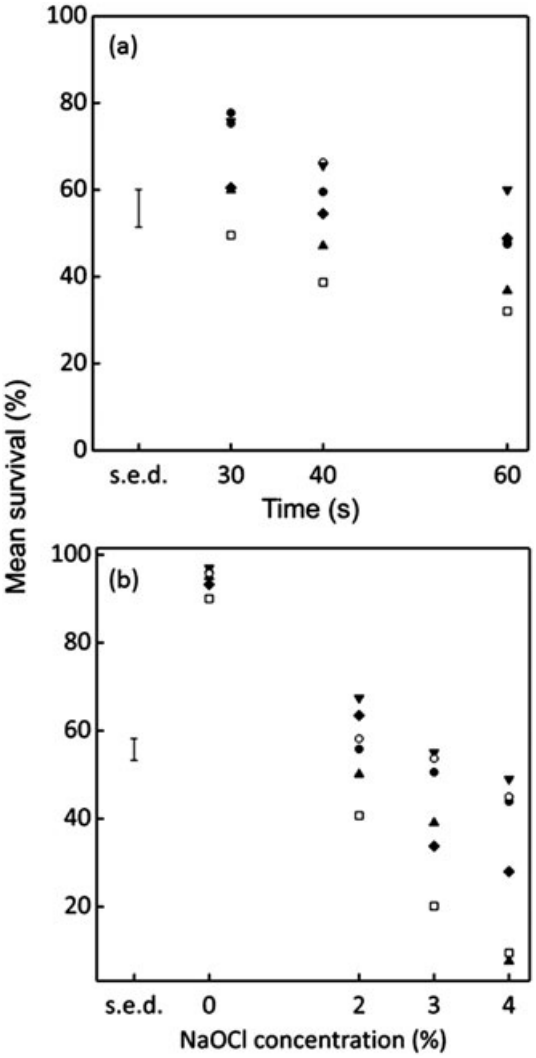

Figure 1. Effect of increasing (a) treatment duration in sodium hypochlorite (NaOCl) solutions and (b) NaOCl concentration, on first instars survival of six grape phylloxera strains, G1 (▲), G19 (●), G20 (▼), G30 (○), G4 (□) and G7 (♦). Data presented are means of first instars combined for the three concentration values and three immersion duration times for treatments that were followed by a 30 s water rinse. s.e.d., standard error of difference between means.

treatments when immersed for 60 s, compared with 55% (±2.6) and 66% (±2.3) that were immersed for 40 and 30 s, respectively (Figure 1a).

Effect of NaOCl concentration on survival of grape phylloxera

Survival of first instars in NaOCl treatments that were followed by a water rinse was highest with the water control (0% NaOCl) and the lowest NaOCl concentration (2%) used ($P < 0.001$) (Figure 1b). Survival of first instars markedly declined as the NaOCl concentration increased (Figure 1b). With the three immersion durations combined across the six genetic strains, 95% (±0.7) first instars survived in the water Control (0% NaOCl) compared with 55% (±1.9) in 2% NaOCl. A mean of 42% (±2.0) and 31% (±2.3) of first instars treated in 3 and 4% NaOCl, respectively, survived treatments (Figure 1b).

Establishment and development of phylloxera strains post-NaOCl treatments

First instars that survived NaOCl treatments and were placed on roots established feeding sites and subsequently laid eggs

with varying degrees of success between the four NaOCl concentration values. There were no significant trends between the six genetic strains ($P > 0.05$) (data not shown). Overall, there was a higher negative impact on establishment of feeding sites with 23–49% first instars treated in NaOCl surviving compared with those of the water Control, where 78% first instars established feeding sites (Table 3). Of the first instars that survived treatments and were placed on roots, at least 4% (range 4–26%) first instars died before establishing feeding sites and mortality significantly differed between the NaOCl concentration ($P = 0.03$). For the first instars immersed in 2 and 3% NaOCl, 8% died before establishing feeding sites, but these were not statistically different from individuals that survived the water Control treatments (Table 3). With first instars that survived 4% NaOCl treatment, 26% that were placed on roots died before establishing feeding sites (Table 3).

For those phylloxera that survived in 2 and 3% NaOCl, 43 and 49%, respectively, established feeding sites compared with 23% that survived in 4% NaOCl (Table 3). Of the first instars that established feeding sites, none of those treated in 4% NaOCl laid eggs; however, 19 and 10% first instars that were treated in 2 and 3% NaOCl, respectively, laid eggs (Table 3).

Discussion

Sodium hypochlorite has been used in numerous industrial applications and plays an important role in disinfestation of a variety of insect pests (Grzegorczyk and Walker 1997, Jacups et al. 2013, Mackay et al. 2015) and disease-causing microorganisms (Winter et al. 2008). Household bleach is routinely used for disinfestation of footwear and handheld tools in vineyards against grape phylloxera (National Vine Health Steering Committee 2009). The current study represents a systematic comparison of six phylloxera genetic strains and their relative susceptibility to treatments in sodium hypochlorite, the active ingredient of household bleach. The overall aims of this study were as follows: first, to validate the recommendation of dipping footwear and handheld tools in 2% for 30 s followed by a water rinse as a disinfestation protocol for use in vineyards (National Vine Health Steering Committee 2009); second, to determine the optimal concentration of NaOCl and minimum exposure period required to achieve 100% mortality across all the six genetically diverse phylloxera strains selected; and third, to investigate the effect of omitting a water rinse on phylloxera survival. Results showed that more than 50% first instars across the six genetic strains survived after 30 s immersion in 2% NaOCl followed by a 30 s rinse in water. These findings differ from a previous study by Dunstone et al. (2003) where 100% mortality was achieved when first instars were immersed in 2% NaOCl treatments at a minimum contact time of 30 s. The genetic strain of phylloxera used in the previous study was, however, not provided, and it is also not clear if a water rinse was included in the treatments.

Results from this study further showed that increasing contact time and NaOCl concentration followed by a water rinse reduced first instar survival, but did not achieve 100% mortality. Survival was directly proportional to the increase in time of immersion as well as the NaOCl concentration, and this was consistent across the six genetic strains. Results indicate that a concentration above the recommended 2% NaOCl does not yield satisfactory disinfestation, and an exposure time longer than 60 s may be required to achieve close to 100% mortality. van Frankenhuyzen et al. (2004) showed that NaOCl causes partial dechorionation to eggs of the eastern spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae), resulting in susceptibility to desiccation and mechanical injury, and the results were more pronounced when contact time and NaOCl concentration were increased. Several additives could be used to enhance the efficacy of NaOCl as a disinfestation protocol for grape phylloxera. One method is to use NaOCl solutions where the pH has been lowered. For instance, the addition of vinegar to reduce the pH to about 6.8 has been shown to raise antimicrobial efficacy (Feirtag 2007). Sodium hypochlorite disinfestation at high temperature has also been shown to have a shrivelling effect on proteins of organisms (Winter et al. 2008). To overcome the limitation of using household bleach for mosquitoes management, Sherman et al. (1998) proposed a mix of powdered detergent to increase viscosity and hence adherence to surfaces. Future studies to explore whether additives in conjunction with the use of hot water when making up NaOCl solutions for a footbath could be effective for grape phylloxera disinfestation are worthwhile.

Omitting a water rinse phase following exposure to 2% NaOCl is a significant result from this study and a critical step in the disinfestation process as it significantly reduced first instar survival with differences between the six genetic strains and time of exposure to the treatment. To achieve 100% mortality, immersion for 60 s in 2% NaOCl without the water rinse afterwards is optimal across all genetic strains. Bloomfield (1996) showed that NaOCl is a strong oxidiser with broad spectrum antimicrobial activity, and its mode of action in disinfestation protocols can be greatly influenced by rinsing off water post-treatments. When dissolved in water, NaOCl ionises to the sodium ion (Na^+) and the hypochlorite ion (ClO^-) forming hypochlorous acid (HOCl). Hypochlorous acid is responsible for damaging the microbes' cell membranes,

Table 3. Effect of sodium hypochlorite treatment on the establishment and development of grape phylloxera.

NaOCl (%)	No. of first instars placed on roots	Proportion of first instars dead (%)	Proportion of first instars that established feeding sites (%)	Proportion of first instars that laid eggs (%)
0	163	4 a	78 c	60 d
2	141	8 a	49 b	19 c
3	57	8 a	43 b	10 b
4	55	26 b	23 a	0 a
df		20	20	20
P		0.03	<0.001	<0.001
SE		7.3	1.4	4.2

There were no statistical differences between the genetic strains; hence, data presented are means (±standard error) pooled across the six genetic strains. Values followed by the same letter are not significantly different (Fisher's protected LSD test $P < 0.05$). NaOCl, sodium hypochlorite.

proteins and nucleic acids through an oxidative degradation process upon contact (McDonnell and Russell 1999). Thus, in this study, omitting a water-rinse step allows HOCl more time to penetrate and react with the insect cuticle causing mortality. The effect of increased survival as a result of rinsing NaOCl treatments in water has been supported in a study by Chakravarty and Kovar (2013) where treatments to eliminate fungal contamination using bleach were ineffective when followed by a water rinse. Chakravarty and Kovar (2013) found that there was a significant inhibitory effect on growth and spore germination of six tested fungi after exposure to NaOCl. The fungal spores, however, recovered and became viable after a 24 h incubation period when they were immersed in water following treatment, a reversible inhibitory effect described as mycostasis.

The finding that rinsing of footwear and vineyard tools in water after treatment in NaOCl enhances survival is clearly an impedance to phylloxera quarantine management efforts. Different types of footwear and equipment that have crevices where phylloxera may hide could potentially make disinfestation treatments less effective, especially when a lower NaOCl concentration and less time the insects are exposed to treatments are applied. The effect of a water rinse following NaOCl disinfestation treatments is likely compromised when soil, mud and other contaminants are trapped on footwear and handheld tools. A possible negative influence of soil and mud is adherence to footwear and tools, thus impeding contact between grape phylloxera and NaOCl. A study examining the effect of NaOCl on the fungal pathogen *Erwinia amylovora,* the causative agent of fire blight, showed that surface crevices on pruning shears made chemical treatments less effective (Kleinhempel et al. 1987). Treatments with NaOCl require more time to penetrate into crevices and soil contaminants where first instars are present, and thus, omitting the water-rinse step would markedly increase the contact time with HOCl.

Results from this study showed differences in susceptibility to NaOCl by the six phylloxera strains tested. Differences in susceptibility to disinfestation treatments using dry heat were observed for the G1 and G4 phylloxera genetic strains (Korosi et al. 2012), suggesting physiochemical and structural differences between strains. Compared with heat treatments, hypochlorite causes proteins to lose their structure and form large aggregates. In bacteria, many of the proteins that come into contact with hypochlorite are essential for growth, and the interaction with NaOCl inactivates those proteins causing death (Valencia et al. 1996, Winter et al. 2008). In mosquitoes, NaOCl digests the egg chorion exposing the egg to desiccation and causing eventual death (Cabrita et al. 2003, Rezende et al. 2008). A potential variability in the cuticle structure could be attributed to the difference in response to the treatments by the six phylloxera genetic strains used in this study. Further studies to investigate the cuticular difference between phylloxera genetic strains would be informative in this respect.

Although our study validated the efficacy of NaOCl as an effective disinfestation treatment, the relatively high survival rates at 2% NaOCl concentration followed by a water rinse and the ability of the surviving insects to develop to fecund adults suggest an urgent need to modify the current phylloxera disinfestation protocol. New phylloxera infestations are often associated with the movement of first instars through poor adherence to quarantine protocols. Our findings also highlight the need to adhere to a robust disinfestation procedure that is effective against multiple endemic grape phylloxera strains to avoid the unintended transfer between vineyards. Powell et al. (2003) showed that first instars originating from radicicolae populations are the most abundant life stage and are the active dispersive stage of phylloxera. Powell et al. (2003) further showed that the first instar populations dispersing at the soil surface are greater than those actively moving along the vine trunk and into the canopy. The risk of transferring first instars through soil particles attached on footwear from one vineyard to another is, therefore, high. Phylloxera strains that are more aggressive (Forneck et al. 2016) would reproduce much faster causing a rapid rise in populations from a few first instars that survive NaOCl treatment. In this study, numbers of first instars that survived NaOCl treatment and went on to establish feeding sites and reproduce were not robust enough to give a statistical separation that would verify differences in survival between the six genetic strains. Future research focusing on post-chemical treatments may provide further information on the virulence status between different genetic strains.

While immersing first instars in 2% NaOCl without a water rinse was an effective treatment at 60 s in the laboratory, several operational limitations must also be considered if this dose and time are to be used as a recommended disinfestation treatment. Household bleach applications may be unsuited in certain situations as direct exposure to NaOCl can cause damage to footwear, handheld equipment and skin if users come into contact with solutions (Lambert et al. 2000). Furthermore, there can be concerns of exposure to chlorine released into the air and hypochlorite on treated surfaces, and thus, the usage of NaOCl could compromise occupational health and safety standards (Agar 2006, Anon. 2016). Despite these concerns, the use of household bleach has its advantages because it is relatively inexpensive, readily available and most people are already familiar with it and more likely to accept its use.

Conclusions

This study highlights the need to recognise that genetic variability between strains of phylloxera influences the efficacy of NaOCl as a disinfestation treatment. The findings, therefore, highlight the need to consider the phylloxera genetic background when developing and recommending disinfestation procedures for footwear and handheld tools in different PIZs. Our results highlight that the current disinfestation protocol requires modification by: (i) omitting the water rinse after 2% NaOCl treatment; and (ii) increasing time of immersion in 2% NaOCl from 30 to 60 s in order to achieve 100% mortality across genetic strains. The challenge posed, however, is that of NaOCl odour and residues and subsequent damage to footwear and handheld tools. There is, therefore, a pressing need to screen alternative disinfestation treatments to NaOCl or the use of surfactants to enhance penetration of NaOCl through soil and other contaminants adhering onto footwear and vineyard tools. Some authors have previously used ethanol as an alternative disinfestation treatment under research conditions for grape phylloxera eggs (Kellow et al. 1999) and first instars (Powell et al. 2006), but further investigations are required to determine the efficacy of ethanol as well as other potential treatments against different phylloxera strains.

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References

Agar, M. (2006) An ethnography by any other name. Forum Qualitative Sozialforschung/Forum: Qualitative Social Research 7 (4). Retrieved 10 July 2012 (http://www.qualitative-research.net/index.php/fqs/article/view/177/395). Date accessed: 15/09/16.

Anon. (2016) Bleach. Material safety data sheet, White King Premium Bleach (Pental Products: Melbourne, Vic., Australia). Issued 14 January 2016 pp. 1–7.

Bloomfield, S.F. (1996) Chlorine and iodine formulations. Ascenzi, J. M. and Dekker, M., eds. Handbook of disinfectants and antiseptics (Academic Press: New York, NY, USA) pp. 133–158.

Buchanan, G.A. (1988) Changes to quarantine of grape phylloxera in Victoria. The Australian Grapegrower & Winemaker (**290**), 9–11.

Buchanan, G. A. (1990) The distribution, biology and control of grape phylloxera, *Daktulosphaira vitifolii* (Fitch), in Victoria. PhD Thesis, La Trobe University, Bundoora, Vic., Australia.

Cabrita, E., Chereguini, O., Luna, M., de Paz, P. and Herráez, M.P. (2003) Effect of different treatments on the chorion permeability to DMSO of turbot embryos (*Scophthalmus maximus*). Aquaculture **221**, 593–604.

Chakravarty, P. and Kovar, B. (2013) Evaluation of five antifungal agents used in remediation practices against six common indoor fungal species. Journal of Occupational and Environmental Hygiene **10**, 11–16.

Corrie, A.M., Crozier, R.H., van Heeswijck, R. and Hoffmann, A.A. (2002) Clonal reproduction and population genetic structure of grape phylloxera, *Daktulosphaira vitifoliae*, in Australia. Heredity **88**, 203–211.

De Castella, F. and Brittlebank, C.C. (1917) Report of the government viticulturalist. Journal of Agriculture Victoria **15**, 685–700.

Deretic, J., Powell, K. and Hetherington, S. (2003) Assessing the risk of phylloxera transfer during post-harvest handling of wine grapes. Acta Horticulturae **617**, 61–66.

Domenico, D.D., Ruggeri, L. and Trentini, M. (2006) The use of sodium hypochlorite as ovicide against *Aedes albopictus*. Journal of the American Mosquito Control Association **22**, 346–348.

Dunstone, R.J., Corrie, A.M. and Powell, K.S. (2003) Effect of sodium hypochlorite on first instar phylloxera *(Daktulosphaira vitifoliae* Fitch) mortality. Australian Journal of Grape and Wine Research **9**, 107–109.

European and Mediterranean Plant Protection Organization (1990) Data sheets on quarantine pests: *Viteus vitifoliae*. No. 106 (European and Mediterranean Plant Protection Organization: Paris, France).

Feirtag, J. (2007) Beyond eradication: can we accept effective forms of sanitation beyond the killer chemical paradigm? Contamination Control **14**, 64–65.

Forneck, A., Walker, M.A. and Blaich, R. (2001) Ecological and genetic aspects of grape phylloxera *Daktulosphaira vitifoliae* (Hemiptera: Phylloxeridae) performance on rootstock hosts. Bulletin of Entomological Research **91**, 445–451.

Forneck, A., Powell, K.S. and Walker, M.A. (2016) Scientific opinion: improving the definition of grape phylloxera biotypes and standardizing biotype screening protocols. American Journal of Enology and Viticulture **67**, 371–376.

Granett, J., Omer, A.D. and Walker, M.A. (2001a) Seasonal capacity of attached and detached vineyard roots to support grape phylloxera (Homoptera: Phylloxeridae). Journal of Economic Entomology **94**, 138–144.

Granett, J., Walker, M.A., Kocsis, L. and Omer, A.D. (2001b) Biology and management of grape phylloxera. Annual Review of Entomology **46**, 387–412.

Grzegorczyk, W. and Walker, M.A. (1997) Surface sterilization of grape phylloxera eggs in preparation for *in vitro* culture with *Vitis* species. American Journal of Enology and Viticulture **48**, 157–159.

Herbert, K.S., Hoffmann, A.A. and Powell, K.S. (2006) Changes in grape phylloxera abundance in ungrafted vineyards. Journal of Economic Entomology **99**, 1774–1783.

Herbert, K.S., Umina, P.A., Mitrovski, P., Powell, K.S., Viduka, K. and Hoffmann, A. (2010) Clone lineages of grape phylloxera differ in their performance on *Vitis vinifera*. Bulletin of Entomological Research **100**, 671–678.

Jacups, S.P., Ball, T.S., Paton, C.J., Johnson, P.H. and Ritchie, S.A. (2013) Operational use of household bleach to "crash and release" *Aedes aegypti* prior to *Wolbachia*-infected mosquito release. Journal of Medical Entomology **50**, 344–351.

Kellow, A.V., Corrie, A.M. and Van Heeswijck, R. (1999) Surface sterilisation of phylloxera eggs for investigating grapevine-phylloxera interactions in tissue culture. Australian Journal of Grape and Wine Research **5**, 27–28.

Kellow, A.V., Sedgley, M. and Van Heeswijck, R. (2004) Interaction between *Vitis vinifera* and grape phylloxera: changes in root tissue during nodosity formation. Annals of Botany **93**, 581–590.

King, P.D. and Buchanan, G.A. (1986) The dispersal of phylloxera crawlers and spread of phylloxera infestations in New Zealand and Australian vineyards. American Journal of Enology and Viticulture **37**, 26–33.

Kingston, K.B., Cooper, P.D. and Powell, K.S. (2007a) Grape phylloxera external morphology observations under scanning electron microscopy. Acta Horticulturae **733**, 107–114.

Kingston, K.B., Powell, K.S. and Cooper, P.D. (2007b) Characterising the root-feeding habits of grape phylloxera using electrical penetration graph. Acta Horticulturae **733**, 33–45.

Kleinhempel, H., Nachtigall, M., Ficke, W. and Ehrig, F. (1987) Disinfection of pruning shears for the prevention of the fire blight transmission. Acta Horticulturae **217**, 211–218.

Korosi, G.A., Mee, P.T. and Powell, K.S. (2012) Influence of temperature and humidity on mortality of grapevine phylloxera *Daktulosphaira vitifoliae* clonal lineages: a scientific validation of a disinfestation procedure for viticultural machinery. Australian Journal of Grape and Wine Research **18**, 43–47.

Lamache, G. and Whelan, P. (2002) Recommended chlorination procedures for receptacles containing mosquito eggs for quarantine purposes. Bulletin of the Mosquito Control Association of Australia **14**, 14–18.

Lambert, H., Manel, J. and Gabrion, I. (2000) Poisoning by household products. La Revue du Praticien **50**, 365–371.

Mackay, A.J., Amador, M., Felix, G., Acevedo, V. and Barrera, R. (2015) Evaluation of household bleach as an ovicide for the control of *Aedes aegypti*. Journal of the American Mosquito Control Association **31**, 77–84.

McDonnell, G. and Russell, A.D. (1999) Antiseptics and disinfectants: activity, action, and resistance. Clinical Microbiology Reviews **12**, 147–179.

National Vine Health Steering Commitee (2009) National phylloxera management protocol: movement procedures for phylloxera risk vectors from a Phylloxera Infested Zone (PIZ). http://www.vinehealth.com.au/bio-security/phylloxera-regulations/. Accessed 06/09/16.

Omer, A.D. and Granett, J. (2000) Relationship between grape phylloxera and fungal infections in grapevine roots. Journal of Plant Diseases and Protection **107**, 285–294.

Omer, A., Granett, J. and Walker, M.A. (2002) Influence of plant growth stage on grape phylloxera (Homoptera: Phylloxeridae) populations. Environmental Entomology **31**, 120–126.

Powell, K.S. (2008) Grape phylloxera: an overview. Johnson, S.N. and Murray, P.J., eds. Root feeders: an ecosystem perspective(Centre for Agriculture and Biosciences International: Wallingford, England) pp. 96–114.

Powell, K. S. (2009) Sustainable phylloxera management. Final Report. DPI 06/02 (Grape and Wine Research and Development Corporation: Adelaide, SA, Australia) p. 170.

Powell, K.S. (2012) A holistic approach to future management of grapevine phylloxera. Bostanian, N., Vincent, C. and Isaacs, R., eds. Arthropod management in vineyards: pests, approaches and future directions (Springer Science+Business Media: London, England) pp. 239–251.

Powell, K.S. and Krstic, M. (2015) Rootstock tolerance and resistance to different genetic strains of phylloxera. Wine and Viticulture Journal **30(5)**, 48–51.

Powell, K.S., Slattery, W.J., Deretic, J., Herbert, K. and Hetherington, S. (2003) Influence of soil type and climate on the population dynamics of grapevine phylloxera in Australia. Acta Horticulturae **617**, 33–41.

Powell, K. S., Korosi, G., Trethowan, C. and White, V. (2006) Sustainable long term stratergies for phylloxera management under Australian conditions. Final Report (Grape and Wine Research and Development Corporation: Adelaide, SA, Australia). p. 158.

Powell, K.S., Cooper, P.D. and Forneck, A. (2013) The biology, physiology and host–plant interactions of grape phylloxera *Daktulosphaira vitifoliae*. Advances in Insect Physiology **45**, 159–218.

Rezende, G.L., Martins, A.J., Gentile, C., Farnesi, L.C., Pelajo-Machado, M., Peixoto, A. and Valle, D. (2008) Embryonic desiccation resistance in *Aedes aegypti*: presumptive role of the chitinized Serosal Cuticle. BMC Developmental Biology **8**, 1–14.

Sherman, C., Fernandez, E.A., Chan, A.S., Lozano, R.C., Leontsini, E. and Winch, P.J. (1998) La untadita: a procedure for maintaining washbasins and drums free of *Aedes aegypti* based on modifications of existing practices. The American Journal of Tropical Medicine and Hygiene **58**, 257–262.

Stanton, J.M. and O'Donnell, W.E. (1994) Hatching, motility, and infectivity of root-knot nematode (*Meloidogyne javanica*) following exposure to sodium hypochlorite. Australian Journal of Experimental Agriculture **34**, 105–108.

Umina, P.A., Corrie, A.M., Herbert, K.S., White, V.L., Powell, K.S. and Hoffmann, A.A. (2007) The use of DNA markers for pest management-clonal lineages and population biology of grape phylloxera. Acta Horticulturae **733**, 183–195.

Valencia, M.D.P., Miller, L.H. and Mazur, P. (1996) Permeabilization of eggs of the malaria mosquito *Anopheles gambiae*. Cryobiology **33**, 149–162.

van Frankenhuyzen, K., Ebling, P., McCron, B., Ladd, T., Gauthier, D. and Vossbrink, C. (2004) Occurrence of Cystosporogenes sp. (Protozoa, Microsporidia) in a multi-species insect production facility and its elimination from a colony of eastern spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). Journal of Invertebrate Pathology **87**, 16–28.

Vinehealth Australia (2016) Phylloxera management zones in Australia. http://www.vinehealth.com.au/resources/maps. Date accessed 04/08/16

Winter, J., Ilbert, M., Graf, P.C.F., Özcelik, D. and Jakob, U. (2008) Bleach activates a redox-regulated chaperone by oxidative protein unfolding. Cell **135**, 691–701.

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