Phytopathologia Mediterranea (2013) 52, 2, 359–368

RESEARCH PAPER

Soaking grapevine cuttings in water: a potential source of cross contamination by micro-organisms

Helen WAITE[1,2], David GRAMAJE[3,4], Melanie WHITELAW-WECKERT[1], Peter TORLEY[1,2] and W. Jim HARDIE[1]

[1] National Wine and Grape Industry Centre, Charles Sturt University, Wagga Wagga NSW 2678, Australia
[2] School of Agriculture and Wine Sciences, Charles Sturt University, Wagga Wagga NSW 2678, Australia
[3] Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain
[4] Institute of Sustainable Agriculture (IAS), Spanish National Research Council (CSIC), Alameda del Obispo s/n, P.O. Box 4084, 14080 Córdoba, Spain

Summary. Grapevine nurseries soak cuttings in water during propagation to compensate for dehydration and promote root initiation. However, trunk disease pathogens have been isolated from soaking water, indicating cross contamination. Cuttings of *Vitis vinifera* cv. Sunmuscat and *V. berlandieri x V. rupestris* rootstock cv. 140 Ruggeri were immersed in sterilized, deionised water for 1, 2, 4, 8 and 16 h. The soaking water was cultured (25°C for 3 days) on non-specific and specific media for fungi and bacteria. The base of each cutting was debarked and trimmed and three 3 mm thick, contiguous, transverse slices of wood cultured at 25°C for 3 days. The soaking water for both cultivars became contaminated with microorganisms within the first hour. Numbers of fungi isolated from the wood slices soaked for one hour were significantly greater than those from non-soaked cuttings. The number of bacterial colonies growing from the wood slices increased after soaking for 2–4 h in Sunmuscat. In a second experiment Shiraz cuttings were soaked for 1, 2, 4, 8 and 24 h. The soaking water became contaminated within the first hour but only the bacterial count increased significantly over time. Microorganisms also established on the container surfaces within the first hour although there were no significant increases over 24 h. These results confirm that soaking cuttings is a potential cause of cross contamination and demonstrate contamination of cuttings occurs after relatively short periods of soaking. Avoiding exposing cuttings to water will reduce the transmission of trunk diseases in propagation.

Key words: trunk diseases, soaking, cross contamination, grapevine propagation.

Introduction

In modern times the grapevine propagation process has been mechanized and streamlined, enabling individual nurseries to reduce costs and produce large numbers of vines over an extended season. Bench mounted grafting machines that automatically cut and join a dormant rootstock cutting and one bud scion prior to rooting, along with cool rooms to hold cuttings in the dormant state until required for grafting, are now common in the grapevine nursery industry. Bench grafting, as this technique is known, has enabled individual nurseries to produce many hundreds of thousands of vines in a single season. However, the efficiency gains resulting from mechanization and the use of cool rooms have been somewhat offset by losses in quality. Incompletely healed graft unions and biotic contamination of the rootstocks, scions and graft unions are common problems in bench grafted vines (Stamp, 2001) and spasmodic, large scale failures of grafted vines in the nursery and vineyards are a serious problem for nurseries and grape growers (Morton, 2000; Halleen *et al.*, 2003; Gramaje and Armengol, 2011; Whitelaw-Weckert *et al.*, 2012).

Corresponding author: H. Waite
Fax: +61269332940
E-mail: hwaite@csu.edu.au

ISSN (print): 0031-9465
ISSN (online): 1593-2095

The large scale vine failures that occur soon after planting have been attributed to infection by trunk disease pathogens (Rego *et al*., 2000; Sidoti *et al*., 2000; Gramaje and Armengol, 2011), pointing to propagation as a potential source of infection in young vines (Whitelaw-Weckert *et al*., 2012). There is indirect evidence that contaminating microorganisms enter the propagation chain in (Halleen *et al*., 2003; Whiteman *et al*., 2007), or on (Whiteman *et al*., 2004a), asymptomatic cuttings from infected mother vines and contaminate other cuttings via water used to soak cuttings during propagation (Rego *et al*., 2001; Edwards *et al*., 2007, 2011; Aroca *et al*., 2010).

In addition, *Rahnella aquatilis*, *Enterobacter intermedius* and *Trichosporon pullulans*, microorganisms that are not recognised grapevine pathogens but are associated with dust and faecal contamination of water, have been isolated from compromised vine tissues and have also been shown to interfere with graft healing and to inhibit shoot growth (Waite and Cole, 2006). Botryosphaeriacea spp. (Phillips, 2002) and *Phaeomoniella chlamydospora* (Wallace *et al*., 2004) are also reported to interfere with graft healing.

Grapevine nurseries routinely soak many hundreds of cuttings together in water as a means of compensating for dehydration that occurs during cutting harvest and transportation to nurseries (Fourie and Halleen, 2006; Waite and Morton, 2007; Gramaje and Armengol, 2011). Soaking occurs for periods typically between 1 and 12 hours, though sometimes for longer, and is often performed more than once during the propagation cycle.

Soaking is thought to facilitate the entry of epiphytes into cutting tissue through the wounds made at each end of the cuttings, disbudding wounds and graft unions (Whiteman *et al*., 2004b; Fourie and Halleen, 2006; Retief *et al*., 2006; West *et al*., 2010) and has been implicated in cross contamination by the trunk disease pathogens *Ca. luteo-olivacea*, *Pa. chlamydospora* and *Phaeoacremonium aleophilum* (Gramaje *et al*., 2009, 2011). Some of these organisms have been detected in nursery environments. *Pa. chlamydospora* DNA has been detected in untreated rainwater collected from the roofs of houses and sheds that is used in Australian nurseries in hydration tanks, hot water treatment tanks and post HWT cool down tanks (Edwards *et al*., 2007) and in pre storage fungicide/rehydration tanks and pre grafting hydration tanks in New Zealand nurseries (Whiteman *et al*., 2004b). Retief *et al*. (2006) also detected *Pa. chlamydospora* in a high percentage of water samples collected after pre-storage hydration (40%) and during grafting (67%) in South African commercial nurseries. *Phaeoacremonium* spp. were also detected using molecular techniques in post-storage hydration tanks in Spanish nurseries (Aroca *et al*., 2010) and species of Botryosphaeriaceae and Petri disease pathogens (*Pa. chlamydospora* and *Phaeoacremonium* spp.) were detected by PCR on the surface of grafted varieties and rootstocks and in hydration and callusing baths in French nurseries (Vigues *et al*., 2009). In Italy, Pollastro *et al*. (2009) detected *Pa. chlamydospora* DNA in 28% of pre-grafting and 23% of pre-callusing hydration tanks. Viable propagules of *Ca. luteo-olivacea* were also obtained from hydration tanks in Spanish nurseries by filtering the water samples and culturing the filtrate on appropriate media (Gramaje *et al*., 2011).

Some nurseries use fungicides in the soaking water, but their efficacy cannot always be relied upon (Fourie and Halleen, 2004), particularly if multiple batches of cuttings are soaked in the same fungicidal dip. Apart from *Pa. chlamydospora* (Pascoe and Cottral, 2000) and Botryosphaeriaceae spp. (Billones-Baaijens *et al*., 2013) many more micro-organisms including bacteria are also found on the surfaces of grapevines (Munkvold and Marois, 1993). The potential for endophytic microorganisms to suppress pathogens in grapevine wood tissue has been raised by several authors (Martini *et al*., 2009; Gonzáles and Tello, 2010), but knowledge of the influence of endophytes on the development of diseases is incomplete (Crous *et al*., 2001; Gonzáles and Tello, 2010) and previously unidentified microorganisms might be opportunistic pathogens when accidentally introduced into the tissues of vines when cuttings are soaked. Although it is probable that the cuttings themselves are a major source of microbial contaminants identified in soaking water, particularly if potable water is used, this has not been tested.

To determine the capacity of epiphytic microorganisms to contaminate soaking water and thus the wood of cuttings, we investigated the rate of movement of microorganisms into soaking water and the woody tissue of cuttings at different soaking intervals. We also investigated the ability of microorganisms to form bio-films on surfaces of soaking vessels and thus present a potential contamination threat to subsequent batches of cuttings; an issue raised by the nursery supplying the cuttings for the first experiment.

Materials and Methods

Microbial contaminants in soaking water and woody tissue

Healthy, dormant cuttings (approximately 8 mm diameter) of *V. vinifera* cv. Sunmuscat and rootstock 140 Ruggeri from the Sunraysia district of the Murray Valley were obtained from a New South Wales vine nursery in September 2010. The cuttings were held in cold storage at 2–3°C at the nursery between harvesting in early winter (June) and shipping in September. Cuttings were trimmed to size (300 mm, approximately 19 g) with fresh cuts 10 mm above the top bud and 5 mm below the bottom bud. The freshly trimmed cuttings were randomly divided between 3 replicates of 6 soaking treatments (0, 1, 2, 4, 8 and 16 h) per variety with 3 cuttings per replicate. Cuttings were completely submerged in sterile (autoclaved at 131°C for 30 min at 2770 mbar) deionised water to a depth of 30 mm (0.5 L/cutting as per industry standard) at ambient room temperature (18°C) in sterile plastic containers (260 × 180 × 90 mm) with loosely fitting lids. Sterile deionised water was used in these experiments to allow the assays to be standardized as domestic supply water varies widely in pH and the ions present over time and between regions (Batarseh, 2006).

A 50 mL sample of water was taken from each treatment replicate using sterile plastic centrifuge tubes (30 mm diameter) after agitation with a sterile spatula to ensure the water was well mixed and all levels of water were sampled. Immediately prior to sub-sampling the 50 mL aliquots were vigorously inverted several times and vortexed for 20 seconds to ensure even mixing. After a preliminary trial to determine appropriate dilutions, three replicate 30 µL aliquots of undiluted sample water and sample water diluted to 10^{-1} in phosphate-buffered saline (PBS) were spread plated onto selective media for fungi [Dichloran Rose Bengal Chloramphenicol (DRBC) agar, Oxoid], bacteria [Nutrient agar (Oxoid) with 30 ppm benomyl (NA-B)] and Pseudomonas agar (Oxoid) for *Pseudomonas* spp., and the non selective fungal medium potato dextrose agar (PDA, Oxoid) and incubated in darkness at 25°C for 3 days and the number of colony forming units (cfu) counted.

Following soaking treatments, the cuttings were debarked and surface sterilized by flaming briefly with ethanol (70%). The base of each cutting was trimmed by 3 mm and three contiguous 3 mm thick cross sections taken from the base of each cutting and incubated in darkness on PDA at 25°C. After 3 days incubation, the numbers of fungal and bacterial colonies emerging from the wood were counted. The cfu g^{-1} (Cg) was calculated using equation 1:

$$Cg = \frac{Cc}{S} \quad (1)$$

where Cc is the number of cfus per slice and S (g) is the weight of the slice.

Formation of biofilms

Arising from the comments of the nursery that supplied the cuttings for the first experiment, a second experiment was performed the following season (2011) to determine the capacity of fungal and bacterial epiphytes to form bio-films on the surfaces of soaking vessels and thus become an additional source of contamination for subsequent batches of cuttings soaked in the same container. Australian nurseries use a variety of large vessels for soaking cuttings, usually plastic, stainless steel or galvanised steel all of which can be colonised by microorganisms (Percival *et al*., 1998; Webb *et al*., 2000; Ilhan-Sungur and Çotuk, 2010) that can be very difficult to remove from container surfaces (Batté *et al*., 2003).

For this experiment, cuttings of *V. vinifera* cv. Shiraz were obtained in late June from the Charles Sturt University vineyard at Wagga Wagga, NSW and held for 3 months in cold storage at 2–3°C in ventilated plastic bags with damp paper to prevent dehydration. Cuttings were removed from cold storage, allowed to come to ambient temperature, trimmed to size (300 mm) with fresh cuts 10 mm above the top bud and 5 mm below the bottom bud and randomly divided between 5 soaking treatments (1, 2, 4, 8 and 24 h) per variety with 3 replicates of 3 cuttings per replicate. The cuttings were soaked in sterile deionised water (0.5 L/cutting as per industry standard) in sterile plastic containers with loosely fitting lids.

Following the method used in experiment 1, a 50 mL sample of water was taken from each treatment replicate after stirring and diluted to 10^{-1} in phosphate-buffered saline (PBS). Three replicate 30 µL aliquots of each diluted sample were spread plated onto selective media for fungi (DRBC) and bacteria (NA-B) and incubated as above before colonies were counted.

Numbers of microorganisms on the inside surfaces of the containers were sampled by draining the

water from the containers and swabbing a 25 cm^2 area with a sterile cotton swab wetted in 10 mL sterile PBS. The swabs were then aseptically returned to the PBS, agitated vigorously and three replicate 30 μL aliquots spread onto selective media for fungi (DRBC) and bacteria (NA-B) and incubated as above and colonies counted.

Statistical analyses

Culturable microbial concentrations from water samples, container surface swabs and colonies emerging from wood samples were subjected to analysis of variance (ANOVA) test and least significant differences test by Genstat for Windows, 15th Edition.

Results

Microorganisms in soaking water and woody tissue

In this experiment the sterile soaking water of both varieties (Sunmuscat and 140 Ruggeri) became contaminated with *Pseudomonas* bacteria, NA-B bacteria, DRBC fungi and PDA fungi and bacteria within the first hour of soaking (Table 1). Microbial populations in the soaking water of Sunmuscat increased significantly ($P<0.05$) over the second hour except in populations culturable on NA-B where the increase was not significant. Thereafter populations generally decreased until climbing again in the time between 8 h and 16 h, although the later increases were significant ($P<0.05$) only for NA-B (bacteria) and DRBC (fungi). In the case of 140 Ruggeri the pattern was somewhat different. The number of microorganisms in soaking water increased in the second hour, but only significantly ($P<0.05$) in populations culturable on DRBC (fungi) and PDA (mixed populations). Numbers reached a maximum after 4 h of soaking in populations culturable on DRBC (fungi) and PDA (mixed populations) and after 8 h of soaking in populations culturable on PS (Pseudomonads) and NA-B (bacteria). After peaking, there were no significant differences over the time of the experiment in all populations except fungi cultured on DRBC where there was a significant ($P<0.05$) drop in numbers before beginning to increase again between 8 and 16 h (Table 1).

The culturable fungal populations in wood samples of both varieties also increased significantly ($P<0.05$) during the first hour of soaking, but then remained

Table 1. Colony forming units (cfu) of bacteria and fungi isolated from soaking water of 140 Ruggeri and Sunmuscat cuttings.

Culture media	Soaking time (hours)[a]				
	1 10^4 cfu mL^{-1}	2 10^4 cfu mL^{-1}	4 10^4 cfu mL^{-1}	8 10^4 cfu mL^{-1}	16 10^4 cfu mL^{-1}
140 Ruggeri					
PS	0.57 ± 0.49	3.34 ± 1.80	6.73 ± 3.80	7.79 ± 4.58	4.11 ± 1.38
NA-B	0.95 ± 0.78	3.66 ± 1.56	6.50 ± 4.17	10.78 ± 8.56	6.21 ± 2.98
DRBC	0.04 ± 0.01 b	5.84 ±3.19 a	6.02 ± 0.53 a	1.12 ± 0.59 b	2.01 ± 1.18 b
PDA	0.50 ± 0.40 c	3.90 ± 2.68 b	7.51 ± 1.34 a	4.49 ± 0.27 ab	6.13 ± 2.14 ab
Sunmuscat					
PS	1.20 ± 0.24 c	10.59 ± 1.55 a	3.11 ± 0.62 b	1.76 ±0.28 bc	2.90 ± 0.45 b
NA-B	1.18 ± 0.20 b	17.90 ± 8.75 ab	5.11 ± 1.06 b	4.09 ± 0.55 b	13.69 ± 2.27 a
DRBC	0.74 ± 0.25 b	5.46 ± 1.45 a	1.83 ± 0.78 b	2.30 ± 1.67 b	7.14 ± 2.71 a
PDA	2.26 ± 0.34 c	14.02 ± 6.52 a	5.77 ± 0.47 bc	7.74 ± 1.14 b	10.15 ± 3.55 ab

[a] For data in a single row, values followed by the same letter are not significantly different ($P<0.05$).

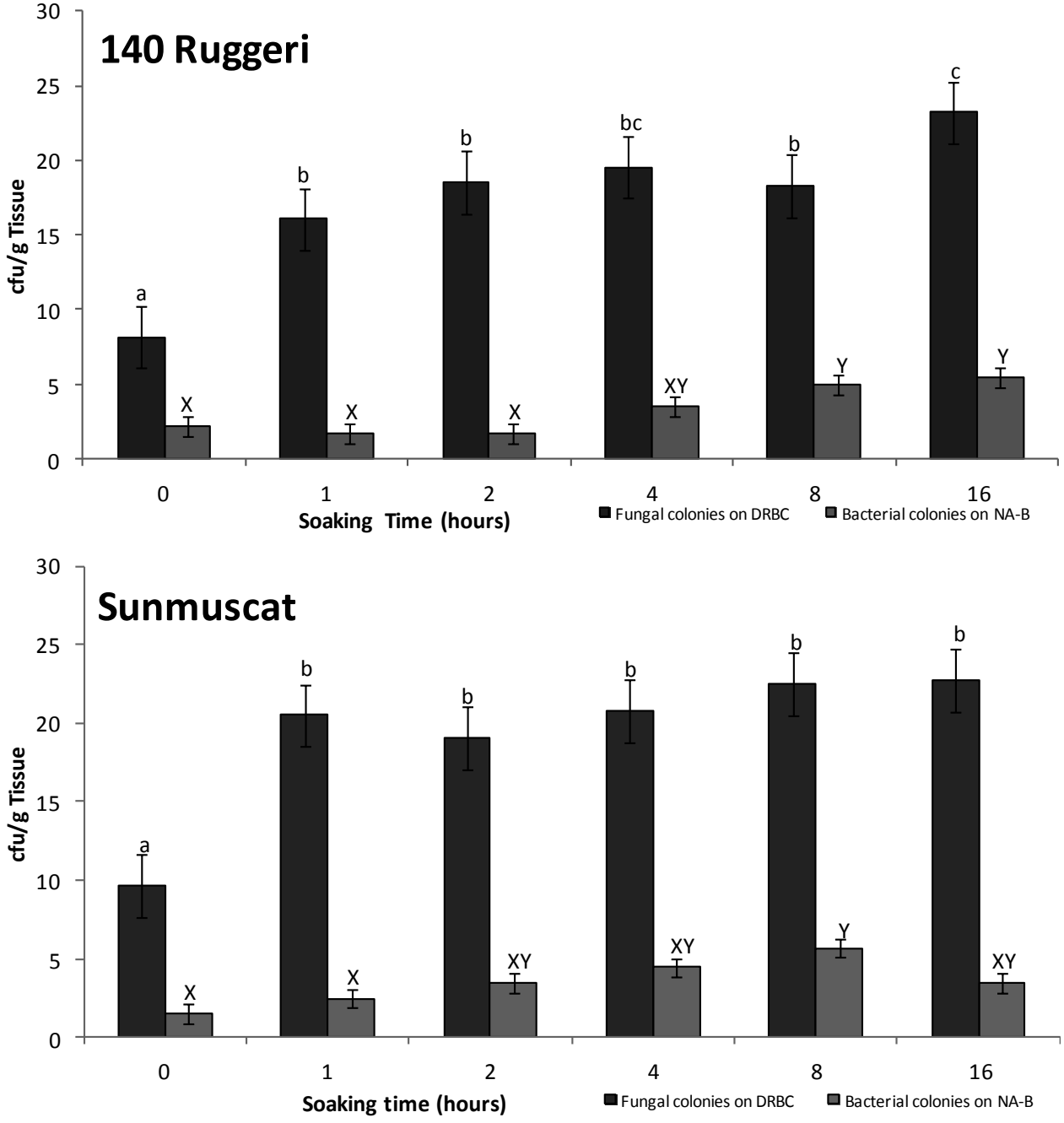

Figure 1. Fungi and bacteria isolated from rootstock 140 Ruggeri and *V. vinifera* cv. Sunmuscat after different periods of soaking. Vertical bars are the standard error of the means. Letters are for comparison of means of fungal colonies (a, b) and bacterial colonies (X, Y). Bars with a different letter are significantly different according to the Student's Least Significant Difference (P<0.05).

largely static for the duration of the experiment, although there was a significant (P<0.05) increase in fungal colonies in the 140 Ruggeri after 16 h (Figure 1).

There were also increases in the number of bacterial colonies isolated from the wood of both varieties, but the increases were not significant until the cuttings had been soaked for 8 h (Figure 1).

Although the titre of all microorganisms in the woody tissue of both 140 Ruggeri and Sunmuscat increased over the time of the experiment, the rate at which the epiphytic microorganisms on the bark of cuttings moved into the soaking water and thus the woody tissue of soaked cuttings varied with variety and type of microorganism. It is interesting to note that despite the high titre of culturable bacteria (NA-B) in the soaking water of both varieties compared to the number of culturable fungi (DRBC) (Table 1), the numbers of fungi isolated from the wood of Sunmuscat and 140 Ruggeri were significantly (P<0.05) greater than the numbers of bacterial isolated from the wood of both varieties (Figure 1). The rate of increase in the titre of bacteria isolated from the wood of both varieties was also slower than the rate of increase in the titre of fungi in the wood.

Microorganisms on container surfaces

In this experiment, only the NA-B bacterial count in the Shiraz soaking water increased significantly (P<0.05) beyond the initial contamination that occurred in the first hour of soaking (Table 2). Bacterial and fungal populations became established on the surface of the soaking container within the first hour of the commencement of soaking, followed by non-significant increases for fungi (P=0.173) and NA-B bacteria (P=0.053) over the time of the experiment (Table 3). These upward trends indicate that the increases in the populations, particularly of bacteria, are likely to have become significant if the experiment had continued for longer.

Discussion

All healthy plants, including grapevines, are colonised by a diverse range of bacteria, fungi and yeasts that inhabit both the phylloplane and the internal tissues (Bell *et al*., 1995; Halleen *et al*., 2003; West *et al*., 2010). These epiphyte and endophyte communities vary between plant species and with environmen-

Table 2. Colony forming units (cfu) of bacteria and fungi isolated from soaking water of Shiraz cuttings.

Culture medium	Soaking time (hours)				
	1 10^4 cfu mL^{-1}	2 10^4 cfu mL^{-1}	4 10^4 cfu mL^{-1}	8 10^4 cfu mL^{-1}	24 10^4 cfu mL^{-1}
NA-B	3.66 ± 2.40 c	10.51 ± 4.96 b	7.82 ± 2.20 bc	9.52 ±3.46 bc	19.65 ± 4.65 a
DRBC	0.26 ± 0.12	0.40 ± 0.12	0.36 ± 0.16	0.50 ± 0.25	0.32 ± 0.13

[a,b] For data in a single row, values followed by the same letter are not significantly different (P<0.05).

Table 3. Colony forming units (cfu) of bacteria and fungi recovered from the surfaces of containers used for soaking Shiraz cuttings.

Culture medium	Soaking time (hours)				
	1 10^2 cfu cm^{-2}	2 10^2 cfu cm^{-2}	4 10^2 cfu cm^{-2}	8 10^2 cfu cm^{-2}	24 10^2 cfu cm^{-2}
NA-B	15.64 ± 10.56	8.57 ± 4.94	9.07 ± 5.16	19.06 ± 13.31	68.70 ± 50.36
DRBC	1.60 ± 0.35	1.68 ± 1.20	2.27 ± 1.01	2.00 ± 1.62	5.91 ± 4.45

tal conditions (Quadt-Hallmann *et. al*., 1997; Yang *et al*., 2001; Compant *et al*., 2005b). Endophytes normally gain entry through roots, and the aerial parts of plants including stomata and flowers (Sette *et al*., 2006) and although some are pathogenic, many form complex symbiotic relationships with the host plants (Compant *et al*., 2005a; Rodriguez *et al*., 2009). In the case of grapevine propagation, the DNA and propagules of known grapevine pathogens have been isolated from water used to soak cuttings at various stages of the propagation process (Whiteman *et al*., 2004a; Retief *et al*., 2006; Edwards *et al*., 2007; Aroca *et al*., 2010), potentially causing cross contamination of entire batches of cuttings by exposing the wounds necessarily made during propagation to the pathogens in the water (Waite and Morton, 2007).

We have now established a direct link between soaking grapevine cuttings in water at the beginning of the propagation chain and immediate increases in the titre of microorganisms in the bases of cuttings and demonstrated that contamination of cuttings occurs after relatively short periods of soaking, thus confirming that soaking cuttings is a cause of cross contamination during propagation.

A number of other interesting observations arising from this work highlight the complex and dynamic nature of microbial epiphyte and endophyte populations (Ramey *et al*., 2004; Whipps *et al*., 2008). Although numbers of culturable fungi isolated from the wood of Sunmuscat and 140 Ruggeri were significantly ($P<0.05$) greater than the numbers of culturable bacterial isolated from the wood of both varieties the bacterial numbers in the soaking water were generally greater than the fungal numbers ($P<0.05$). This was also true for the second experiment where numbers of bacteria in the soaking water were higher than the numbers of fungi (Table 2). The higher levels of wood colonization by fungi than bacteria may have been a reflection of lower suitability of the woody tissue as a substrate for bacteria, greater capacity of fungi to adhere to and colonize the wood of the cuttings, or antagonistic interactions between the two groups of microorganisms (Ramey *et al*., 2004). Suppressive interactions within and between fungal and bacterial endophyte populations are well known (Compant *et al*., 2005a; Sette *et al*., 2006; Berg, 2009; Rodriguez *et al*., 2009).

The factors causing the fluctuations in the size of soaking water microbial populations within and between sampling times are likely to have arisen from a number of sources including: differences in microbial populations between the cuttings used in each replicate; microbial settling due to gravity between the end of agitation and sample collection (Hornby, 1969); variations in solute concentrations due to substrates leaching from the cuttings into the soaking water; and changes in population dynamics resulting from interactions between the different groups within the microbial populations (Mian *et al*., 1997; Jordan *et al*., 1999; Yang *et al*., 2001; Whipps *et al*., 2008).

Culturable fungal concentrations in the Sunmuscat soaking water were generally greater than those of 140 Ruggeri. This may be a reflection of different inoculum loads in the source vineyards or the general variability in epiphytes that occurs between plant species (Whipps *et al*., 2008; Yang *et al*., 2001). Differences in spray regimes and variations in the suitability of the bark as a habitat may have caused these differences (Yang *et al*., 2001; Lindow and Brandl, 2003). Rootstock mother vines are not normally trained on trellises in the manner of *V. vinifera* scions, but are instead allowed to sprawl on the ground (Stamp, 2003; Hunter *et al*., 2004) and it is likely that the differences reflect different fungal populations.

It is possible that much of the epiphyte population could have consisted of fastidious and unculturable microflora that did not grow on the media used in this experiment (Yang *et al*., 2001). Large populations of unculturable epiphytic and endophytic microorganisms associated with grapevines have been revealed by molecular techniques (West *et al*., 2010) and the possibility of these organisms impacting on the results of this work cannot be excluded.

The significant decline in culturable fungal and bacterial populations in Sunmuscat soaking water after the early initial maxima might be explained by both adsorption to the container surfaces and the death of microbial populations due to the osmotic effects of the sterile water used as the soaking medium and/or the lack of suitable substrate to enable the population of microorganisms associated with this variety to persist (Straka and Stokes, 1957; Mian *et al*., 1997; Jordan *et al*., 1999). With the exception of DRBC culturable fungi, the microbial populations associated with 140 Ruggeri generally increased over time. Possible causes may be the increase in microbial substrate and reduced osmotic stress caused by leaching of soluble carbohydrates from the ends of the cuttings that is often observed by the author in rootstock varieties in nurseries following soaking. Lower levels

of suitable microbial substrates may explain the slow increase in the microorganisms in the water of the Shiraz cuttings in the second experiment (Straka and Stokes, 1957; Mian *et al*., 1997; Jordan *et al*., 1999).

There is a trend in nurseries to shorten soaking times to less than 8 h to avoid cross contamination, but the fungal concentrations in the water after soaking for 1 hour were sufficient to result in significant increases in endophytic fungi, demonstrating that enough fungi move into the water from soaked cuttings for cross contamination to occur earlier in the soaking cycle than is generally thought.

Most nurseries change the soaking water between batches, but microorganisms established on the container surfaces are a potential source of cross contamination for subsequent batches of cuttings. The upward, but not significant trend in bacterial and fungal populations on container surfaces over the 24 h of this experiment is typical of the colonization patterns that are reported by authors investigating the establishment and growth of biofilms in other situations. Hood and Zottola (1997) reported that after initial colonization of stainless steel surfaces in the first hour of exposure, there was little increase in colonization over time (0–80 h). The authors noted that the adherence of microorganisms to stainless steel surfaces over time varies between species and is influenced by factors such as the growth stage of the organism, substrate availability and other substances, including proteins in the liquid environment. The upward trend in biofilm populations in this experiment is in contrast to the fluctuating populations in the water (Tables 1 and 2). However, biofilm species represent only a subset of the species in the liquid matrix (Costerton *et al*., 1994; Tait and Sutherland, 2002) and the characteristics and behaviour of microorganisms in an established biofilm are independent of the same species in the liquid matrix. Biofilm populations interact differently and are more stable than the population of origin (Costerton *et al*., 1994; Hansen *et al*., 2007).

The slow colonization of the container surfaces reported here may also indicate that plastic is a relatively poor surface for colonization by microorganisms compared to the bark of the cuttings. Factors such as the competition for space between the colonizing microorganisms, the lack of suitable substrate in the water and the osmotic stress imposed by using distilled deionised water may have also affected the rate of colonization (Webb *et al*., 2000; Chmielewski and Frank, 2003). However, microorganisms are particularly difficult to dislodge from container surfaces with common biocides (Costerton *et al*., 1994; Batté *et al*., 2003; Harding *et al*., 2009) and are therefore potential source of cross contamination in nurseries where successive batches of cuttings or vines are soaked in the same container. This is particularly likely to be the case if soaking time is prolonged (Waite and Morton, 2007; Aroca *et al*., 2010) and the level of substrates increases (Hood and Zottola, 1997; Chmielewski and Frank, 2003), containers are not thoroughly cleaned between batches and the soaking water is not treated with an active biocide. Commonly used fungicides tested in the vine nursery context can reduce inoculum of known pathogens, particularly *Pa. chlamydospora* and *P. aleophilum* in soaking water (Fourie and Halleen, 2006) and cuttings (Retief *et al*., 2006; Gramaje *et al*., 2009; Rego *et al*., 2009) but, these and other general purpose biocides have not been evaluated for the specific purpose of cleaning soaking containers.

The results of this study support the growing body of evidence that soaking water is a source of cross contamination and trunk disease transmission during propagation (Whiteman *et al*., 2004b; Retief *et al*., 2006; Edwards *et al*., 2007; Aroca *et al*., 2010). If untreated water is used there is also potential for other microorganisms that are not known grapevine pathogens, to enter the cutting tissue during soaking and impede shoot growth (Waite and Cole, 2006).

Although fungicides and general biocides used in nurseries have been evaluated against a number of known trunk disease pathogens (Groenewald *et al*., 2000; Jaspers, 2001; Fourie and Halleen, 2006; Gramaje *et al*., 2009; Rego *et al*., 2009), the research on biocides in the nursery context has been outpaced by the identification of pathogens implicated in trunk diseases. Therefore, development of nursery management strategies that avoid exposing cuttings to water during propagation is an essential first step in reducing the transmission of trunk diseases in propagation.

Acknowledgements

Financial support by the 'Programa de Apoyo a la Investigación y Desarrollo (PAID-00-10) de la Universidad Politécnica de Valencia' for David Gramaje during his three months in Australia is gratefully acknowledged.

Financial support by Charles Sturt University and the Grape and Wine Research and Development

Corporation for Helen Waite is also gratefully acknowledged.

Literature cited

Aroca A., D. Gramaje, J. Armengol, José García-Jiménez and R. Raposo, 2010. Evaluation of the grapevine nursery propagation process as a source of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* and occurrence of trunk disease pathogens in rootstock mother vines in Spain. *European Journal of Plant Pathology* 126, 165–174.

Batarseh M.I., 2006. The quality of potable water types in Jordan. *Environmental Monitoring and Assessment* 117, 235–244.

Batté M., B.M.R. Appenzeller, D. Grandjean, S. Fass, V. Gauthier, F. Jorand, L. Mathieu M. Boualam, S. Saby and J.C. Block, 2003. Biofilms in drinking water distribution systems. *Reviews in Environmental Science and Bio/Technology* 2, 147–168.

Bell C.R., G.A. Dickie, W.L.G. Harvey and J.W.Y.F. Chan, 1995. Endophytic bacteria in grapevine. *Canadian journal of Microbiology* 41, 46–53.

Berg G., 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology* 84, 11–18.

Billones-Baaijens R., H.J. Ridgway, E.E. Jones and M.V. Jaspers, 2013. Inoculum sources of *Bortyosphaeriaceae* species in New Zealand grapevine nurseries. *European Journal of Plant Pathology* 135, 159–174.

Chmielewski R.A.N. and J.F. Frank, 2003. Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety* 2, 22–32.

Compant S., B. Duffy, J. Nowak, C. Clément and E. Ait Barka, 2005a. Use of plant-growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* 71, 4951–4959.

Compant S., B. Reiter, A. Sessitsch, J. Nowak, C. Clement and E. Ait Barka, 2005b. Endophytic colonization of *Vitis vinifera* l. by plant growth promoting bacterium *Burkholderia* sp.strain PsJN. *Applied and Environmental Microbiology* 71, 1685–1693.

Costerton J.W., Z. Lewandowski, D. deBeer, D. Caldwell, D. Korber and G. James, 1994. Biofilms, the customized microniche. *Journal of Bacteriology* 176, 2137–2142.

Crous P.W., L. Swart and S. Coertze, 2001. The effect of hot-water treatment on fungi occurring in apparently healthy grapevine cuttings. *Phytopathologia Mediterranea* 40, S464–S466.

Edwards J., F. Constable, T. Wiechel and S. Salib, 2007. Comparison of the molecular tests-single PCR, nested PCR and quantitative PCR (SYBR®Green and TaqMan®) – for detection of *Phaeomoniella chlamydospora* during grapevine nursery propagation. *Phytopathologia Mediterranea* 46, 58–72.

Fourie P.H. and F. Halleen, 2004. Proactive control of Petri disease through treatment of propagating material. *Plant Disease* 88, 1241–1245.

Fourie P.H. and F. Halleen, 2006. Chemical and biological protection of grapevine propagation material from trunk disease pathogens. *European Journal of Plant Pathology* 116, 255–265.

Gonzáles V and M.L. Tello, 2010. The endophyte mycota associated with *Vitis vinifera* in central Spain. *Fungal Diversity* 497, 29–42.

Gramaje D. and J.Armengol, 2011. Fungal trunk pathogens in the grapevine propagation process: potential inoculum sources, detection, identification, and management strategies. *Plant Disease* 95, 1040–1055.

Gramaje D., A. Aroca, R. Raposo, J. García-Jiménez and J. Armengol, 2009. Evaluation of fungicides to control Petri disease pathogens in the grapevine propagation process. *Crop Protection* 28, 1091–1097.

Gramaje D, L. Mostert and J. Armengol, 2011. Characterization of *Cadophora luteo-olivacea* and *C. melinii* isolates obtained from grapevines and environmental samples from grapevine nurseries in Spain. *Phytopathologia Mediterranea* 50, S112–S126.

Groenewald M., S. Denman and P.W. Crous, 2000. Fungicide sensitivity of *Phaeomoniella chlamydospora*, the causal organism of Petri grapevine decline. *South African Journal of Enology and Viticulture* 21, 59–61.

Halleen F., P.W. Crous and O. Petrini, 2003. Fungi associated with healthy grapevine cuttings in nurseries, with special reference to pathogens involved in the decline of young vines. *Australasian Plant Pathology* 32, 47–52.

Hansen S.K., P.B. Rainey, J.A.J. Haagensen and S. Molin, 2007. Evolution of species interactions in a biofilm community. *Nature* 445, 533–536.

Harding M.W., L.L.R. Marques, R.J. Howard and M.E. Olson, 2009. Can filamentous fungi form biofilms? *Trends in Microbiology* 17 (11), 475–480.

Hood S.K. and E.A. Zottola, 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *International Journal of Food Microbiology* 37, 145–153.

Hornby D., 1969. Gravimetrical and mycological investigations of soil suspensions in the soil dilution plate technique. *Journal of Applied Bacteriology* 32, 244–258.

Hunter J. J., C. G. Volschenk, D. J. Le Roux, G. W. Fouché and L. Adams., 2004. *Plant Material Quality, a compilation of research*. Research Reports, ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa.

Ilhan-Sungur E. and A. Çotuk, 2010. Microbial corrosion of galvanized stainless steel in a simulated recirculating cooling tower system. *Corrosion Science* 52, 161–171.

Jaspers M.V., 2001. Effect of fungicides, *in vitro*, on germination and growth of *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 40, S453–S458.

Jordan K.N., S. Hall and P.J. McClure, 1999. Osmotic stress on dilution of acid injured *Escherichia coli* 0157:H7. *Letters in Applied Microbiology* 28, 389–393.

Lindow S.E. and M.T. Brandl, 2003. Microbiology of the phyllosphere. *Applied Environmental Microbiology* 69, 1875–1883.

Martini M., R. Musetti, S. Grisan, R. Polizzotto, F. Pavan and R. Oster, 2009. DNA-dependent detection of the grapevine fungal epiphytes *Aureobasidium pullulans* and *Epicoccum nigrum*. *Plant Disease* 93, 993–998.

Mian M.A., G.H. Fleet and A.D. Hocking, 1997. Effect of diluent type on viability of yeasts enumerated from foods or pure culture. *International Journal of Food Microbiology* 35, 103–107.

Morton L., 2000. Viticulture and grapevine declines: lessons of black goo. *Phytopathologia Mediterranea* 39, 59–67.

Munkvold G.P. and J.J. Marois, 1993. Efficacy of natural epiphytes and colonizers of grapevine pruning wounds for biological control of eutypa dieback. *Phytopathology* 83(6), 624–629.

Pascoe I. and E. Cottral, 2000. Developments in grapevine trunk diseases research in Australia. *Phytopathologia Mediterranea* 39, 68–75.

Percival S.L., J.S. Knapp, R.G.J. Edyvean and D.S. Wales, 1998. Biofilms, mains water and stainless steel. *Water Resources* 32, 2187–2201.

Phillips A.J.L., 2002. *Botryosphaeria* species associated with diseases of grapevines in Portugal. *Phytopathologia Mediterranea* 41, 3–18.

Pollastro S., W. Habib, A. Pichierri, N. Masiello and F. Faretra, 2009. Potential sources of *Phaeomoniella chlamydospora* inoculum in grapevine nurseries in southern Italy. *Phytopathologia Mediterranea* 48, 174.

Quadt-Hallmann A., J. Hallmann and J.W. Kloepper, 1997. Bacterial endophytes in cotton: location and interaction with other plant-associated bacteria. *Canadian Journal of Microbiology* 43, 254–259.

Ramey B.E., M. Koutsoudis, S.B. von Bodman and C. Fuqua, 2004. Biofilm formation in plant-microbe associations. *Current Opinion in Microbiology* 7, 602–609.

Rego C., H. Oliveira, A. Carvalho and A. Phillips, 2000. Involvement of *Phaeoacremonium* spp. and *Cylindrocarpon destructans* with grapevine decline in Portugal. *Phytopathologia Mediterranea* 39, 76–79.

Rego C., A. Carvalho, T. Nascimento and H. Oliveira, 2001. First approach to understanding the inoculum sources of *Cylindrocarpon destructans* and *Phaeomoniella chlamydospora* concerning grapevine rootstocks in Portugal. *IOBC/WPRS Bulletin* 24, 67–72.

Rego C., T. Nascimento, A. Cabral, M.J. Silva and H. Oliveira 2009. Control of grapevine wood fungi in commercial nurseries. *Phytopathologia Mediterranea* 48, 128–135.

Retief E., A. McLeod and P.H. Fourie, 2006. Potential inoculum sources of *Phaeomoniella chlamydospora* in South African grapevine nurseries. *European Journal of Plant Pathology* 115, 331–339.

Rodriguez R.J., J.F. White Jr., A.E. Arnold and R.S. Redman, 2009. Fungal endophytes: diversity and functional roles. *Tansley Review, New Phytologist* 1–17.

Sette L.D., M.R.Z. Passarini, C. Delarmelina, F. Salati and M.C.T. Duarte, 2006. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. *World Journal of Microbiology and Biotechnology* 22, 1185–1195.

Sidoti A., E. Buonocore, T. Serges and L. Mugnai, 2000. Decline of young grapevines associated with *Phaeoacremonium chlamydosporum* in Sicily (Italy). *Phytopathologia Mediterranea* 39, 87–91.

Stamp J., 2001. The contribution of imperfections in nursery stock to the decline to the decline of young vines in California. *Phytopathologia Mediterranea* 40, S369–S375.

Stamp J. A., 2003. Pathogenic Status of High Quality Grapevine Nursery Stock. *Wine Business Monthly* 10(2), 30–35.

Straka R.P. and J.L. Stokes, 1957. Rapid destruction of bacterial in commonly used diluents and its elimination. *Applied Microbiology* 5, 21–25.

Tait K. and I.W. Sutherland, 2002. Antagonistic interactions amongst bacteriocin-producing enteric bacteria in dual species biofilms. *Journal of Applied Microbiology* 93, 345–352.

Vigues, V., O. Yobregat, B. Barthélémy, F. Dias, M. Coarer and P. Larignon, 2009. Fungi associated with wood decay diseases: Identification of the steps involving risk in French nursery. *Phytopathologia Mediterranea* 48, 177–178.

Waite H. and M. Cole, 2006. *An investigation of the role of endogenous and exogenous bacteria in grapevine propagation and development*. Project MU01/01 Report, Grape and Wine Development Corporation.

Waite H. and L. Morton, 2007. Hot water treatment, trunk diseases and other critical factors in the production of high-quality grapevine planting material. *Phytopathologia Mediterranea* 46, 5–17.

Wallace J., J. Edwards, I.G. Pascoe and P. May, 2004. *Phaeomoniella chlamydospora* inhibits callus formation by grapevine rootstock and scion cultivars. *Phytopathologia Mediterranea* 43, 151–152.

Webb J.S., M. Nixon, I.M. Eastwood, M. Greenhalgh, G.D. Robson and P.S. Handley, 2000. Fungal colonization and biodeterioration of plasticized polyvinyl chloride. *Applied Environmental Microbiology* 6, 3194–3200.

West E.R., E.J. Cother, C.C. Steel and G.J. Ash, 2010. The characterization and diversity of bacterial endophytes of grapevine. *Canadian Journal of Microbiology* 56, 209–216.

Whipps J.M., P. Hand, D. Pink and G.D. Bending, 2008. Phyllosphere microbiology with special reference to diversity and plant genotype. *Journal of Applied Microbiology* 105, 1744–1755.

Whitelaw-Weckert MA, L. Rahman, L.M. Appleby, A. Hall, A.C. Clark, W.J. Hardie H. Waite, 2012. Young Vine Decline in the Riverina is caused by co-infection by *Botryosphaeri*a and *Ilyonectria* at different propagation stages. *Phytopathologia Mediterranea* 51(2), 442.

Whiteman S. A., M.V. Jaspers, A. Stewart and H. J. Ridgway, 2004a. Identification of potential sources of *Phaeomoniella chlamydospora* in the grapevine propagation process. *Phytopathologia Mediterranea* 43(1), 152–153.

Whiteman S. A., M.V. Jaspers, A. Stewart and H. J. Ridgway, 2004b. *Phaeomoniella chlamydospora* detection in the grapevine propagation process by species-specific PCR. *Phytopathologia Mediterranea* 43, 156.

Whiteman S.A., A. Stewart, H.J. Ridgway and M.V. Jaspers, 2007. Infection of rootstock mother-vines by *Phaeomoniella chlamydospora* results in infected young grapevines. *Australasian Plant Pathology* 36, 198–203.

Yang C., D.E. Crowley, J. Borneman and N.T. Keen, 2001. Microbial phyllosphere populations are more complex than previously realized. *PNAS* 98, 3889–3894.

Accepted for publication: March 15, 2013