

Overwintering and presence of *Colletotrichum acutatum* (ripe rot) on mummified bunches, dormant wood, developing tissues and mature berries of *Vitis vinifera*

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Summary

Colletotrichum acutatum, the casual agent of ripe rot in tropical and sub-tropical grape growing regions of Australia, affects crop yield and wine quality. To elucidate the epidemiology of the fungus, its presence was studied on shoots, pea sized and mature berries, mummified bunches, spurs and canes during two consecutive growing seasons and one winter season. The presence of *C. acutatum* was analysed by both traditional and real-time PCR methods. A protocol was developed to achieve PCR amplification for fungal DNA extracted from 'difficult' wood samples. *C. acutatum* was detected on mature berries, mummified berries, the peduncles of mummified berries, winter spurs and canes but not on the remaining analysed tissues. Thus the fungus overwinters on *Vitis vinifera* plant tissues and the conidia on spurs and dormant wood are likely to be the most important source of primary inoculum for the next growing season.

Key words: Ripe rot, overwintering, real-time PCR.

Introduction

Colletotrichum acutatum (J.H. Simmonds ex J.H. Simmonds) syn. *C. simmondsii* (SHIVAS and TAN 2009), a filamentous fungus with worldwide importance, develops on a broad range of host plants (FREEMAN *et al.* 1998, HYDE *et al.* 2009). First described by SIMMONDS (1965) as a fruit-rot pathogen in Australia, the fungus causes ripe rot of grapes, and is characterised by the production of orange conidial masses. The disease occurs predominantly in warm, wet and humid sub-tropical climates. The disease has been reported on muscadine grapes (*Muscadinia rotundifolia*) in south eastern USA (DAYKIN and Milholland 1984, KUMMUANG *et al.* 1996), wine grapes (*Vitis vinifera*) in coastal New South Wales (NSW) and south east Queensland, Australia (MELKSHAM *et al.* 2002, STEEL *et al.* 2007, WHITELAW-WECKERT *et al.* 2007), and table grapes in Japan (YAMAMOTO *et al.* 1999, SHIRAISHI *et al.* 2007).

Correct taxonomic identification of *Colletotrichum* spp. is essential for both research and disease management purposes. Traditional methods rely predominantly on morphological observations such as the shape and size of conidia, colony growth rate, colour, pigmentation on artificial media, and to a lesser extent fungicide sensitivity,

optimal growth temperature and vegetative compatibility. While traditional methods of taxonomic fungal identification are important, these techniques are time consuming. Results can be influenced by environmental factors and the way in which organisms are grown. Recently, various molecular approaches have been successfully used for differentiating species and phylogenetic relationships within one species (BROWN *et al.* 1996, WHITELAW-WECKERT *et al.* 2007, HYDE *et al.* 2009). Real-time PCR methods based on species-specific primers have gained popularity in this aspect as these methods are relatively simple and do not require isolation of the fungus from infected tissue and expert identification. However, the quantity of secondary metabolites such as polysaccharides, polyphenols and tannins are abundant in grape tissues (COLLINS and SYMONS 1992, LODHI *et al.* 1994, HANANIA *et al.* 2004, IANDOLINO *et al.* 2004). The amount of these substances, and particularly flavonoids, is enhanced during pathogen infection (WINKEL-SHIRLEY 2002). These compounds come into contact with nuclei during cell disruption which affects DNA purification, makes samples viscous, promotes DNA degradation, and hampers restrictability and PCR amplification (NEWBURY and Possingham 1977, HANANIA *et al.* 2004). Several protocols using hexadecyltrimethylammonium (CTAB), Trizol (Invitrogen), and others, have been developed to circumvent this problem (for a review, see HANANIA *et al.* 2004).

C. acutatum has been reported to overwinter on infected mummified bunches and spurs of the *Muscadinia rotundifolia* (syn. *V. rotundifolia*) cultivars 'Carlos', 'Magnolia', 'Sterling', 'Doreen' and 'Coward' with mummies on the vine acting as an important source of inoculum (DAYKIN and MOLHOLLAND 1984, KUMMUANG *et al.* 1996). Severe pruning during the dormant season is recommended to remove the mummified fruit and dead or damaged wood. There is little epidemiological information in the literature regarding overwintering of *C. acutatum* on *V. vinifera* (PEARSON and GOHEEN 1994) which hampers ripe rot control. The aim of this study was to use traditional and improved PCR-based approaches to detect the presence of *C. acutatum* from different tissues of the grapevine in order to better understand the epidemiology of the pathogen.

Material and Methods

Infection studies of *C. acutatum*: This study was conducted in a commercial vineyard situated in

the Hunter Valley, 200 km north of Sydney, NSW, Australia, elevation 56 m with a known history of ripe rot. Samples were collected from 20 year old, own rooted 'Chardonnay' vines, planted in 3.0 m wide rows, with 1.4 m distance between vines. Twenty bunches were collected randomly at maturity (22 °Brix) in the summer of 2009/2010 and 2010/2011 (Table). Ten berries were cut from each bunch and surface-sterilised with 1 % w/v hypochlorite (Biolab) and 0.05 % Tween 80 for 2 min, rinsed three times in sterile water and placed onto Dichloran Rose Bengal Chloramphenicol agar (DRBC, Oxoid Australia Pty Ltd). Subsequent fungal growth was transferred to potato-dextrose agar (PDA, Oxoid Australia Pty Ltd) and identified after 5-7 d of incubation at 25 °C. In the winter of 2010 (August in the Southern Hemisphere) 18 spurs, approximately 5-10 cm in length, were collected randomly from 18 vines; 33 mummified bunches and 33 canes (approximately 1 m long) were collected randomly from the ground in the middle of the rows (Table). Ten mummified berries per bunch and 10 peduncles were analysed as described for mature berries while canes and spurs were cut into 1 cm long sections, surface sterilised as described above and plated onto DRBC agar.

Additionally, 10 developing shoots (50-120 cm lengths) were collected randomly from 10 vines when the berries were pea size. For this study, the 5 cm section of shoot attached to the cane was considered as a spur at pea size berry stage. Each shoot had a bunch at pea size berry stage. Shoots, leaves, and tendrils were cut into 2.5-3 cm long sections and plated onto DRBC together with 10 berries per shoot. Results were expressed as percentage of infected samples where the pathogen was detected, e.g. occurrence on mummified, mature or pea-size bunches, canes, spurs, developing shoots, and leaves. Morphological identification was confirmed by real-time PCR (SAMUELIAN *et al.* 2011). From each shoot five samples were taken from leaves, five from stems, three from tendrils (2.5-3 cm long sections) and the material from each tissue was pooled for subsequent PCR

analyses; five berries per bunch were treated independently for DNA extraction and consequent PCR analyses.

Real-time PCR: Total genomic DNA was extracted from berry skins, spurs, canes, mummified berries and peduncles, and pea size berries without prior surface sterilisation. Skins were peeled from mature berries and excess pulp removed by quick touch to a lint free Kimwipe (Kimberly-Clark Professional). Skins were ground with a mortar and pestle, while the remaining samples were ground with a Grinding Jar Set (Qiagen) in a TissueLyser (Qiagen) at 20 hrz for 1 to 2 min to fine powder under liquid nitrogen. DNA was extracted with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. DNA extracted from woody tissue was purified a second time with the same kit in an attempt to reduce the presence of PCR inhibitors. Real-time PCR amplification was performed on a Corbett Rotor-Gene™6000 5-plex Thermocycler (Qiagen) using either the SYBR Green JumpStart Taq ReadyMix Kit (Sigma-Aldrich) or Quantitech SYBR Green PCR Kit (Qiagen). The CaITS_F701/CaITS_699 primer pair developed by DEBODE *et al.* (2009), and tested for *C. acutatum* on *V. vinifera* (SAMUELIAN *et al.* 2011), was used in this study to perform real-time PCR. Each PCR reaction was carried out in a 25 µL reaction mix containing 1 µL sample DNA (5-10 ng), 5 pmol of each primer and 1 x SYBR Green JumpStart Taq ReadyMix buffer or Quantitech SYBR Green PCR buffer. The final profile consisted of an initial denaturation for 2 min at 95 °C for the SYBR Green JumpStart Taq ReadyMix Kit or 15 min for the Quantitech SYBR Green PCR Kit. The following 40 cycles were identical for both kits: denaturation at 94 °C for 30 s, annealing temperature of 60 °C for 30 s, and extension for 60 s at 72 °C. Control reactions contained water instead of template DNA. All real-time PCR experiments were repeated twice and the data normalised as previously described (SAMUELIAN *et al.* 2011). Comparison of relative DNA concentrations was determined using the $2^{-\Delta(C_{T, target} - C_{T, subq10})}$ equation, where C is

Table

Detection and monitoring of *Colletotrichum acutatum* on *V. vinifera* tissues at different time points and phenological stages. Number of samples where *C. acutatum* was identified is presented in percentage

Time of collection	Growth stage ¹⁾	Plant tissue	<i>C. acutatum</i> detected	
			on DRBC ²⁾ (%)	by real-time PCR (%)
Summer 2009/2010	E-L 38, harvest, berries ripe	berries	50	70
Winter 2010	E-L 1, dormancy	mummified bunches (ground)	15	85
		dormant canes (ground)	15	85
		spurs (vine)	17	67
Summer 2010/2011	E-L 31, berries per size	developing shoots		
		- green tissue (excluding spurs)	n.d.	n.d.
		- berries	n.d.	n.d.
		- leaves	n.d.	n.d.
		- spurs	60	80
	E-L 38, harvest, berries ripe	berries	40	50

¹⁾ Grapevine growth stages are presented according to Coombe (1995). n.d. – not detected.

²⁾ DRBC – Dichloran Rose Bengal Chloramphenicol agar plates.

the threshold cycle that represents the PCR cycle at which the copy number passes the fixed first detected threshold (LIVAK and SCHMITTGEN 2001). Real-time PCR results were confirmed by visualisation of the amplicons on 1 % agarose gels stained with ethidium bromide.

Results and Discussion

Approximately 10 ng DNA was extracted per milligram of berry skins and 3 ng/mg from all remaining plant samples. Real-time PCR performed with the SYBR Green JumpStart Taq ReadyMix Kit provided approximately 35% higher intensity of relative signal levels ($C_{T,mean} = 21.18 \pm 1.88$) when analysing presence or absence of *C. acutatum* on berry skins than the Quantitech SYBR Green PCR Kit ($C_{T,mean} = 25.83 \pm 2.12$) based on 10 berries. However, when DNA from woody samples were analysed, amplification was achieved with the SYBR Green JumpStart Taq ReadyMix Kit for only one fifth of the samples where presence of the fungus was detected with the Quantitech SYBR Green PCR Kit (Figure). It might be speculated that failure to achieve amplification with the SYBR Green JumpStart Taq ReadyMix Kit was due to the presence of PCR inhibitors in the DNA samples. The use

of the DNeasy Plant Mini Kit to purify the DNA samples a second time did not lead to amplification of the samples where detection failed the first time. However, heating DNA extracted from "difficult" samples such as wood at 95 °C for 15 min appeared to degrade PCR inhibitors. When amplification products were visualised on an agarose gel, a single DNA band of approximately 585 bp was observed for all samples where *C. acutatum* was detected confirming the reliability of the real-time PCR procedure. Characteristic salmon-coloured spore masses are produced on ripe berries during the life cycle of *C. acutatum*. Plating of mature berries onto artificial media and subsequent fungal morphological identification revealed the presence of viable *C. acutatum* spores in 50 and 40 % of the analysed bunches during the summers of 2009/2010 and 2010/2011, respectively, while real-time PCR detected the pathogen in 70 % and 50 % of the bunches during the same period (Table). *C. acutatum* was revived from 15 % of mummified berries, peduncles and winter canes, 17 % of winter spurs, 60 % of spurs at pea size but not on the remaining parts of developing shoots when plated on DRBC. In contrast, real-time PCR detected *C. acutatum* in 85 % of mummified berries, peduncles and winter canes, 67 % of winter spurs and 80 % of spurs at pea size berry stage. This result is consistent with other findings

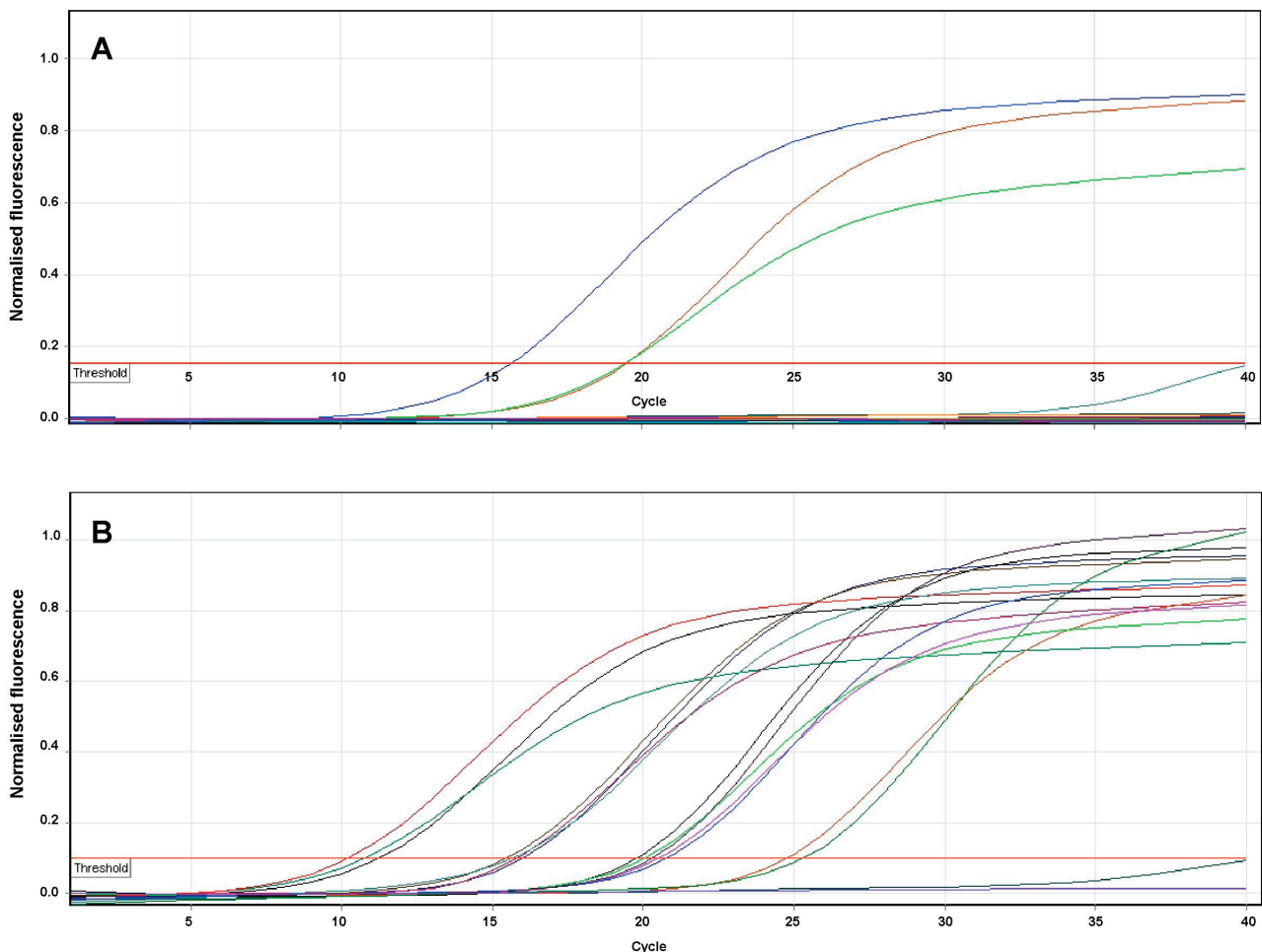


Figure: Detection of *Colletotrichum acutatum* in seven wood and seven rachis samples by real-time PCR using (A) the SYBR Green JumpStart Taq ReadyMix Kit and (B) the Quantitech SYBR Green PCR Kit. The same amounts of DNA and primers were used with both approaches.

(PARIKKA and LEMMETTY 2004, SAMUELIAN *et al.* 2011) as molecular biology techniques are also able to detect non-viable spores. This suggests that a large proportion of the spores do not survive during the winter period and that the remaining ones are sufficient to inoculate almost half of the bunches at berry maturity in the next growing season. *C. acutatum* has been reported to overwinter in infected strawberry crowns and fruit (FREEMAN *et al.* 2002) and on living leaves of Valencia orange (*Citrus sinensis*) (ZULFIQAR *et al.* 1996). On sweet cherry buds, it was observed often at high frequency (BØRVE and STENSVAND 2006); superficially or slightly internally on detached shoots and particularly on winter buds and wounds on the bark of mulberry tree (YOSHIDA and SHIRATA 1999); in dormant buds and twigs but not in dropped leaves or fruit mummies of apple fruit (CRUSIUS *et al.* 2002). The pathogen was recovered at a high rate on almond mummies in California (FØSTER and ADASKAVEG 1999), probably due to the protection provided by the mummified fruits. On blueberry bushes, *C. acutatum* survives the winter in or on blighted twigs, spent fruit trusses and live buds, as well as in other symptomless tissues (YOSHIDA *et al.* 2007). The fungus appears to prefer bud scales and bark as general overwintering sites in woody plants. Even though dormant plant tissues have been identified as the main source of inoculum for several tree species, the contribution of *C. acutatum* conidia in the soil to disease outbreaks should also be taken into consideration. Conidia have been found to survive for 9 months in soil attached to strawberry plants (FREEMAN *et al.* 2002). YOSHIDA and SHIRATA (1999) reported that conidia remain viable in the soil for up to 6 months while NORMAN and STEANDBERG (1997) reported that survival of conidia on leather leaf fern in diseased debris buried in the soil declined rapidly under moist conditions but some remained viable for up to 12 months. The role of the conidia that survive in the soil remains unknown for grapevines. To the best of our knowledge there are no studies published concerning the overwintering of *C. acutatum* on *V. vinifera*. Our attempts to isolate the pathogen from mummified berries, winter wood samples and spurs was successful for approximately 17 % of the samples investigated even though it was detected in significantly larger portion of the material by real-time PCR. This is due to the amplification of non-viable pathogen propagules (PARIKKA and LEMMETTY 2004), suggesting that a large proportion of the pathogen population cannot survive winter conditions. However, the remaining portion of the population, especially on spurs, is a sufficient inoculum source to cause infections during the next growing season (Table). Furthermore, secondary conidia were reported to be produced from the conidial and hyphal phialides within 6 h after infection of symptomless strawberry leaves and were responsible for up to threefold increase in the total number of conidia within 7 d (LEANDRO *et al.* 2001) which could explain the greater percentage of *C. acutatum* isolates detected on spurs at pea size berry stage. Detection of *C. acutatum* on different plant tissues indicates that the pathogen may overwinter on different parts of the plant. At present it is not clear when spurs and other plant parts are infected and what fungicides should be applied to reduce or prevent such infection. Fungicide applications during winter months were rather inefficient in eradication

of the *Colletotrichum* complex on apple (CRUSIUS *et al.* 2002). Future research is necessary to address this issue and to provide improved management strategies for ripe rot of grapevine.

Conclusions

Dead wood and mummified bunches from the previous year, as well as wood tissues on the vines are potential sources of inoculum for ripe rot. Conidia surviving on plant material from the ground have the potential to be dispersed via wind and rain water splashes. However, in this study *C. acutatum* was not identified on green shoots, leaves and berries at pea size stage but only on the spurs. In addition, the fungus was not found from 10 spore traps analysed weekly for the duration of this study (data not shown). Based on these findings it might be concluded that the *C. acutatum* conidia that remain on spurs are a likely source of inoculum under appropriate climatic conditions during the next growing season. The amount of viable conidia on the spurs was almost equivalent to the amount of conidia on the debris. Therefore, the remaining debris may also be a source of inoculum and removal of this may reduce the presence of the pathogen in the vineyard. Secondly, heating the PCR mix for 15 min at 95 °C allows reliable identification of fungi in various *V. vinifera* tissues. This is a very simple, quick and straightforward approach to overcome the necessity of further purification of genomic DNA from 'difficult' samples such as grapevine wood.

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