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Research Note

The isolation and characterisation of *Xylophilus ampelinus*

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S u m m a r y : Bacterial blight, caused by *Xylophilus ampelinus* (PANAGOPOULUS) WILLEMS *et al.* is a destructive disease of grapevine (*Vitis vinifera*). Incubation of cuttings at 15 °C for three days under moist conditions significantly increased the number of isolated *X. ampelinus* colonies. This treatment facilitates the isolation of the pathogen from infected material from which it could previously not be isolated.

K e y w o r d s : bacterial necrosis, Xylophilus ampelinus, Vitis vinifera, detection.

Introduction: Bacterial blight of grapevine, caused by *Xylophilus ampelinus* (Panagopoulus) WILLEMS *et al.* is a destructive disease mostly of table grape cultivars in the Mediterranean region and in South Africa but may also occur in Switzerland, Austria, Bulgaria, the Canary Islands, Sardinia, Argentina, Tunisia, Turkey and the former Yugoslavia (BRADBURY 1973; GARAU *et al.* 1988). The pathogen survives in the vascular tissues of infected plants (PANAGOPOULUS 1987). The disease can be transmitted by propagation material, during grafting and by pruning knives. Bacteria are spread by moisture to plants where infection may take place through wounds, leaf scars and other sites. Infection may also occur without wounding (BRADBURY 1991). Panagopoulus (1987) found that 50 % of apparently healthy canes from diseased vineyards in Crete were latently infected. No selective medium is available for the isolation of *X. ampelinus* and its slow growth rate on Nutrient Agar (NA) hampers its recovery because of interference by saprophytes. We found it difficult to isolate the bacterium from infected material with distinct symptoms collected during hot and dry periods. Material collected from the same plants during cool and wet periods yielded large numbers of the bacterium. DU PLESSIS (1940) was also unable to isolate *X. ampelinus* from diseased material (PANAGOPOULUS 1996) and GARAU *et al.* (1988) also failed to isolate the pathogen from material with bacterial blight symptoms that had immunofluorescent antigenic cells. To enable us to screen propagation material, it was necessary to develop a suitable enrichment technique for the isolation of *X. ampelinus* from infected and latently infected material.

Materials and methods: B a c t e r i a l s t r a i n s : Twenty-eight South African isolates, 23 from the Paarl district and 5 from the De Doorns district were used in this study. Three of the isolates from the Paarl district were isolated from symptomless plants. Reference cultures obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB) in England: *X. ampelinus* (NCPPB 2217; NCPPB 2218; NCPPB 2590) were included in the identification tests. Isolations were made in September 1993 as described by PANAGOPOULUS (1969) on Difco NA. Cultures were maintained at room temperature on NA supplemented with 2 % $CaCO_3$. Stock cultures were freeze dried. All the strains were tested for catalase and Kovacs oxidase activity, urease production, lipolysis of Tween 80, production of H_2S from cysteine, production of acid from glucose in Hugh and Leifson's medium and the production of acid in Dye's medium C from glucose, sucrose, lactose, maltose, galactose and arabinose (BRADBURY 1991).

Pathogenicity was determined according to PANAGOPOULUS (1969) on *Vitis vinifera* cultivar Sultana grown in a greenhouse. Young shoots and leaves were inoculated.

M e t a b o l i c f i n g e r p r i n t s o f s t r a i n s u s i n g B i o l o g™ G N m i c r o p l a t e s : Six representative strains were subcultured on NA by picking a few colonies onto cotton swabs and streaking it on media covering the entire plate. NA was used instead of the recommended Sucrose Peptone Agar, as the isolates failed to grow on the latter medium. Plates were incubated for 48 h at 26 °C. Ten plates of NA were reinoculated by the same method and incubated for an additional 48 h. A cell suspension with a density of 0.30 ± 0.05 at 600 nm was made in sterile saline (0.83 % NaCl) and Biolog™ GN microplates (150 µl per well) were inoculated as recommended. Plates were read visually after 62 h.

E n r i c h m e n t o f b a c t e r i a i n p l a n t s a m p l e s : Suspensions of 4 *X. ampelinus* strains were made in sterile saline and counted by dilution plating. 1.5 ml of the bacterial suspension were sucked through 8 cm long cuttings of greenhouse-grown young grapevine shoots (cultivar Sultana) that included a node. The cuttings were placed in plastic bags containing wet cotton plugs, closed with rubber bands and incubated in the dark at 10, 12.5, 15, 17.5, 20 and 22.5 °C. After 3 d incubation, the basal ends of the shoots were placed in sterile saline and a vacuum was applied at the distal end until 0.75 ml was extracted from each shoot. Extracts from two shoots which were not inoculated were also analysed for the presence of *X. ampelinus* before and after incubation. For the control the extracts of two inoculated shoots were analysed before incubation. A number of cuttings with natural infections, showing bacterial blight symptoms from which the pathogen could not be isolated by conventional methods, were also included. Dilutions of the extracts were plated in triplicate on NA. Colonies were counted after 7 d incubation. The experiment was repeated twice.

Results: B a c t e r i a l s t r a i n s : All the strains were catalase positive, Kovacs oxidase negative, strongly urease positive within 2-3 d, lipolysed Tween 80, produced H_2S from cysteine, did not produce acid from glucose in Hugh and Leifson's medium, did not produce acid from glucose, sucrose, lactose or maltose in Dye's medium C but produced acid from galactose and arabinose. The colonies were all

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light yellow on NA and approximately 1 mm in diameter after 7 d. All the strains were uniform in appearance and in reactions.

Typical symptoms were produced by all the strains on shoots and leaves. The symptoms on the shoots took 3-4 weeks to develop and on the leaves 10-14 d.

Metabolic fingerprints of strains using Biolog™ GN microplates: All the *X. ampelinus* strains tested in the Biolog™ GN microplates utilised only acetic acid, propionic acid, L-aspartic acid, L-glutamic acid and L-pyroglutamic acid.

Enrichment of bacteria in plant samples: Recovery of *X. ampelinus* from inoculated shoots was 100 to 1000 times greater than from the control (no incubation) after a 3-d incubation at temperatures below 17.5 °C (Table). Data were transformed by taking natural logarithms of the values. Differences between treatments and the control were subjected to Dunnett's T test. Treatments below 17.5 °C differed significantly from platings of shoots before incubation. At temperatures of 17.5 °C and above, saprophyte populations increased to levels that interfered with the detection of the pathogen on plates. From the naturally infected cuttings, the best recovery rate was obtained after incubation of the shoots for 3 d at 15 °C. No *X. ampelinus* colonies were cultured from shoots which were not inoculated.

Table

Colony forming units recovered of *X. ampelinus* from inoculated cuttings on nutrient agar before and after 3 days of incubation at different temperatures

Treatment temp., °C	Replicate 1	Replicate 2	Mean[a]
10	$1.0x10^2$	$1.6x10^5$	
	$5.3x10^4$	no growth	9.1553822[b]
12.5	$1.6x10^3$	$5.3x10^5$	
	$6.6x10^3$	$1.5x10^6$	10.8935479[b]
15	$3.9x10^2$	$6.0x10^4$	
	$1.7x10^4$	$1.3x10^5$	9.6211262[b]
17.5	$3.3x10^6$	saprophytes	
	saprophytes	saprophytes	[c]-
20	saprophytes	$4.6x10^4$	
	$1.1x10^5$	saprophytes	[c]-
22.5	saprophytes	saprophytes	
	saprophytes	saprophytes	[c]-
Control[d]	2	3	
	8	$3.0x10^3$	2.9693921[b]
Suspension	$8.6x10^5$	$3.4x10^5$	

[a] Data were transformed by taking natural logarithms of the values.

[b] Treatments below 17.5 °C differed significantly from the control (no incubation). Differences between treatments and the control were subjected to Dunnett's T-test.

[c] No average could be determined as the plates were overgrown by saprophytes.

[d] The control was colony forming units recovered from infiltrated cuttings before any incubation.

Discussion: Difficulty was experienced in culturing *X. ampelinus* from diseased grapevine material onto medium. GERAU *et al.* (1988) also failed to isolate the organism from material in which the presence of the organism was confirmed by immunofluo-rescent microscopy. The enrichment technique significantly improved the detection of *X. ampelinus* from grapevine shoots by direct isolation. The organism has also been detected in shoots from vine plantings without symptoms, previously thought to be free of the disease, using this enrichment method. Although the optimum incubation temperature of shoots at which the largest number of *X. ampelinus* colonies were recovered seemed to be 17.5 °C, problems were experienced with saprophyte interference, especially from naturally infected cuttings. An incubation temperature of between 10 and 15 °C, lowered saprophyte interference.

It is important to note that other slow growing bacteria are also regularly isolated from grapevine material which can easily be confused with *X. ampelinus*. Confirmation of identity is therefore always necessary. A large proportion of the slow growing organisms were found to be Gram-positive. Confirmation tests are generally time consuming and in our experience, Gram reaction, catalase test, oxidase test, urease production and the lypolisation of Tween 80 are some of the less time consuming tests that are helpful in preliminary identification of the organism. Serological (GORRIS *et al.* 1997) and polymerase chain reaction (BOTHA *et al.*, unpublished data) techniques are available for the detection of *X. ampelinus* in grapevine material. If the number of bacteria in the plant extracts is increased, the sensitivity of these techniques will be improved.

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