

Grapevine Heat Treatment – Xanthomonas ampelina

FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **DPI 3V**

Principal Investigator: **Dr W P Roberts**

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TREATMENT OF GRAPE VINE CUTTINGS TO ELIMINATE *XYLOPHILUS AMPELINUS* (*XANTHOMONAS AMPELINA*).

Final Report

Project No: DPI 3

Introduction

Bacterial blight of grapevine was first reported in Italy in 1879 and France in 1895 (Panagopoulos, 1987) but the bacterial nature of the causal agent was not established until 1969 (Panagopoulos, 1969).

The main characteristics of the disease are summarised below. A fuller description can be found in Panagopoulos (1969, 1987). The name of the causal organism was changed from *Xanthomonas ampelina* to *Xylophilus ampelinus* by Willems *et al.*, (1987).

Grape vine propagating material imported into Australia must undergo a period in post-entry quarantine to check for the presence of a variety of diseases. Bacterial blight is one of the diseases that requires a lengthy period of observation in post-entry quarantine. Hot water treatment has the potential to reduce this time.

Professor P G Psallidas at the Benaki Phytopathological Institute, Greece was contracted to test the effect of hot water treatment on *Xylophilus ampelinus*. This work was supported by the Grape and Wine Research Council.

Disease symptoms and losses

The disease is characterised by vascular bacteriosis and leaf spotting with the formation of cracks and cankers on spurs, branches, young shoots, petioles and peduncles. The range and severity of the symptoms depends very much on the cultivars and the environmental conditions and bacterial blight can sometimes be confused with other diseases. The disease results in losses in productivity and in some cases vineyards have been abandoned (Panagopoulos, 1987).

Distribution

The distribution of the disease reported in the IMI Descriptions of Fungi and Bacteria (Bradbury, 1991) includes: South Africa, France, Greece (including Crete), Italy (including Sardinia and Sicily), Spain and Turkey. An earlier publication (Panagopoulos, 1987) also reports the disease as being present in Portugal and the USSR and suspected to occur in Bulgaria, Yugoslavia, Austria, Switzerland, Tunisia, Canary Islands and Argentina.

Control

Despite trials with a wide range of bactericides none were successful in controlling field infections. Therefore, control must be based on preventing the spread of the disease and minimising disease incidence and severity by manipulation of the environment and the use of more resistant grape varieties. Control of the disease is complicated by the tendency for symptoms to vary depending on the seasonal conditions and by the possibility of latent infections being present in material that is symptom free.

Post entry quarantine conditions for grapevine imports

Following a review of the quarantinable diseases of grapevines in 1990 (McLean, 1990) the Australian Quarantine and Inspection Service (AQIS) modified its post entry requirements for grave vine imports (Cartwright, personal communication).

The following summarises the current practices.

All vegetative material is treated in hot water at 50°C for 20 minutes for the elimination of Pierce's disease agent and Flavescence doree mycoplasma.

Handling after this stage then depends on the source of the material.

Grapevines from accredited North American virus indexed sources.

One year under glasshouse conditions to detect quarantinable fungal diseases.

Grapevines from other accredited virus indexed sources.

One year under glasshouse conditions to detect quarantinable fungal diseases plus one year outside to detect latent bacterial infections of *X. ampelinus*. Total time 2 years.

Grapevines from non-accredited sources.

One year under glasshouse conditions to detect fungal diseases plus one year outside to detect *X. ampelinus*. Additional screening for Corky bark and other virus testing requires woody indexing over 3 seasons. Total time in post-entry quarantine is 3-4 years.

The two diseases determining the length of time in post-entry quarantine are corky bark and *X. ampelinus*. There is some potential for other techniques (green grafting and serology) to be used for corky bark. These techniques are being evaluated and if effective could substantially reduce the time needed for corky bark screening. If these prove to be satisfactory the major factor determining the time in post-entry quarantine will be *X. ampelinus*.

Project objective

To investigate the use of hot water treatment for the elimination of *X. ampelinus* (*X. ampelina*) from grape vine cuttings in order to reduce the risk of this disease establishing in Australia.

Results

Grape vine cuttings artificially and naturally infected with *X. ampelinus* were tested under a range of temperature and treatment times. Development of symptoms can be variable with this disease so observations on some material was continued for several seasons to confirm the findings.

Detailed results are presented in the final report from Professor Psallidas (Appendix 1).

The principal findings were:

All infected cuttings (artificially and naturally infected) treated with hot water at 50°C for at least 20 minutes were found to be free of *X. ampelinus* by the three methods employed. These methods included sap extraction followed by plating on bacterial growth media, direct isolation from the cuttings and observations of symptom development.

Infected cuttings (artificially infected) treated at 45°C for 30 minutes were all found to be free of *X. ampelinus*.

Some infected cuttings treated at 45°C for 20 minutes were found to still carry *X. ampelinus* bacteria.

Treatment of dormant cuttings (cv Sultana) at 50°C for 20 or 30 minutes did not have any obvious effect on the survival of buds.

Five minutes treatment of bacteria suspended in culture fluid at 50°C was sufficient to kill populations of 10^7 bacteria.

Symptoms in infected material were not always visible in the first year of growth.

Discussion

One of the effective hot water treatments (50°C for at least 20 minutes) is equivalent to that being currently used by AQIS to treat all imports of grapevine vegetative material. This would suggest that the current practice of placing grapevine material outside to allow expression of latent *X. ampelinus* infections is unnecessary. In fact it was observed in this current project that not all infected canes develop symptoms during the first 2-3 seasons and it was suggested that observations for the detection of *X. ampelinus* should be continued for

at least four years to guarantee freedom from the disease. This suggests that the current practice of hot water treatment (intended to eliminate Pierce's disease) may be a much more important safeguard against *X. ampelinus* than the period of observation outside the glasshouse.

It is difficult to estimate the margins for error in the temperature and time that could be tolerated. A longer time could be preferable but this may be limited by the tolerance of the cuttings to hot water. The results indicate that hot water treatment at 45°C for 20 minutes would not be satisfactory but that treatment at 45°C for 30 minutes would be satisfactory.

Although the rooting ability of the cutting varied these experiments did not show any relationship between temperature/treatment time and rooting ability. However, experience suggest (Cartwright, personal communication) that material in poor condition, such as the material sometimes submitted for post-entry quarantine, may not be as tolerant to hot water treatment as material in good condition.

If hot water treatment is accepted for the elimination of *X. ampelinus* then quarantine clearance of grapevine material from accredited virus tested non North American sources would only take 1 year. Clearance of material from non-accredited sources would still be limited by the need to index for corky bark. However, if faster methods for corky bark become available then the period in post entry quarantine could be considerably reduced.

Recommendation

That the Australian Quarantine and Inspection Service consider the use of hot water treatment as the primary quarantine protection against *X. ampelinus*.

Publications arising from this research project

Psallidas P.G. & Argyropoulou A. Effect of hot water treatment on Xylophilus ampelinus in dormant grape cuttings. Proceedings of the 8th International Conference on Plant Pathogenic Bacteria, 1992. (In press). Copy of paper in Appendix 2.

References

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APPENDIX 1.

Grapevine: Heat Treatment - *Xylophilus ampelinus*

FINAL REPORT

General information

During 1992-1993 growing season observations were made on the grapevine plants of the various experiments as they have been described in our previous progress reports. The observations aimed to investigate the presence of *Xylophilus ampelinus* in the grapevine plants planted in pots in spring of 1991 and 1992.

In order to facilitate the detection of *Xylophilus ampelinus* into the xylem vessels of the plants apart from the methods used previously (isolations, symptoms) this year we delayed pruning in order to have sap exudation from the pruning cuts. The sap was collected by sacking it with a syringe in which instead of a needle, a plastic tip was used. The collected sap was put into Eppendorf tubes and stored in deep freeze for further examination. Up to now the presence of the bacterium in the sap was examined by streaking it on Difco Nutrient Agar medium. In the future we plan to produce antiserum against *Xylophilus ampelinus* and use immunofluorescence and ELISA techniques for detection.

In practice, the cut ends of grapevine canes intended to be used as propagation material, are covered with paraffin. Since this treatment may have an effect on the heat treatment of these canes, in the experiments of 1992-1993 this parameter was also investigated. The canes in each treatment were divided in equal numbers.

Observations-Results

a. 1991-1992 experiment

The results of the 1991-1992 experiment are presented in the combined tables 1 and 2. From these tables it is obvious that all plants found healthy during the first investigation remained healthy 3 years after planting. Some of the plants found to be infected died but most of them are still survived carrying the pathogen. As it happens in naturally infected plants in the field not all the xylem vessels are infected. Some are healthy giving good vegetation and growth to the buds on their size while the buds corresponding to the infected vessels exhibit typical symptoms (not sprouting, desiccation and death of the new shoots, cankers etc.).

b. 1992-1993 experiments

The results of 1992-1993 experiments are shown in tables 3 and 4. The results could be summarized as follows: in artificial inoculated canes the results obtained are in agreement with those of previous experiments that is that the heat treatment at 50°C for 20 min frees the dormant grapevine cuttings from the systemic existence of *Xylophilus ampelinus*, while the treatment at 45°C for 20 min is not effective. The heat treatment at 45°C for 30 min is quite effective as that of 50°C for 20 min. In naturally infected canes the results are identical with the ones obtained with artificially infected canes.

All the cuttings treated at 50°C for 20 min were found to be free of *Xylophilus ampelinus* with all three methods of detection employed (prunning sap, isolation and symptom development). The treatment at 45°C for 20 min proved again ineffective. Because of the small number of naturally infected canes we could acquire the treatment at 45°C for 30 min was excluded from the experiment with naturally infected canes.

From all the experiments carried out concerning the freeing of dormant grapevine canes from systemic existence of *Xylophilus ampelinus* there are some points that merit to be mentioned.

1. The infection of the canes by *Xylophilus ampelinus* does not affect their rooting capability, since the percentage of rooted canes among the infected (artificially or naturally) is not lower than the percentage among the apparently healthy ones.
2. The use of paraffin to cover the cut ends of the cuttings does not affect the heat treatment effect on systemically present *Xylophilus ampelinus*.
3. The typical disease symptoms usually appear the second year after rooting.
4. The use of naturally exudating xylem sap during pruning is a good method to detect *Xylophilus ampelinus* infection in mother plants. From our investigations we found that in all cases where *Xylophilus ampelinus* bacteria were detected, by isolation or symptom development, it has also been detected by the xylem sap method. The reverse did not happen since from the 68 canes in which the bacterium was detected by the xylem sap method only 48 were found positive with the direct isolation method.
5. The method of *Xylophilus ampelinus* detection based on symptom development is not safe because not all infected canes develop symptoms during the first 2-3

years after planting for rooting.

So in cases where grapevine plants are planted under quarantine conditions, they should be inspected for symptom development at least for four years.

Although the investigations on the potted plants will be continued for at least two additional years, from the results obtained, both with artificially and naturally infected grapevine (cv Sultana) canes it could be concluded that:

1. The hot water treatment at 50°C for 20 min of dormant canes, guarantees that they are free of the bacterium *Xylophilus ampelinus*.
2. The hot water treatment at 50°C for 20 min or 30 min does not affect the rooting and vegetative capability of the canes at least as far as the cultivar "sultana" is concerned. Since some variability in heat resistance occurs among different grape varieties it should be checked if the variety to be treated is resistant to this temperature.



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Senior Researcher

Table 1. Cuttings rooted after treatment at 45°C and 50°C for 20 min. - Experiment 1991-1992

Observations in 1992									
	45°C			50°C			Control		
Treatment	No of rooted per total	Healthy	Diseased	No of rooted per total	Healthy	Diseased	No of rooted per total	Healthy	Diseased
Artificial inoculated	13/15	11	2	34/45	34	-	13/30	2	11
From diseased vine yards	-	-	-	9/27	9	-	3/5	3	-
Infiltrated with Knop's solution	-	-	-	-	-	-	19/20	19	-
Observations in 1993	No of survived per rooted			No of survived per rooted			No of survived per rooted		
Artificial inoculated	11/13	11	-	33/34	33	-	12/13	2	10
From diseased vine yards	-	-	-	8/9	8	-	10	3	-
Infiltrated with Knop's solution	-	-	-	-	-	-	19/19	19	-

Table 2. Cuttings rooted after treatment at 45°C and 50°C for 30 min. - Experiment 1991-1992

Observations in 1992									
	45°C			50°C			Control		
Treatment	No of rooted per total	Healthy	Diseased	No of rooted per total	Healthy	Diseased	No of rooted per total	Healthy	Diseased
Artificial inoculated	21/29	21	-	17/31	17	-	13/30	2	11
From diseased vine yards	-	-	-	12/23	12	-	3/5	3	-
Infiltrated with Knop's solution	-	-	-	9/10	9	-	19/20	19	-
Observations in 1993	No of survived per rooted			No of survived per rooted			No of survived per rooted		
Artificial inoculated	21/21	21	-	17/17	17	-	12/13	2	10
From diseased vine yards	-	-	-	12/12	12	-	3/3	3	-
Infiltrated with Knop's solution	-	-	-	9/9	9	-	19/19	19	-

Table 3. Heat treated cuttings artificially inoculated - Experiment 1992-1993

Treatment	No of rooted per total	Diseased per rooted	Healthy per rooted
50° for 20' without parafin	41/45	0/41	41/45
50° for 20' with parafin	44/45	0/44	44/44
45° for 20' without parafin	46/48	5/46	41/46
45° for 20' with parafin	44/45	3/44	41/44
45° for 30' without parafin	25/25	0/25	25/25
45° for 30' with parafin	26/26	0/26	26/26
Control (artificial inoculated) with parafin	40/41	33/40	7/40
Control (artificial inoculated) without parafin	31/35	25/31	6/31
Control (without any treatment)	19/20	0/19	19/19
Control (50° for 20')	20/20	0/20	20/20

Table 4. Heat treatment - Naturally infected canes

Treatment	No of infected	Rooted		Healthy		Diseased	
		Infected	Total	Infected	Total	Infected	Total
50° for 20'	7	7/7	24/48	0/7	24/24	0/7	0/24
45° for 20'	8	5/8	21/48	1/5	17/21	4/5	4/21
Control							
22° for 20'	7	4/7	21/46	2/4	23/25	2/4	2/25

Effect of Hot Water Treatment on *Xylophilus ampelinus* in Dormant Grape Cuttings[1]

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ABSTRACT

The effect of hot water on the survival of *Xylophilus ampelinus* both in different suspending media and in artificially and naturally infected grapevine canes of the cv. Sultana was investigated. The suspending medium played an important role on the survival of the bacterium to the exposure at different temperatures. Suspending media rich in nutrients supported the survival. The bacterium tolerated 90 min exposure at 45°C when Nutrient broth or Yeast Salt media were used while it was killed after 20 min exposure when suspended in half strength Knop's solution. Exposure at 50°C for 5 min killed a bacterial population of $7.5X10^6$ cfu/ml irrespectively of the medium used.

Artificially inoculated canes with approximately $3.2X10^8$ cfu/ml were freed from the bacterium, or its population was reduced to below the level of detection after exposure at 45°C for 20 min. However, exposure at 45°C for 20 min does not guarantee the eradication of the bacterium.

Exposure of dormant cuttings at 50°C for 30 min did not have any obvious effect on the survival of the buds.

From the results obtained it could be concluded that the hot water treatment offers a simple, effective, economical and environmentally safe method for eradicating *Xylophilus ampelinus* from dormant grape cuttings.

The method could be recommended to prevent the introduction of the disease in countries which are free, through the importation of propagating material.

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KEYWORDS: *Xylophilus ampelinus*, grapevine, hot water treatment, dormant cuttings, eradication.

INTRODUCTION

Bacterial blight or bacterial necrosis of grapevine caused by *Xylophilus ampelinus* (Panagopoulos) Willems is a serious, chronic vascular disease of grapes. In Greece the disease is endemic in the island of Crete, where it was described by Sarejanni in 1939. Its etiology was clarified by Panagopoulos in 1969. The causal agent, *Xanthomonas ampelina*, was reclassified as *Xylophilus ampelinus* by Willems et al. in 1987. The most characteristic symptom of the disease is the formation of cankers on branches canes, young shoots, and petioles. Diseased plant parts upon longitudinal section show a red-brown discoloration of the xylem tissues. However, the extent of symptom development and the type of symptoms vary and depend on different parameters as the cultivar, the environmental conditions, and the time of infection.

Canes which were infected later in the growing season may not show any visible symptom, or vascular discoloration. These canes which are latently infected are the most significant sources for disseminating the pathogen in long distances. It has been shown (PANAGOPOULOS, 1987) that a proportion of 32-50% of apparently healthy canes collected from an infected vineyard of the cv. Sultana, were infected by the pathogen.

To prevent the spread of the disease in an unaffected region it is very important that all planting and grafting material to be pathogen-free. Since visual examination of the propagating material does not cover the latently infected ones the use of hot water to eradicate the pathogen from artificially and naturally infected dormant canes was investigated.

MATERIALS AND METHODS

Bacterial cultures

Two strains of *X. ampelinus* from our collection were used. The strain BPIC 48 which is the Type strain and the BPIC 620. The bacteria were maintained on NA (Nutrient agar, Difco) slants and for inoculum preparation they were grown on GYCA (Galactose, Yeast, Corbonate, Agar) at 26°C.

Thermosensitivity of *Xylophilus ampelinus*

The effect of exposure time at 45 and 50°C on the survival of the bacterium was studied with two methods. a) From an initial suspension containing $1.8X10^8$ cfu/ml decimal dilutions up to 10^{-7} were made in test tubes containing NB (Nutrient Broth, Difco) or YS (Yeast Extract, Salts). Six sets of tubes were placed in a water bath at 45°C and 50°C. One set of tubes was removed after exposure for 10, 20, 25, 30, 60 and 90 min, at 45°C and 5, 10, 15,

20 and 30 min at 50°C. One set of tubes was used as control (no heat treatment). The tubes, after the exposure, were incubated at 26°C for 10 days and checked for bacterial growth (turbidity). From the tubes showing growth a loopful of suspension was streaked onto NA to check possible contamination. b) Decimal dilutions up to 10^{-7} were made in half strength Knop's solution. From each dilution 1 ml was placed into lyophilization tubes and six sets of the tubes were placed each time into a water bath at 45° and 50°C. One set of tubes was removed after exposure of the time scheduled. 50 µl of suspension was removed from each tube, streaked on NA containing petri dishes and incubated at 26°C for 10 days. The dishes were checked for colonies of *Xylophilus ampelinus*.

Artificial inoculation of plant material

One year old canes of the cv. Sultana were collected from different locations at different times. The canes were cut in 40 cm long pieces and kept at 4 to 6°C in a refrigerator into polyethylene bags until use. The artificial inoculation of the canes was performed by infiltration with a bacterial suspension in half-strength Knop's solution containing $5X10^6$ cfu/ml. The infiltration was achieved either by sucking or by forcing the bacterial suspension to pass through the cane. In the first case the uper end of the cane was connected to the inlet tube of a vacuum pump while the base of the cane was immersed into the bacterial suspension. One ml of suspension was sucked. In the second case the base of the cane was connected with a syringae through a thick-walled elastic tube and the bacterial suspension was forced to pass through the cane, until a drop was formed at the end of the cane. This method was developed by Dr A.S. Alivizatos in our laboratory.

Hot water treatments

The cuttings were grouped according to the treatment they had received and submersed in the water bath in groups of 50. No change in the temperature was observed when the cuttings were submersed. A time interval of 5 min was left for the temperature into the tissues to reach that of the water bath. The cuttings were treated at 45°C and 50°C for 20 and 30 min. After treatment the cuttings were stratified in sand and subsequently part of them were potted for rooting and the remaining were used to make isolations in order to check the presence of *X. ampelinus*. The isolations were made from the middle internode of each cutting.

RESULTS AND CONCLUSIONS

The results are summarized in tables 1,2 and 3. From the results obtained it could be concluded that:

1) *Xylophilus ampelinus*, in suspension, tolerated 45°C at time more than $1^1/_2$ hours in YS medium. There is an evidence that the suspending medium has some effect on the heat

tolerance. 2) The infiltration methods used for artificial inoculation of dormant grape canes proved efficient. Most of the inoculated cuttings did not show any visible symptom on the first year after planting. Typical symptoms of the disease are obvious the second year but not in 100% of the inoculated canes. 3) Hot water treatment at 45°C for 20 min does not guarantee eradication of *Xylophilus ampelinus* from dormant grape canes. 4) Hot water treatment at 50°C for 20 min seems to be effective in eradicating the pathogen from infected dormant canes. Treatment at 50°C for 30 min did not have any lethal effect on the dormant grapevine tissues.

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Table 1. Lethal effect of hot water treatment at 45°C and 50° on dilutions of Xylophilus ampelinus[1] in different suspending media.

Medium	Highest population of bacteria killed at			
	45°C		50°C	
	Cfu/ml	Treatment 2 time	Cfu/ml	Treatment 2 time
NB (Difco)	$6,0 \times 10^6$	60 min	$7,5 \times 10^6$	5 min
YS	$6,0 \times 10^6$	90 min	$6,0 \times 10^7$	5 min
(1/2) Knop's solution	$1,7 \times 10^6$	20 min	$1,7 \times 10^7$	5 min

1. Five day old growth on GYCA (Galactose, Yeast, Carbonate, Agar) slants of the strains BPIC 48 and 620 were each suspended in 5ml of water, and combined (initial population 6×10^8 cfu/ml).

2. The time required for the suspending medium to reach the test temperature was checked by a thermometer placed in a test tube containing the suspending medium.

Table 2. Isolation of X. ampelinus from treated canes at 45 and 50°C for 20 and 30 min and untreated controls

Treatment	45°C			50°C			Control
	No of cuttings tested	No of cuttings with X.a/ total		No of cuttings tested	No of cuttings with X.a/ total		No of cuttings with X.a/ total
		20	30		20	30	
Artificial inoculated	95	0/39	0/56	132	0/87	0/45	21/42
From diseased vineyards	18	0/18	-	69	0/46	0/23	7/51
Infiltrated with Knop's solution	-	-	-	21	0/6	0/15	0/51

Table 3. Cuttings rooted after treatment at 45 and 50°C for 20 and 30 min and untreated controls

Treatment	45°C				50°C				Control	
	No of rooted per total		No of healthy cuttings per total		No of rooted per total		No of healthy cuttings per total		No of rooted per total	No of healthy cuttings per total
	20min	30min	20min	30min	20min	30min	20min	30min		
Artificial inoculated	13/15	21/29	11/13	21/21	34/45	17/31	34/34	17/17	13/30	2/13
From diseased vineyards	-	-	-	-	9/27	12/23	9/9	12/12	3/5	3/3
Infiltrated with Knop's solution	-	-	-	-	-	9/10	-	9/9	19/20	19/19