Pathogen eradication using the pistachio dieback bacterium as a model

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Pistachio nuts

Dedicated to my parents,

Vu Tu Hung

&

Thanh Thi Yen My

with all my love, respect and gratitude

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Abstract

Pistachio dieback is a bacterial disease involving internal staining, trunk and limb lesions, decline, dieback and, in some instances, death of trees. The causal agent is *Xanthomonas translucens* pv. *pistaciae* (*Xtp*), a vascular pathogen that provides a local model to assess the effectiveness of existing eradication strategies for systemic bacterial pathogens of woody perennials which are likely to be introduced into Australia. Burning and burial are two accepted means of disposal of diseased plant material for eradication purposes. However, there is little or no information on the survival of bacterial pathogens following burning or burial of infected wood. The aim of the project was to evaluate the efficacy of burning and burial as means of safe disposal of diseased wood.

Burning of pistachio wood, naturally infected with *Xtp*, was conducted twice in field conditions. Controlled laboratory experiments with pure cultures of *Xtp* and with naturally and artificially infected pistachio wood were performed to support the results of the burns. Viable *Xtp* was detected in some non-burned wood, but not in charcoal, ash or partially burned wood. In liquid culture, 65°C was lethal to *Xtp* whereas survival at 60^{o}C or less varied with culture medium and duration of exposure. In infected wood *Xtp* survived exposure to 40 - 55°C for at least 60 min *in vitro* but was killed by exposure to 60°C for 15 min or more. These data corroborated the burning experiment.

Survival of *Xtp* in infected pistachio wood placed on the soil surface or buried 10 cm deep was evaluated in an open environment at the University of Adelaide, Waite Campus orchard, South Australia. The experiment was conducted from August 2008 to March 2011 using naturally infected pistachio wood segments and mulched wood, and was partially repeated in 2010 over 5 months using naturally infected pistachio wood segments and artificially infected twigs. Viable *Xtp* was isolated from branch segments and from

mulched wood buried for 31 and 23 months, respectively. Viable *Xtp* was not detected in branch segments placed on the soil surface at any time, but was detected in one mulched wood sample at 20 months and in artificially infected twigs for up to 3 months after the placement of wood on the soil surface. Prevailing dry weather conditions during the study might have contributed to the quick decline in *Xtp* population in the wood on the soil surface. Infrequent isolation of *Xtp* from buried materials might have been due to the entry of the pathogen into a dormant state, such as viable but nonculturable, in response to changing environments during burial.

The ability of copper to induce *Xtp* to become viable but nonculturable was investigated. Copper induced nonculturability in *Xtp* at 0.05 mM but this effect was not obvious at 0.005 or 0.01 mM. *Xtp* exhibited some ability to adapt to the presence of copper at 0.05 mM and there was some indication that spontaneous mutants existed in the *Xtp* population prior to exposure to copper. Further research is required to confirm the existence of the viable but nonculturable state in *Xtp* as well of spontaneous copper-resistant mutants in the population.

In conclusion, burning is an appropriate eradication technique to dispose of infected debris, providing the pathogen is exposed to a temperature of 60°C or greater for at least 15 minutes. Decomposition of woody material and loss of viability of the pathogen were slow and influenced by environmental conditions. In addition, the pathogen might enter a nonculturable state or evolve in response to changing conditions during burial and become a possible source of inoculum for new infections. Overall, knowledge gained from this study provides information to support and extend existing eradication response strategies for newly introduced or emerging pathogens.

Declaration

I, Vu Thanh Tu Anh, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Vu Thanh Tu Anh

Statement of the contributions to jointly authored paper

T. A. Vu Thanh, M. R. Sosnowski, D. Giblot-Ducray, C. Taylor and E. S. Scott, 2012. Effect of burning and high temperature on survival of *Xanthomonas translucens* pv. *pistaciae* in infected pistachio branches and twigs. Doi: 10.1111/j.1365-3059.2012.02596.x. Presented in Chapter 4.

Author contributions: TAVT and MRS designed and conducted the first burning experiment. TAVT designed and conducted the second burning experiment and *in vitro* temperature experiments, analysed the data and drafted/constructed the manuscript. ESS, MRS and DGD contributed to the research ideas, design, interpretation of experiments and the editing of the manuscript. CT liaised with local authorities and pistachio growers for materials and assisted with the burning experiments. All co-authors contributed to the final version of the manuscript.

The paper presented in this thesis is in submission form according to the instructions to authors of the journal available on the following website: http://www.wiley.com/bw/submit.asp?ref=0032-0862&site=1. *This thesis has been prepared according to the University of Adelaide's specifications for 'combination conventional/publication format'.*

The following authors agree that the statement of the contributions to jointly authored paper accurately describes their contribution to the research manuscript and give consent to its inclusion in this thesis.

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Abbreviations

ABSPA	antibiotic benlate sucrose peptone agar
ANOVA	analysis of variance
BOM	Bureau of Meteorology
bp	base pair
BSPA	benlate sucrose peptone agar
CFU	colony forming units
dNTPs	deoxynucleotide triphosphates
EPP	emergency plant pest
h, min, s	hour, minute, second
LB	Luria-Bertani broth
LSD	least significant difference
NA	nutrient agar
NA+A	nutrient agar plus antibiotics
NB	nutrient broth
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
SDW	sterile distilled water
SPA	sucrose peptone agar
SPB	sucrose peptone broth
SPC	spread plate count

Chapter 1 Introduction and literature review

1.1 Introduction

In Australia, pistachios (*Pistacia vera* L.) are grown mainly along the River Murray where the borders of New South Wales, Victoria and South Australia meet. The Australian pistachio industry is relatively young, as commercial planting started in the early 1980s and the first commercial orchards came into production in 1995 (Taylor & Edwards, 2000). Over the last two decades, the Australian pistachio industry has been challenged by a new bacterial disease, which causes limb and trunk lesions, internal wood staining, decline and dieback (Taylor & Edwards, 2000). The causal organism was identified as X*anthomonas translucens* (Facelli *et al.*, 2002; Facelli *et al.*, 2005) and recently classified as a new pathovar of the species, namely *Xanthomonas translucens* pv. *pistaciae* pv. nov. (Giblot-Ducray *et al.*, 2009), hereafter referred to as *Xtp*.

Research on the epidemiology and management of pistachio dieback has been conducted at the University of Adelaide since 1999, in association with the Department of Primary Industries Victoria at Mildura and the South Australian Research and Development Institute (SARDI) in Adelaide. *Xtp* resides mainly in the xylem of infected trees. Although the natural means of survival, dissemination and inoculation remain unknown, transmission of the bacteria on contaminated pruning tools was demonstrated experimentally (Scott *et al.*, 2009). A polymerase chain reaction (PCR)-based diagnostic assay was also developed (Marefat *et al.*, 2006a). Although several management measures have shown potential in prevention or reduction of infections, eradication of the pathogen from infected trees is considered improbable (Sedgley *et al.*, 2004).

As the disease cycle is poorly understood and measures to prevent or cure the disease are lacking, growers may benefit from strategies to reduce the amount of inoculum that may persist in infected wood. This study was intended to provide information that may help to prevent establishment of the disease in new areas. Also, as *Xtp* appears to be endemic in Australia and is known to survive in wood for a number of years, it provides a local model to assess the effectiveness of existing eradication strategies for systemic bacterial pathogens of woody perennials which are considered high-priority emergency plant pests (EPP) in Australia, such as *Erwinia amylovora*, which causes fire blight of pome fruit.

In this review, pistachio dieback and its causal agent will be examined. Management measures which have been developed and tested for the disease, as well as eradication methods for other systemic bacterial pathogens of woody tissue will also be discussed.

1.2 Pistachio production

1.2.1 The pistachio tree

The pistachio, *Pistacia vera* L., is native to western Asia and Asia Minor (Ferguson *et al.*, 2005a) and commercial growing of pistachio started in Iran in the 1800s (Pistachio Growers' Association Incorporated, 2008). Pistachio is grown in Lebanon, Syria, Iran, Turkey, Southern Europe, the desert countries of Asia and Africa, and California (Crane & Maranto, 1988). The largest pistachio producer is Iran, followed by the United States of America, Turkey, Syria and China (Anonymous, 2009). Pistachio belongs to the family Anacardiaceae and the genus *Pistacia*, which comprises 11 species, and only *P. vera* produces edible nuts and has economic value (Ferguson *et al.*, 2005a). The tree is

dioecious; male and female flowers are borne on different trees, thus both trees are required to produce nuts. Full bearing is achieved when trees reach 10 - 12 years of age. When the trees mature, they develop an alternate bearing pattern, which produces a heavy crop of nuts in one year (on-year) and reduced or no crop in the next year (off-year) (Crane & Maranto, 1988; Ferguson *et al.*, 2005a). Pistachio trees are propagated by budding (shield or T-budding and chip budding) or grafting (Crane & Maranto, 1988). *P. vera* is grafted or budded onto rootstocks of other *Pistacia* species, such as *P. terebinthus*, *P. atlantica*, *P. integerrima* (Pioneer Gold I) and two hybrids of *P. atlantica* and *P. integerrima*, namely Pioneer Gold II (PGII) and UC Berkeley I (UCB I) (Ferguson *et al.*, 2005b). Selection of rootstocks is based on resistance to nematodes, soil-borne diseases such as *Verticillium* wilt and specific growing conditions (Ferguson *et al.*, 2005b). Locations which have long, hot, dry summers and moderate winters are appropriate for pistachio production (Ferguson *et al.*, 2005a).

1.2.2 Pistachio cultivation in Australia

Pistachio was first introduced into Australia in 1935 (Robinson, 1997) and commercial planting started in the early 1980s after CSIRO's Merbein laboratories were successful in breeding and releasing a new female cultivar, Sirora (Pistachio Growers' Association Incorporated, 2002). The main commercial orchards have been established along the River Murray in New South Wales, Victoria and South Australia (Fig. 1.1). By 2011, the pistachio industry in Australia consisted of about 40 growers with about 750 ha of trees planted (Pistachio Growers' Association Incorporated, 2011). Current average Australian pistachio production is approximately 1,200 tonnes per year and is expected to increase to an average of 1,600 to 1,700 per annum, with maximum of 2,500 tonnes in an on-year and 800 tonnes in an off-year by 2020 (Fig. 1.2).

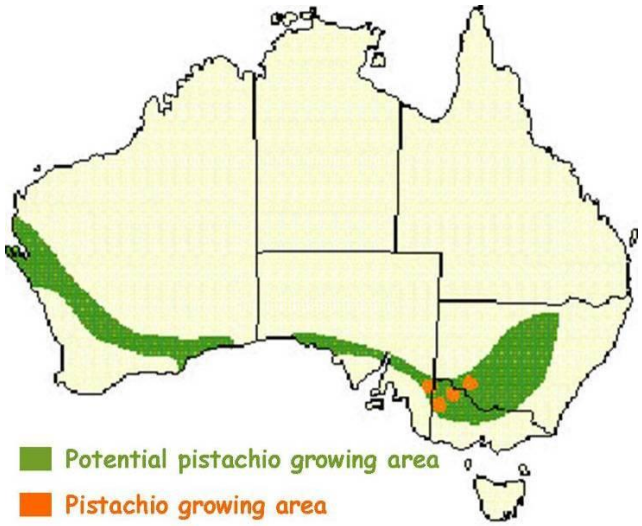

Figure 1.1 Actual and potential areas of pistachio production in Australia in 1997 (Robinson, 1997)

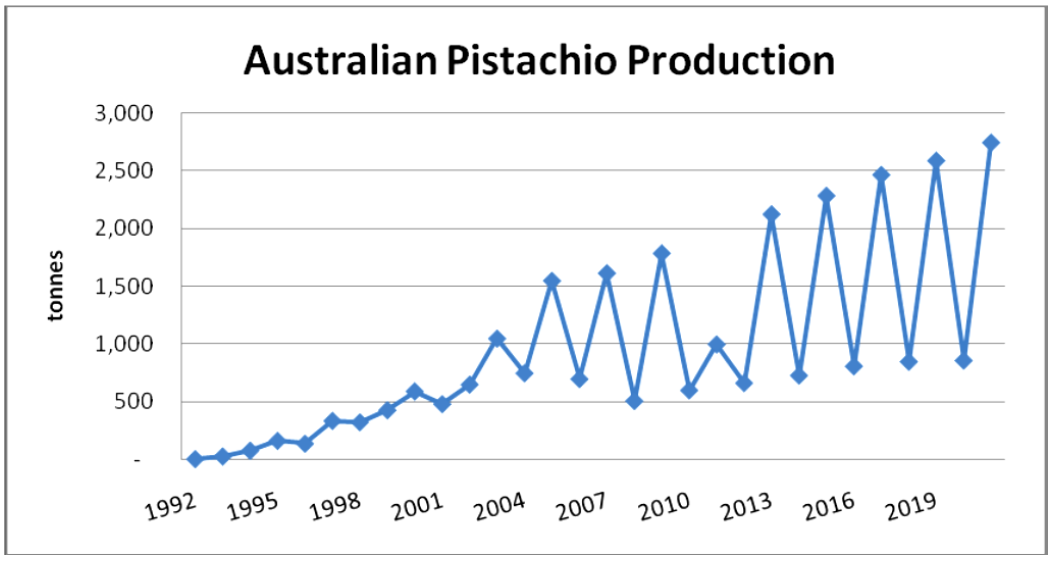

Figure 1.2 Australian pistachio production, previous and projected to 2021 (Pistachio Growers' Association Incorporated, 2011).

Kerman, a cultivar selected by the United States Department of Agriculture, and Sirora are the two female cultivars commonly recommended in Australia (Maggs, 1982). However, Sirora is grown more widely because it requires a shorter chilling period and has a more uniform nut split rate (Taylor & Edwards, 2000). *P. atlantica*, *P. terebinthus* and *P. integerrima* (Pioneer Gold I) are the most popular rootstocks used in Australia. Numerous male cultivars were selected by Maggs (1982), but the most commonly used are 14.4 and 15.2, propagated by Sunraysia Nurseries, Mildura, and sold as 'blue male' and 'red male', respectively. Various male cultivars are typically planted in each orchard to make sure sufficient pollen is produced during the extended flowering period of Sirora.

During the establishment of a pistachio orchard, trees are trained and pruned to ensure that they have the desired framework shape and are suitable for mechanical harvesting (Bass *et al.*, 1986; Robinson, 1997). Mature bearing pistachios trees are pruned annually during winter and it is the common practice by Australian growers to mulch the prunings and leave them on the orchard floor. Orchards are irrigated as required through under-tree sprinklers or drip irrigation systems.

1.2.3 Diseases

Several important fungal diseases affect the growth of pistachio trees in California, such as Alternaria leaf blight caused by *Alternaria alternata*, panicle and shoot blight caused by *Botryosphaeria dothidea*, and Verticillium wilt caused by *Verticillium dahliae* (Ferguson *et al.*, 2005a). Of these, *Alternaria* and *Botryosphaeria* are considered threats as they appear more often and cause significant loss (Pistachio Growers' Association Incorporated, 2011). Anthracnose disease, first reported in New South Wales in 2001 by Ash and Lanoiselet (2001) and which occurred again in 2011, is also considered a possible threat to production (Pistachio Growers' Association Incorporated, 2011). To date, dieback is the

only bacterial disease reported for pistachio. This disease, which reduces yield and kills trees, is one of the main factors limiting the expansion of the Australian pistachio industry (Horticulture Australia Limited, 2006; Scott *et al.*, 2009).

1.3 Pistachio dieback

Pistachio dieback is a relatively new bacterial disease and has been reported only in Australia. The first symptoms of twig dieback and internal staining were observed in pistachio orchards in 1989 and trunk lesions were first observed on male trees in 1992 (Facelli *et al.*, 2001). Losses due to the disease have not been documented comprehensively, but the disease is reported to have caused the death of more than 10% of trees in some areas (Edwards & Taylor, 1998).

1.3.1 Symptoms of pistachio dieback

Disease symptoms have been observed in more than 40% of pistachio orchards, some of which had 10-20% of trees with symptoms (Taylor *et al.*, 2005). Characteristic symptoms may include shoot death and dieback, decline with little or no current season growth, limb and trunk lesions (often covered with black, superficial fungal growth), excessive resinous exudates, discolouration of woody tissue in shoots over 2 years old, and, in some instances, tree death (Fig. 1.3) (Taylor & Edwards, 2000). These symptoms have been observed on the scions Sirora and Kerman, and on a range of male varieties (Taylor & Edwards, 2000), but not on the rootstocks (*P. terebinthus, P. atlantica* or *P. integerrima* cv. Pioneer Gold) (Facelli *et al.*, 2001). In addition, the symptoms are often seen on mature, bearing trees but have rarely been observed on young trees (Facelli *et al.*, 2002). Leaf symptoms have not been reported (Facelli *et al.*, 2005; Facelli *et al.*, 2009). It is difficult to determine the progression of the symptoms due to the lack of evidence on timing of initial infection. The

Figure 1.3 Pistachio dieback symptoms: (a) dieback (black arrow), (b) trunk lesion, (c) resinous exudate, (d) xylem staining (brown arrows) and (e) dead, diseased tree. (Photographs courtesy of Taylor C. and Facelli E.)

bacteria are likely to be present in the tree for several years before external symptoms can be seen (Taylor & Edwards, 2000).

1.3.2 Causal agent

Even though twig dieback and internal staining were first observed in 1989, sampling and isolation of pathogens from diseased trees were initiated only in 1997 for identification of a potential causal agent of the disease (Facelli *et al.*, 2001). A bacterium, identified as being closely related to *Xanthomonas translucens* using Fatty Acid analysis and Biolog®, was consistently isolated from diseased pistachio trees (Taylor & Edwards, 2000; Facelli *et al.*, 2002; Facelli *et al.*, 2005). In subsequent studies, repetitive extragenic palindromic PCR, gas chromatography of fatty acid methyl esters and denaturing-polyacrylamide gel electrophoresis analyses, distinguished two genetically distinct groups of *X. translucens*, designated A and B, that appeared to differ in their host range and severity of symptoms (Marefat *et al.*, 2006a; Marefat *et al.*, 2006b). The two groups were named by Giblot-Ducray *et al.* (2009) as two variants of *X. translucens* pv. *pistaciae*, or *Xtp*, through DNA/DNA hybridisation, screening of integrons and Gyrase B gene-based phylogeny.

Like other *Xanthomonas* bacteria, *Xtp* is motile, Gram-negative and has rod-shaped cells of about 0.4 x 1.5 µm in size (Facelli *et al.*, 2005). In culture, the two variants are indistinguishable and produce small, pale yellow, defined colonies on nutrient agar (NA), and pale yellow, mucoid, domed and non-defined colonies on sucrose peptone agar (SPA) (Sedgley *et al.*, 2004; Facelli *et al.*, 2005). In PCR, *Xtp* A and B yield fragments of 331 bp and 120 bp, respectively (Marefat *et al.*, 2006a).

Sedgley *et al.* (2004; 2006) investigated location of the pathogen within the tree, entry points, pathways of spread and transmission of the pathogen. The pathogen occurred mainly in xylem tissues and has been isolated only rarely from leaves, flowers and nuts. It

has been isolated mostly from stained and unstained young sapwood of main trunks, primary and younger branches and current season growth, rarely from old and stained heartwood, and not from roots (Sedgley *et al.*, 2004; Facelli *et al.*, 2009). It can be experimentally introduced to the trees via openings such as pruning wounds, leaf scars and trunk injection, and invades the vascular system. Leaves and lenticels are unlikely entry points (Sedgley *et al.*, 2006). The pathogen moves up and down from the point of inoculation and can cross the graft union, but appears to be confined to the xylem present in the year of inoculation with little or no lateral spread (Sedgley *et al.*, 2004). It can be transmitted among trees via pruning tools (Sedgley *et al.*, 2004; 2006). The authors were unable to detect dissemination in air, rain and irrigation run-off and there was no evidence of insects as vectors. Bark and lesions were considered unlikely sources of inoculum as the pathogen has not been isolated from surfaces of these parts, inner bark or cortex associated with lesions (Facelli *et al.*, 2009). In addition, resinous exudate appears to be bacteriostatic (Sedgley *et al*. 2004). The pathogen was sporadically isolated from leaves and nuts (Facelli *et al.*, 2009) and not detected in buds collected from infected trees (Taylor *et al.*, 2007). Thus, the natural means of spread remains unknown.

Further studies on the origin of the pathogen, how it causes disease in pistachio as well as on biological and or environmental factors affecting the spread and symptom expression of pistachio dieback are still required. However, accumulated knowledge of the pathogen over the decade has facilitated the development of management practices for the disease.

1.3.3 Management of pistachio dieback

Management of bacterial diseases of plants is challenging and often requires a multifaceted approach that utilises a diverse set of management tools. Management of diseases caused

by *Xanthomonas* species can be achieved by cultural practices such as field sanitation, use of clean planting materials and resistant crop cultivars, by chemical and biological control, and through quarantine regulations for intercountry or interregion movement of plant materials (Mew & Natural, 1993). A review of the literature on previous and current management strategies for bacterial diseases of relevance to pistachio dieback is given below. In addition, relevant information on the management of other bacterial diseases is also presented, which helps to address gaps in knowledge of pathogens of woody tissues and to shape the scope of the project.

1.3.3.1 Sanitation

Many xanthomonad pathogens have a life cycle largely dependent on their host plant for survival, and crop residues and debris often serve as a source of inoculum (Mew & Natural, 1993). Thus removal of these residues from infected sites may prevent or reduce infection of subsequent crops. This practice has been suggested for the management of pistachio dieback although no data have been published to support this.

1.3.3.2 Use of antibiotics

Several antibiotics have been reported to inhibit the growth of bacteria, including *Xanthomonas* species. Streptomycin and ampicillin inhibited the growth of *X. campestris* pv. *oryzicola* (Adhikari & Mew, 1985) and *X. campestris* pv. *campestris* (Chang *et al.*, 1991). Rifamycin killed *X. campestris* pv. *malvacearum* (Al-Mousawi *et al.*, 1983). Growth of *Xtp* was inhibited by rifamycin, ampicillin and streptomycin *in vitro*; of these, streptomycin was inhibitory at the lowest concentration tested (Taylor & Edwards, 2000). This seemed a promising result for the management of pistachio dieback. However, the emergence of streptomycin-resistant strains of *E. amylovora* (Moller *et al.*, 1973; Manulis *et al.*, 1999), *X. campestris* pv. *citri* (Mew & Natural, 1993) and *X. campestris* pv.

vesicatoria (Knauss, 1972; Ritchie & Dittspongpitch, 1991) indicates the unsustainability of controlling diseases by antibiotics. In addition, the use of antibiotics to control bacterial plant disease is not permitted in northern Europe (European and Mediterranean Plant Protection Organisation, 2008) and Australia (R. Emmett, pers. comm., 2000) due to the risk of transfer of antibiotic resistance genes into human pathogens.

1.3.3.3 Use of inducers of resistance

Induced resistance is a natural defence mechanism in plants that is activated when the plant is attacked by a pathogen, non-pathogen or treated with chemicals (Van Loon *et al.*, 1998). It is first localised around necrotic lesions associated with pathogen infection or chemicals, then spreads systemically to the remaining parts of the plant. This spread of resistance throughout the plant's tissues is called systemic acquired resistance (SAR) (Agrios, 1997) and it confers a broad spectrum and long lasting immunity upon the whole plant (Sticher *et al.*, 1997). Salicylic acid is an important signal molecule involved in SAR (Ryals *et al.*, 1996; Durner *et al.*, 1997; Gullino *et al.*, 2000).

Taking advantage of these natural defence mechanisms, various chemical compounds that do not directly affect the pathogen, but trigger the plant defence reactions, have been used to activate the SAR response. This has proved to be effective against plant pathogens in a number of crops. For example, acibenzolar-S-methyl has been used to control a broad range of diseases of various crops caused by *Xanthomonas* spp. (Campbell & Wilson, 1999; Romero *et al.*, 2001), *E. amylovora* (Zeller & Zeller, 1999; Hassan & Buchenauer, 2007) and *Pseudomonas syringae* pv. *tabaci* (Cole, 1999). Also, phosphorous acid increased or induced host defence responses in citrus (Afek & Sztejnberg, 1989) and in tobacco, capsicum and cowpea (Guest & Bompeix, 1990) to *Phytophthora* spp..

Acibenzolar-S-methyl, phosphorous acid and salicylic acid have been tested as a means of eradicating *Xtp* from potted trees, but none appeared to eradicate the pathogen already present in the plants (Taylor & Edwards, 2000). The experiments failed to prove a role for these compounds in preventing infection of potted trees by *Xtp* due to a failure in the inoculation technique. However, phosphorous acid increased yield and reduced symptom expression when applied to foliage of mature trees in the orchard and provided short-term control of some symptoms when applied to foliage or by means of trunk injections.

1.3.3.4 Use of bactericides

Quaternary ammonium-based bactericides have proved effective in protecting pruning wounds from infection when used at the rate of 25% or higher (Sedgley *et al.*, 2006; Horticulture Australia Limited, 2006). Copper-based bactericides have also been widely used in the control of diseases caused by *Xanthomonas* species, but the application of copper after inoculation of pruning wounds with *Xtp* did not prevent infection of pistachio (Sedgley *et al.*, 2004; Taylor *et al.*, 2005). In addition, development of copper-resistant strains in *X. arboricola* pv. *juglandis* (Lee *et al.*, 1994), *X. axonopodis* pv. *vesicatoria* (Voludakis *et al.*, 2005), *X. campestris* pv. *vesicatoria* (Marco & Stall, 1983; Adaskaveg & Hine, 1985; Bender *et al.*, 1990; Cooksey *et al.*, 1990; Ritchie & Dittspongpitch, 1991), *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* (Behlau *et al.*, 2011) limits the use of this management strategy. Copper compounds have been used routinely and effectively in the control of fire blight (Loper *et al.*, 1991; Psallidas & Tsiantos, 2000). Resistance to copper has not been detected in *E. amylovora*, but tolerant strains have been reported in Syria (Al-Daoude *et al.*, 2009). In addition, the occurrence of spontaneous tolerance to low copper concentration amongst almost all *E. amylovora* strains isolated from fire blight cankers of pear trees in Washington suggested that development of copper resistance is

probable (Loper *et al.*, 1991). Furthermore, the observation that copper could induce a viable but nonculturable state (VBNC) in bacteria, including *Xanthomonas* (Ghezzi & Steck, 1999; Del Campo *et al.*, 2009) and *E. amylovora* (Ordax *et al.*, 2006; Ordax *et al.*, 2009), questions the effectiveness of copper-containing bactericides in the control of bacterial diseases in the field. The possibility that plant pathogenic bacteria may enter this VBNC state is discussed in more detail in section 1.5.

1.3.3.5 Hygiene during pruning

As *Xtp* has been reported to enter trees via openings such as pruning wounds and to be transmitted via pruning tools, an appropriate management strategy may be to prevent infection by strict hygiene during pruning. The current recommendation is to prune with simultaneous application of bactericide to reduce the spread of the pathogen. Felcomatic®-type pruners, which dispense bactericide when cutting, have been recommended and quaternary ammonium disinfectants have shown promise (Sedgley *et al.*, 2006). Disinfection of pruning tools between cuts has also been practised to prevent spread of *E. amylovora* from infected trees to healthy ones. Dipping cutting shears or knives in 70% ethanol for 20 min, or 15 seconds then flamed on both sides (Hasler *et al.*, 1996), 70% isopropanol (Beer & Rundle, 1987), 3% sodium hypochlorite, 4% lisetol or in hot water at 70°C for 5 min (Hasler *et al.*, 1996), effectively prevented spread of *E. amylovora.* However, sodium hypochlorite was reported to be corrosive and irritant and, for high concentration of pathogens on contaminated knives, only 50% sodium hypochlorite or concentrated Dettol® is effective (Psallidas & Tsiantos, 2000). In addition, hot water treatment cannot be applied in the field (Hasler *et al.*, 1996).

1.3.3.6 Drastic and clean pruning

The fact that the pistachio dieback pathogen appears to remain confined to the xylem present at the time of inoculation, with little or no lateral spread, suggested that severe pruning and disinfection of pruning cuts may control the disease (Sedgley *et al.*, 2004). In practice, severe pruning appears to be a promising management tool for infected trees as it promotes new growth, but it did not prevent the gradual re-establishment of the pathogen in the new structure of the trees (Taylor *et al.*, 2007). This practice may, however, be used together with other strategies to provide an integrated management strategy for pistachio dieback. Queiroz-Voltan *et al.* (2006) studied the efficacy of three pruning methods (traditional, "skeleton cut" and trunking) in the control of *Xylella fastidiosa* in commercial cultivars of coffee and suggested that drastic pruning methods ("skeleton cut" and trunking) might be helpful in controlling the pathogen when disease incidence is high.

1.3.3.7 Bud wood repository

Xtp has not been detected in buds of infected pistachio trees, suggesting that the incidence of *Xtp* in bud wood is rare or below the detection threshold (Taylor *et al.*, 2007) and thus propagation of trees through budding might be a safe method (Scott *et al.*, 2009). However, *Xtp* was detected, although at very low concentrations, in some healthy trees identified as candidates to establish a bud wood repository (Scott *et al.*, 2009). This precluded use of the trees from as a source of healthy material for propagation. Bud wood repositories are currently used for some tree crops in Australia, such as almond (http://www.australianalmonds.com.au, accessed 12/2011), apple and pear (http://www.apfip.com.au, 12/2011), and citrus (http://www.auscitrus.com.au, 12/2011).

1.3.3.8 Biological control

Research on microorganisms as biological control agents has revealed the potential of non-pathogenic fungi and bacteria in controlling plant diseases. Among antagonistic fungi, *Trichoderma* spp. have been extensively studied and reported to control soil fungi such as *Pythium* spp., *Rhizoctonia solani* and *Sclerotium rolfsii* (Weindling, 1932; Chet *et al.*, 1981) and fungal diseases, such as Eutypa dieback in vineyards (John *et al.*, 2005). Bacteria, such as *Agrobacterium* (Kerr, 1980; Li *et al.*, 2009), *Bacillus* (Jock *et al.*, 2002; Broggini *et al.*, 2005), *Pseudomonas* (Loper *et al.*, 2007; McSpadden Gardener, 2007) and *Streptomyces* (Emmert & Handelsman, 1999; Neeno-Eckwall *et al.*, 2001; Schottel *et al.*, 2001) have also shown potential in controlling fungal and bacterial diseases. Of these genera, *Bacillus* has been reported to inhibit growth of *Xanthomonas* pathogens, such as *X. campestris*, causing black rot in cabbage (Wulff *et al.*, 2002a; Wulff *et al.*, 2002b; Massomo *et al.*, 2004; Monteiro *et al.*, 2005) and *X. oryzae*, causing bacterial blight of rice (Lin *et al.*, 2001). *Pseudomonas* was also reported to inhibit growth of *X. oryzae in vitro* and suppress bacterial blight of rice in net-house and field experiments (Velusamy *et al.*, 2006). Both *Bacillus* spp. and *Pseudomonas* spp. may be endophytes, thus can grow in the vascular tissues of plants and have been reported to produce antibiotics (Emmert & Handelsman, 1999; Velusamy *et al.*, 2006; Nagórska *et al.*, 2007), siderophores (Leong, 1986; Marten *et al.*, 2000) and antimicrobial peptides (by *Bacillus*) (Lin *et al.*, 2001). As such, they might be promising biological control agents for pistachio dieback. Recently, eight bacterial isolates were assessed for their antagonism of *Xtp* and ability to reduce the colonisation of pistachio wood by *Xtp* at the University of Adelaide (Salowi, 2010). One isolate, tentatively identified as a *Pseudomonas* sp., had potential to inhibit *Xtp* and survive in excised twigs of pistachio. Another isolate, likely to be a *Bacillus* sp., reduced colonisation of twigs by *Xtp*.

In addition to antagonistic bacteria and fungi, antimicrobial proteins and peptides have also been explored for their use in plant disease control. These antimicrobial compounds are produced by plants (Badosa *et al.*, 2007), as part of their specific defence mechanisms, and by microorganisms such as bacteria (Monteiro *et al.*, 2005). However, properties of natural peptides, such as their long amino acid sequences, poor bioavailability and susceptibility to protease degradation, low stability and nonspecific toxicity, limit their use (Badosa *et al.*, 2007; Marcos *et al.*, 2008). Synthetic peptides were designed to overcome these limitations. Ferrer *et al*. (2006) reported four analogues of a natural peptide, which completely inhibited *X. axonopodis* pv. *vesicatoria* and one analogue with a strong bactericidal effect against this pathogen and *E. amylovora.* Recently, one synthetic peptide, BP 100, was reported to be effective in inhibiting infection of apple and pear flowers by *E. amylovora* (Badosa *et al.*, 2007) and *in vitro* growth of *X. axonopodis* pv. *vesicatoria*. When tested for ability to inhibit *Xtp in vitro,* BP100 delayed or reduced multiplication of *Xtp* in liquid medium (Salowi, 2010). However, its efficacy *in planta* warrants further studies.

1.3.3.9 Prospects for management

The above measures, even if proved to be effective, only help to protect pistachio trees against new infections or, in the case of drastic pruning, reduce, but do not eradicate the pathogen from infected trees. Although bacteria and peptides inhibited the growth of *Xtp in vitro*, their efficacy in controlling the pathogen *in planta* requires further investigations. It is, therefore, appropriate and timely to explore methods for the eradication of the pathogen.

1.4 Eradication of plant pathogens

Eradication is defined as "the elimination of every individual of a species from a geographic area that is sufficiently isolated to prevent reinvasion" (Myers *et al.*, 1998). It is one of three alternative responses, namely eradication, containment or no action, in the management of an incursion and may be attempted where export markets will be affected and success is likely (Sosnowski *et al.*, 2009). Eradication of plant pathogens often involves a number of steps, of which disposal of infected and suspect plant materials by burning and burial is recommended by the Australian Quarantine and Inspection Service (http://www.iwgq.com.au, 04/2007). Burning is preferred as it is thought to destroy affected materials and kill any pathogens the materials may harbour, while burial is employed when burning is not practical, such as for disposal of large volumes of juicy and fleshy produce (Ebbels, 2003). Hardison (1976), in his review on the control of plant disease by thermo-sanitation through application of fire and or flame, indicated a number of cases where burning had effectively controlled plant diseases. There have also been cases of successful eradication of plant pathogens involving burial (Daly & Rodoni, 2007). However, no information is available concerning the eradication of the pistachio dieback pathogen. Thus literature on the eradication of other pathogens of perennial crops by burning or burial following removal of infected plants, as well as on factors influencing mortality of pathogen during burning and burial, will provide a basis for research on the efficacy of the two eradication methods using pistachio dieback.

1.4.1 Eradication by burning infected plant materials

Fire blight is a bacterial disease of pome fruit that affects many plants in the family Rosaceae. The disease is wide-spread and has been reported from 40 countries around the world (Bonn & Van der Zwet, 2000). The causal agent is *E. amylovora*, a wood-inhabiting

bacterium and an exotic pathogen that is now listed as a high priority EPP in the Australian Apple and Pear Industry Biosecurity Plan (Plant Health Australia, 2011), thus it is a useful source of information for this project.

Destruction of diseased host plants from fire blight-affected areas has been reported as the only option to eradicate and contain the disease. Fire blight-affected plants have been removed and burned in Poland (Zandarski *et al.*, 2001) and Sweden (Gråberg, 1993). In addition, in Sweden, all non-symptomatic host plants within 5 m of an infected plant were destroyed. Reuveni *et al.* (1999) reported that burning the infected parts of apple trees *in situ* with propane gas was efficient in eliminating *E. amylovora*, and prevented the spread of the bacteria to the main limbs. Thus, burning before *E. amylovora* spreads to the main limbs might provide a safe and rapid measure to control fire blight. However, this strategy was not recommended as part of the fire blight management programme, called "Fire-Man", in Israel, as the heat created wounds on surviving limbs, which increased the risk of re-infection with *E. amylovora* (Shtienberg *et al.*, 2002). This direct burning method might not be appropriate for pistachio dieback as the disease occurs in the trunks. Therefore, conventional burning, that is removal of infected trees and tree materials from infected sites prior to burning, should be examined.

Another example of successful eradication of a bacterial pathogen from a woody perennial crop by burning is the case of citrus canker caused by *X. citri* in the USA. Citrus canker was first detected in Florida in 1910, and declared eradicated from Florida in 1933 after uprooting and burning all suspected and infected trees (Schoulties *et al.*, 1987; Das, 2003). The disease was discovered again in another county of Florida in 1986 and successfully eradicated in 1994 using the same method (Schubert *et al.*, 2001). Following an incursion in Northern Territory, *X. campestris* pv. *citri* (syn. *X. citri*) was successfully eradicated from Australia by burning pummelo trees (Broadbent *et al.*, 1992). In 1991, it

was again eradicated from Thursday Island in the Torres Strait region of Queensland by burning infected trees and all citrus trees within 15 m of diseased trees (Jones, 1991), and from Lambell's Lagoon near Darwin by burning all trees in an affected pummelo orchard *in situ* (Broadbent, 1995). Another outbreak occurred in 2004-2005 near Emerald in Queensland, and all movement of plant and plant-related material was immediately restricted. The disease was again eradicated by burning of infected citrus plants (Queensland Department of Primary Industry and Fisheries, 2004).

Despite the apparent success of burning of infected or exposed trees as an eradication strategy for bacterial pathogens, it is possible that pathogens may survive in ashes, unburnt plant debris or other air-borne debris (Murray, 1998; Sosnowski *et al.*, 2012). The fact that data on the survival of pathogens after burning are not readily available supports the need to demonstrate the effectiveness of this eradication method using the pistachio dieback pathogen as model.

A good understanding of the effect of temperature on the survival and growth of the pathogen is of crucial importance for the success of pathogen eradication by burning. Dye and Lelliot (1974) reported that the majority of xanthomonads grew at 35°C, but none grew at 40°C. Similarly, Koizumi (1976) found that bacteria could not be detected when inoculated leaves were incubated at 40°C for 48 h. For *X. campestris* pv. *citri*, temperatures of 48°C for 60 min or 51°C for 15 min are lethal to the bacteria in leaf lesions (Stall *et al.*, 1993). In a preliminary study of the effect of temperature on the growth of *Xtp* from pistachio, Taylor and Edwards (2000) found that the pathogen appeared to grow slowly at 40°C and 50°C. However, no data were provided on whether the pathogen could survive above 50°C, or what constitutes a lethal temperature for the pathogen. An understanding of the effect of higher temperatures and duration of exposure to these temperatures on the

survival of *Xtp* could be useful for eradication programmes by burning to avoid survival of the pathogen in unburned infected materials.

1.4.2 Eradication by burial of infected plant materials

Burial of infected plant materials has also been employed as a means of pathogen eradication. There is, however, limited literature on this eradication method for diseases of perennial crops. Successful eradication of *E. amylovora* in Australia by deep burial was reported (Daly & Rodoni, 2007). The disease was first detected in Australia in the Royal Botanic Gardens (RBG) of Melbourne in 1997 (Rodoni *et al.*, 1999; Jock *et al.*, 2000) and posed a threat to the Australian apple and pear industries. The outbreak was eradicated by destruction of all possible hosts in the RBG and within 250 m of the RBG boundary; foliage and wood were placed in sealed bags and buried 2 m deep at a site 30 km away from the RBG (Daly & Rodoni, 2007). Burial was also part of an integrated approach for prevention and control of citrus canker in Florida (Graham *et al.*, 2004). That eradication campaign involved cutting down infected or potentially exposed trees within 579 m before chipping wood into pieces. The debris was transported in a covered trailer to be buried in a landfill site. Escaping debris with viable cells was detected during the chipping process and emptying the trailers at the landfill, but the duration of survival of the bacteria in such debris was not tested. However, infected debris was thought not to spread the disease due to the absence of susceptible citrus in the vicinity as well as the small number of viable cells in the escaped debris (Graham *et al.*, 2004). Citrus canker spread widely in Florida after Hurricane Wilma in 2005 and became endemic (Gottwald, 2007).

Burial of infected plant materials leads to decomposition and exposes the pathogen to the soil environment. Thus, survival of the pathogen will depend on the rate of decay of infected material and its ability to live in soil. In general, *Xanthomonas* pathogens can

survive for a relatively long time when protected within plant debris (Boosalis, 1952; Brinkerhoff & Fink, 1964; Schaad & White, 1974; Graham *et al.*, 1987; Duffy, 2000). Populations eventually become undetectable in decomposed debris (Brinkerhoff & Fink, 1964). In addition, xanthomonads are generally considered not to survive or multiply in soil in a free state, for example, populations of *Pseudomonas citri* (syn. *X. citri*) declined rapidly and disappeared in non-sterile soils within 6 days (Lee, 1920) to 2 weeks (Fulton, 1920). Therefore, burial of infected wood could be a promising strategy to eradicate *Xtp*.

Available literature indicates that burying crop debris in soil contributes to greater decline in pathogen survival than when the debris is left on the soil surface (Brinkerhoff & Fink, 1964; Graham *et al.*, 1987; Duffy, 2000). A number of factors were shown to affect the survival of bacteria when debris was buried in soil. For instance, soil moisture played a significant role in reducing the survival of *X. campestris* pv. *citri* in diseased leaves, as bacterial survival was significantly reduced when soil was moistened compared with dry conditions (Duffy, 2000). Soil water potential directly affected leaf decomposition, possibly by promoting microbial activity, which accelerated the decline in bacterial populations in the leaf. Higher temperatures were also reported to speed up decomposition of plant debris by influencing microbial activity, so that *X. campestris* pv. *campestris* became less protected by host debris and therefore the population declined faster (Kocks *et al.*, 1998). In addition, survival of plant pathogenic bacteria in soil is likely to be inhibited by antagonistic microbiota (Fulton, 1920; Lee, 1920). Normal soil microbiota tend to be antagonistic to bacteria added to soil (Katznelson, 1940; Waksman & Woodruff, 1940). Patrick (1954) compared the antibiotic activity of 120 antagonistic isolates of bacteria, actinomycetes and fungi against a group of 28 selected bacterial plant pathogens. The results demonstrated that the number of isolates antagonistic to the different pathogens varied greatly and the ability of a species to survive in the soil might be related to the

difference in the number of antagonists. Patrick (1954) also found that the *Xanthomonas* species tested were sensitive to antagonistic soil microbiota.

There appears to be no published information on the duration of bacterial survival following burial of infected wood of a perennial crop. However, previous studies indicated that *X. campestris* pv. *campestris* survived for more than 334 days in cabbage stem residues (Schultz & Gabrielson, 1986). Using regression analysis, Schultz & Gabrielson (1986) estimated that the maximum survival of the pathogen in buried cabbage stems could be 507 days. Schaad and White (1974) found that the pathogen survived in buried cabbage stems for up to 224 days and the half-life of the pathogen was 13.7 days. Based on this, they estimated that the pathogen could survive in cabbage stems buried in soil for 615 days. Therefore, a burial period of at least 2 years for wood infected by *Xtp* should be considered to assess the effect of burial on the survival of the pistachio dieback pathogen. In addition, as *Xanthomonas* pathogens may survive for as long as the wood persists, the size of pieces of wood to be buried, soil moisture and temperature should be taken into consideration, as they are likely to influence decomposition of the infected wood and, as a result, could be manipulated to shorten survival time of the pathogen.

1.4.3 General considerations for disposal of affected materials

It is recommended that affected materials be disposed of as soon as possible and if this requires transportation to locations other than the affected site, the affected materials should be enclosed in plastic bags and sealed, then disposed of without prior opening (Ebbels, 2003). Sealable containers or high-side trucks, securely sheeted with impermeable covers, are recommended when transporting large amounts of materials, to avoid escape or spread of any affected materials and pathogens they may contain. Materials should be

buried at legally approved sites and covered with at least 2 m of soil to avoid disturbance by birds, animals or the elements (Ebbels, 2003).

1.5 Survival mechanisms of bacteria

Climatic conditions during burial may vary greatly between seasons or even day and night, and nutrient availability for pathogens in materials buried in or placed on soil might become limited over time. Plant pathogens must develop a mechanism to carry on survival under such adverse conditions. There is a growing body of evidence demonstrating ability of pathogens to adapt or cope with unfavourable conditions. Production of polysaccharide exudates, or ooze, and reduced metabolism are discussed below as possible mechanisms employed by plant pathogenic bacteria in response to changing or unfavourable conditions during burial. Understanding these survival mechanisms in bacteria might facilitate the decision on where to bury and duration required to eradicate the pathogen.

1.5.1 Production of polysaccharide exudate

Few plant pathogenic bacteria form endospores and many of them are not able to survive free in soil (Burkholder, 1948; Schuster & Coyne, 1974), so they depend on protection and food supply from infected plant tissues when in soil, or have evolved other mechanisms to survive. Many plant pathogenic bacteria produce polysaccharide exudate, or ooze, that is thought to behave like a hydrophilic colloid (Feder & Ark, 1951; Hedrick, 1956; Corey & Starr, 1957; Leach *et al.*, 1957). The water holding capacity of the polysaccharide might help bacteria survive in unfavourable conditions and seasons. Included in this group are *E. amylovora* (Hildebrand, 1939; Feder & Ark, 1951; Moore & Hildebrand, 1966) and *Xanthomonas* pathogens (Corey & Starr, 1957; Wilson *et al.*, 1965; Stall *et al.*, 1993), which produce polysaccharide exudate in infested host plants and in culture. *Xtp* is known

to produce polysaccharide slime *in planta* (E. Facelli, pers. comm., 2008), which might facilitate survival of *Xtp* in pistachio plant tissues until the tissues are completely decomposed.

1.5.2 Reduction in metabolism

1.5.2.1 Hypobiosis

Pathogenic bacteria might remain in a reduced metabolic state that allows them to survive long term in unfavourable conditions, such as desiccation. Leben (1981) called cells in this state "hypobiotic" and proposed that they could survive in soil due to their deep location within tissues that are resistant to decomposition. When a cell enters this state, it does not require nutrients (Stall *et al.*, 1993). Such a state could be induced by loss of water and unfavourable temperature, conditions which are likely to occur during burial or placement of infected pistachio wood on the soil surface.

1.5.2.2 Viable but nonculturable state

Another mechanism that may confer long-term survival for non-endospore-forming bacteria is the viable but nonculturable (VBNC) state, in which bacteria are alive but unable to divide sufficiently in or on routine bacteriological media to form colonies (Roszak & Cowell, 1987). A typical VBNC response is that cells lose culturability, but the total cell counts do not change and cells may remain viable for a long period of time (Oliver, 2010).

1.5.2.2.1 Factors inducing the VBNC state

Most of the information regarding physical, chemical and biological factors that induce the VBNC state is derived from *in vitro* experiments with human pathogens and aquatic

bacteria. Of the factors examined, temperatures outside the normal range for growth (Maalej *et al.*, 2004; Wong & Wang, 2004), nutrient deprivation (Cook & Bolster, 2007), aeration in the case of a microaerophile (*Campylobacter jejuni*) (Rollins & Colwel, 1986) and desiccation (Pedersen & Jacobsen, 1993) are reported to induce the VBNC state in human pathogens and aquatic bacteria. These factors are relevant for consideration in studying the possibility that plant pathogenic bacteria might enter the VBNC state and also for those in infected plant materials buried in or placed on soil.

1.5.2.2.2 Characteristics of VBNC cells

The first and most noticeable characteristic of a VBNC cell is the loss of culturability on artificial culture media. In addition, VBNC cells were reported to decrease in size, for example, from rod shape (culturable cells) to small cocci (nonculturable cells) (Nilsson *et al.*, 1991), which was thought to be a strategy to minimise cell maintenance requirements (Morgan *et al.*, 1991) or might represent a spore-like stage for non-endospore-forming bacteria (Roszak & Cowell, 1987). Density of ribosomal and nucleic acid material of VBNC cells in *Vibrio vulnificus* was observed to be reduced, but VBNC cells retained a normal cytoplasmic membrane and an external acidic polysaccharide capsule (Linder & Oliver, 1989). In addition, a number of metabolic and physiological changes were reported to occur in VBNC cells (Porter *et al.*, 1995; Oliver, 2000a; Signoretto *et al.*, 2002; Day & Oliver, 2004). However, the only information available on the characteristics of VBNC cells in plant pathogenic bacteria was reported for *E. amylovora*, in which partial increase in the thickness of the "external layer" of the VBNC cells was observed (Ordax *et al.*, 2006).

1.5.2.2.3 Detection of VBNC

Various methods have been used to demonstrate the nonculturable state in bacteria. Kogure *et al.* (1979) employed a direct viable count (DVC) method in which small amounts of yeast extract and nalidixic acid were added to sea water containing bacteria, which was then incubated for 6 h. In these conditions, bacteria did not divide but elongated, thus demonstrating the viability of cells. Another method used to assess the VBNC state was through the ability of the cells to reduce soluble 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan as an indication of metabolic activity (Zimmerman *et al.*, 1978). This method was reported to have advantages compared with the DVC method in that it did not involve nutrients and could be used to detect all cells with an active electron transport chain (Oliver, 1993). A third method, considered to be a potentially powerful tool in detecting nonculturable cells in natural environment, was the combination of DVC and the use of fluorescently labelled monoclonal antibodies (Xu *et al.*, 1982; Grimes & Colwel, 1986). In addition, flow cytometry (Morgan *et al.*, 1991) was used to detect and characterise cells of *Aeromonas hydrophila* during their nonculturable response to sterile river water. The authors observed a reduction in size and a change in shape of nonculturable cells. In the late 1990s, a fluorescence staining method, using the LIVE/DEAD® *Bac*light™ Bacterial Viability Kit, was used to estimate both total and viable counts of bacteria in drinking water (Boulos *et al.*, 1999). The method was reported to be reliable, rapid and easy to use and to give both total and viable counts in a single step. However, some factors, such as sensitivity of SYTO® 9, a nucleic acid binding stain that penetrates all bacterial membranes, to low pH and decreased membrane permeability to this stain or insufficient accumulation of stain in cells might influence the enumeration of viable bacteria using the *Bac*light™ kit. The method was employed in a number of studies to enumerate total and viable cells counts in VBNC populations of plant pathogens

(Alexander *et al.*, 1999; Grey & Steck, 2001; Ordax *et al.*, 2006), including *Xanthomonas* spp. (Ghezzi & Steck, 1999). This provides useful information for studies of VBNC in *Xtp*.

1.5.2.2.4 Resuscitation of cells in the VBNC state

The keystone of the VBNC hypothesis is the ability to recover culturability of non-culturable cells. However, whether the culturable cells appearing after removal of the inducing stress are a result of true resuscitation or represent regrowth of a few persistent culturable cells has long been debated. There has been evidence to support true resuscitation. Whitesides and Oliver (1997) conducted dilution studies, and clearly demonstrated that VBNC cells of *Vibrio vulnificus* were resuscitated following a temperature increase. Other examples, where resuscitation was claimed to occur, were for VBNC cells of *Salmonella enteritidis* following addition of heart fusion broth (Roszak *et al.*, 1984), *E. amylovora* after adding 10-fold diluted King's B broth (Ordax *et al.*, 2006) and *Ralstonia solanacearum* (Grey & Steck, 2001) in soil adjacent to plant roots. However, other researchers have demonstrated that the recovery of culturable cells of *V. cholerae* (Ravel *et al.*, 1995) and *V. parahaemolyticus* (Jiang & Chai, 1996) was due to regrowth. There are also examples in which resuscitation did not occur (Ghezzi & Steck, 1999; Arana *et al.*, 2007) or occurred only in the presence of culturable cells (Morgan *et al.*, 1991).

1.5.2.2.5 Virulence of cells in the VBNC state

Generally, pathogens are not able to initiate infection when in the VBNC state, but retain virulence and are able to cause infection after being resuscitated to the actively metabolising state (Oliver, 2010). For example, VBNC cells of *Vibrio harveyi* did not cause death when inoculated into zebra fish, but the resuscitated cells did (Sun *et al.*, 2008). However, other investigators have reported VBNC cells of *V. vulnificus* (Oliver &

Bockian, 1995) and *V. cholerae* (Cowell *et al.*, 1996) to be able to cause infection but that infectivity was exhibited only for a short time after the cells had become nonculturable. Similarly, Ordax *et al.* (2009) reported that VBNC cells of *E. amylovora* induced by copper could produce symptoms on inoculated pear in the first 5 days of being in a VBNC state. Resuscitated cells were pathogenic all the time, but the pathogenicity varied depending on the concentrations of copper. Virulence of VBNC cells, induced by copper, was also reported for *X. axonopodis* pv. *citri*, although only 1% of VBNC cells were able to produce canker lesions after infiltrating grapefruit plants (Del Campo *et al.*, 2009).

1.5.3 VBNC state in plant pathogenic bacteria

The number of bacterial species reported to enter the VBNC state has increased significantly since the first paper by Xu *et al.* (1982) on this physiological response of *Escherichia coli* and *V. cholerae* in the estuarine and aquatic environment. However, to date, only five species of plant pathogenic bacteria have been reported to enter this state, namely *Agrobacterium tumefaciens*, *Rhizobium leguminosarum* (Alexander *et al.*, 1999), *Ralstonia solanacearum* (Grey & Steck, 2001), *E. amylovora* (Ordax *et al.*, 2006; Ordax *et al.*, 2009) and two pathovars of *X. campestris* namely pv. *campestris* and pv. *citri* (Ghezzi & Steck, 1999; Del Campo *et al.*, 2009). These bacteria entered the VBNC state in the presence of copper in nutrient-free microcosms (for all), autoclaved soil (*X. campestris* and *R. solanacearum*), *in planta* (*R. solanacearum*) or mature apple fruit calyces (*E. amylovora*). *R. solanacearum* was the only plant pathogen reported to enter the VBNC state following incubation of cells in a water microcosm at low temperature (van Overbeek *et al.*, 2004). Whether *Xtp* is able to enter the VBNC state requires investigation.

1.6 Summary and objectives

Xtp is a new pathogen that, so far, has been reported only in Australia. Techniques for detection and management have been developed, but the origin of the pathogen and the means of disease spread and survival in nature are still to be explained. However, its endemic status and ability to persist long-term in wood make it a good candidate as a model system for developing an eradication programme for plant bacterial pathogens. Furthermore, safe means of disposal of infected materials might protect the Australian pistachio industry by preventing further spread and loss caused by the disease. There are various examples to draw upon in considering eradication methods for plant pathogens that might arrive in Australia as well as for *Xtp* should it occur and affect previously unaffected or newly established pistachio orchards. However, information from past eradication programmes on temperature and duration of incineration, duration of burial and whether the pathogens were completely eradicated during burning and burial processes, is insufficient. Therefore, the overall aim of this study was to evaluate the effectiveness of burning and burial as means of disposal of diseased woody materials, using pistachio wood infected by *Xtp*. The objectives were to examine if *Xtp* could (i) survive in burned or partially burned wood; (ii) survive in infected plant materials buried in or placed on the soil; (iii) become viable but nonculturable as a response to changing conditions during burial.

Chapter 2 General materials and methods

2.1 The dieback pathogen

Xtp strain DAR 75532, belonging to Group A and obtained from a diseased pistachio tree in Kyalite, New South Wales (Facelli *et al.*, 2005), was used in experiments where a pure culture of the pathogen was required. The strain had been stored in sucrose peptone broth (SPB) supplemented with 30% glycerol at -80°C since 2005. Prior to each experiment, *Xtp* was revived from storage at -80°C by streaking a loopfull of the stock suspension on sucrose peptone agar (SPA) (Appendix A) in duplicate and plates were incubated at 28°C in the dark for 3 days. The strain was confirmed to be pathogenic to pistachio in 2007 (Scott *et al.*, 2009) and later produced internal staining in woody tissues of confirmed disease-free pistachio twigs when artificially inoculated by vacuum filtration or applied as cell suspension to fresh wounds on branches of young trees (Kritzman, 2010, personal communication). *Xtp* was subsequently re-isolated from the inoculated twigs and trees.

2.2 Culture media

Two culture media, SPA and nutrient agar (NA) (Appendix A) were used at the same time or individually depending on experiments. SPA is a suitable medium for the genus *Xanthomonas* (Hayward, 1960; Moffett & Croft, 1983) and NA is a general medium for culturing bacteria (Dhingra & Sinclair, 1995). SPA amended with 150 mg L^{-1} Benlate® (BSPA) (Facelli *et al.*, 2005) and supplemented with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin (ABSPA) (Sedgley *et al.*, 2004) was initially used for isolation of *Xtp* from wood taken from infected trees and wood buried in soil or placed on the soil surface for a few months. Subsequently, NA supplemented with antibiotics

(NA+A) (Appendix A) was developed to facilitate the isolation of viable *Xtp* with minimal growth of other microorganisms associated with buried and burned wood (see Section 3.2.5).

In aseptic conditions, approximately 18 - 20 mL of molten medium were dispensed into each 90-mm diameter plastic Petri dish and allowed to cool. Plates without antibiotics were stored at room temperature and those with antibiotics were stored at 4°C in a cold room.

2.3 Methods for enumerating *Xtp*

Two methods were used to enumerate *Xtp* depending on the experiment, namely standard plate count (SPC) (Brown, 2009) and the method of Miles and Misra (1938). For the SPC method, aliquots of 100 μL of suspension were pipetted onto a culture medium and spread evenly on the agar surface using a glass spreader. For the Miles and Misra method (1938), three drops of 10-μL aliquots were pipetted individually onto a culture medium and left to dry in a laminar air flow cabinet. The plates were then incubated inverted at 28°C in the dark and colony forming units (CFU) were enumerated at 3, 7 and 14 days.

2.3.1 Enumeration of *Xtp* in suspension

Suspensions of *Xtp* for use in experiments were prepared by transferring two loopsfull of 3-day-old colonies growing on SPA into sterile 10-mL screw-capped centrifuge tubes (Sarstedt, Australia) containing 10 mL of an appropriate culture broth, depending on the experiment. The tubes were shaken gently by hand before incubating on an orbital mixer (Ratex Instruments, Australia) at 100 rpm at 28°C overnight. Where appropriate, total cell number in the resulting suspensions was estimated with a haemocytometer (Weber,

England) before use and CFU were determined by culturing as described above. The suspensions were used immediately after preparation.

2.3.2 Enumeration of *Xtp* in wood

Enumeration of *Xtp* was used to confirm the presence of the pathogen in wood prior to each experiment and to assess survival of *Xtp* in samples from experiments involving burial and burning of wood. The wood was processed using a technique modified from Facelli *et al*. (2005) as follows. Suspensions were obtained by soaking wood tissue (0.5 - 0.7 g) in 9 mL of sterile distilled water (SDW) overnight at room temperature. CFU were enumerated on NA+A using the SPC method for samples from burial and burning experiments, or the Miles and Misra (1938) method for determining initial populations in experiments on effect of temperature on survival. Pale yellow colonies were counted where appropriate and representative colonies were confirmed as *Xtp* by streaking on SPA for characteristic yellow mucoid colonies (Facelli *et al.*, 2005) or by PCR (see below).

2.4 PCR-based technique for detection of *Xtp*

2.4.1 Detection of *Xtp* in wood

Suspensions from infected wood were prepared as described in section 2.3.2. The suspensions were then transferred into sterile centrifuge tubes (1.5 mL) and centrifuged at 10,000 rpm for 10 min to yield a pellet, which was then resuspended in 15 µL of SDW. Aliquots (1 µL) of the resulting extracts, undiluted or diluted in SDW at 1:100 or 1:500, were used in PCR with strain-specific primers as described by Marefat *et al*. (2006a), and or using genus-specific primers as described by Maes *et al.* (1996). A pure culture of *Xtp* isolate DAR 75532 was used as a positive control. The PCR was performed in a total

volume of 20 μL, which consisted of 19 μL of master mix (see below) and 1 μL of extract. All reactions were carried out in a Peltier Programmable Thermal Controller model PTC-100™ (MJ Research Inc., CA, USA). The PCR products were analysed by electrophoresis on a 2% agarose gel for 20 min at 200V, stained with ethidium bromide and visualised under UV light.

2.4.1.1 Detection of *Xtp* using genus-specific primers

This PCR was designed to detect all *Xanthomonas* yielding a specific product of 139 bp (Maes *et al.*, 1996). The master mix (Maes *et al.*, 1996) contained 2 μL 10x buffer, 0.16 μL deoxynucleotide triphosphates (dNTPs) mixture (25 mM each, Fisher Scientific, Australia), 0.8 and 0.6 T1 and T2 primers (10 μM each) (Geneworks Pty Ltd, Australia), respectively, 0.6 μL $MgCl_2$ (50 mM), 0.2 μL HotStarTaq™ Polymerase (5U μL L^{-1}, Qiagen Pty Ltd, Australia) and 14.64 μL water. PCR was performed with the following conditions: 1 x 95°C for 15 min, 35 x (93°C for 30 s, 60°C for 45 s, 72°C for 1 min) and 1 x 72°C for 10 min. The sequences of the *X. translucens*-specific primers are T1, 5'-CCGCCATAGGGCGGAGCACCCCGAT-3', and T2, 5'-GCAGGTGCGACGTTTGCAGAGGGATCTGCAAATC-3'.

2.4.1.2 Detection of *Xtp* using strain-specific primers

The PCR was designed for the detection of both *Xtp* A and B in a multiplex assay, each strain giving a different size of PCR product (Marefat *et al.*, 2006a). In this experiment, only *Xtp* A was used, which is expected to give a specific product of 331 bp. The master mix contained 2 μL 10x buffer, 0.16 μL deoxynucleotide triphosphates (dNTPs) mixture (25 mM each, Fisher Scientific, Australia), 0.8 μL each of forward primer specific to the A strain (PAf), forward primer specific to the B strain (PBf) and reverse primer specific to

the A and B strains (PABr) (Geneworks Pty Ltd, Australia), 1.6 μL polyvinylpyrrolidone (PVP), 0.1 μL HotStarTaq™ Polymerase (5U μL L^{-1}, Qiagen Pty Ltd, Australia) and 12.74 μL sterile mili-Q water. PCR was performed with the following conditions: 1 x 95°C for 15 min, 40 x (94°C for 45 s, 60°C for 45 s, 72°C for 2 min) and 1 x 72°C for 10 min. Primer sequences for the specific detection of *Xtp* are presented in Table 2.1.

Table 2.1 Sequences of primers for detection of pistachio dieback pathogens (Marefat *et al.*, 2006a)

Primer	Direction	Sequence
PAf	Forward primer for A strain	5'-CCTCCTTTTGAGCATGAGAA-3'
PBf	Forward primer for B strain	5'-ACAGTCTAAGGGACCTGCG-3'
PABr	Reverse primer for both strains	5'-TCACTGCTGGCGCATCTTA-3'

2.4.2 Confirmation of colonies as *Xtp*

The PCR-based technique using strain-specific primers was used to confirm the identification of *Xtp*-like colonies isolated from inoculated, naturally infected wood, burning and burial samples on culture media. The PCR-based technique with genus-specific primers was used, when required, to confirm *Xtp*-like colonies to genus level. *Xtp*-like colonies were transferred, using a sterile toothpick, into 1.5 mL sterile centrifuge tubes containing 15 μL of SDW. Aliquots of 1 μL of the suspensions were then used in the PCR as described above.

2.5 Artificial inoculation of pistachio twigs

Pistachio twigs (7 to 10 mm diameter) were collected from healthy, non-inoculated grafted trees (cultivar Sirora, propagated in 2006) growing in pots in the shade-house at the University of Adelaide's Waite Campus, Urrbrae (34°58'.76" S, 138°38.38" E, elevation 145 m), South Australia or from disease-free trees (cultivar Sirora, aged 33 years) in a commercial orchard at Saddleworth (34°05'.02" S, 138°46'.70" E, elevation 324 m), South Australia. An inoculation method developed by Professor Giora Kritzman (per. comm., 2009) and described in Salowi (2010) was used with modifications as follows. The twigs were first surface sterilised by spraying with 70% ethanol, left to dry on paper towel in a laminar flow cabinet, then cut into pieces 70 mm long. The twigs were inoculated twice. First, 125-µL aliquots of *Xtp* suspension were placed into a sterile plastic Petri dish. Then the proximal end of an excised twig was attached to a plastic tube of 10 mm diameter, itself attached at the other end to a vacuum pump (HETOSUC®, Scandinavia). The distal end was placed in contact with the *Xtp* suspension and the vacuum was applied for a few seconds to allow the entire aliquot to be taken up and liquid was first seen at the other end of the twig. The twigs were then placed in Petri dishes for at least 30 minutes in order for the suspension to be absorbed before the second inoculation was conducted. In the second inoculation, the twigs were inverted and 125 µL of *Xtp* suspension was applied in the same way, through the proximal end of each twig. However, this time, a shorter period of vacuum pressure was applied so that the suspension was sucked in slowly to avoid it leaking from the other end. For twigs less than 10 mm diameter, the twigs were attached to the tube using Parafilm (Pechiney Plastic Packaging Company, Chicago, USA) to improve suction. The inoculated twigs were placed in plastic Petri dishes, sealed with Parafilm and incubated at 28°C for 10 days in darkness. CFU were enumerated using the method of Miles and Misra (1938) to confirm the presence of *Xtp* prior to each experiment as

described in section 2.3. Twigs confirmed to be infected were used in burial experiments and temperature assays conducted in 2010.

2.6 Statistical analysis

Data from experiments were subjected to analysis of variance (ANOVA) using GenStat version 11.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2010) unless otherwise stated. Where appropriate, other statistical analyses such as paired t-test (Sokal & Rohlf, 2000), geometric mean (Crawley, 2007) or G-test (Sokal & Rohlf, 2000) were used.

Chapter 3 Development of a culture medium for isolation of *Xanthomonas translucens* pv. *pistaciae* from wood exposed to soil

3.1 Introduction

Recovery of *Xanthomonas* from various types of samples, particularly plant materials exposed to soil, requires selective or semi-selective media. Evaluation of a semi-selective or selective medium is often based on its efficacy in recovering a target pathogen on the medium (plating efficiency) and the ability of the medium to exclude or suppress growth of unwanted microorganisms (selectivity) (Klement *et al*., 1990). A good semi-selective or selective medium should possess high plating efficiency and good selectivity. Most selective media involve the use of antibiotics alone (Randhawa & Schaad, 1984; Schaad & Forster, 1985) or in combination with other inhibitors such as surfactant (McGuire *et al*., 1986) and or dye (Claflin *et al*., 1987; Graham *et al*., 1987; Duffy, 2000) or fungicide (Sikirou & Wydra, 2004). Others are based on the ability of a particular material to alter the permeability of the cell membrane and cell wall preferentially to allow the growth of the target microorganism over that of others (Kado & Heskett, 1970). A list of semi-selective and selective media developed for the isolation of *Xanthomonas* is presented in Table 3.1.

Table 3.1 Semi-selective and selective media for isolation of *Xanthomonas* from different types of material.

Medium	Components	Target pathogen	Types of samples	References
Tween C medium	Peptone KBr $CaCl_2$ Boric acid Tween 80 Cycloheximide Cephalexin 5-Fluorouracil Tobramycin Methyl green	*X. campestris* pv. *vesicatoria* *X. campestris* pv. *citri*	Soil Infected citrus leaves and infested soil	McGuire *et al*. (1986) Graham *et al.*(1987)
D5 medium for *Xanthomonas*	Cellobiose K_2HPO_4 NaH_2PO_4 NH_4Cl $MgSO_4.7H_20$	*Xanthomonas* spp. *X. fragariae* *X. campestris*	Mixed population of bacteria Diseased leaves Diseased chinese cabbage leaves, soil and diseased tissue	Kado & Heskett (1970) Kado & Heskett (1970) Kado & Heskett (1970)

XTS	Nutrient agar Glucose Cycloheximide Gentamycin Cephalexin	*X. campestris* pv. *translucens*	Seeds of wheat	Schaad & Forster (1985)
YDC (Yeast extract dextrose carbonate)	Yeast extract D-glucose $CaCO_3$	*Xanthomonas* spp.	Isolation and maintenance	Lelliot & Stead (1987)
Benlate sucrose peptone agar (BSPA)	Benlate Sucrose Bacteriological peptone K_2HPO_4 $MgSO_4.7H_2O$	*Xanthomonas translucens* pv. *pistaciae*	Diseased pistachio materials	Facelli *et al.* (2005)
Antibiotic benlate sucrose peptone agar (ABSPA)	BSPA Ampicillin Cephalexin Gentamycin	*Xanthomonas translucens* pv. *pistaciae*	Diseased pistachio materials	Sedgley *et al.* (2004)

To study the effect of burial of infected materials on survival of *Xtp*, an experiment was set up in 2008 in which infected branch segments were enclosed in mesh bags prior to burying in or placing on the surface of pots filled with orchard soil in the Waite Campus orchard at the University of Adelaide (reported in Chapter 5). The wood was retrieved monthly to assay for survival of *Xtp* over time. Initial attempts to isolate *Xtp* from wood in contact with soil for the first 3 months of the experiment using ABSPA (Sedgley *et al.*, 2004) were confounded by overgrowth of the pathogen by soil- or wood-associated microorganisms. Therefore, experiments were conducted with the aim of developing a medium for the isolation of *Xtp* from pistachio wood segments that had been exposed to soil.

3.2 Materials, methods and results

For all experiments, wood samples were taken from mesh bags buried in or placed on the soil in pots located in the Waite Campus orchard.

3.2.1 BSPA, Tween C medium and NA plus crystal violet

BSPA (Table 3.1), Tween C medium (McGuire *et al.*, 1986) without 5-fluorouracil and tobramycin, and NA (Appendix A) amended with crystal violet (Merck) (NA-CV) at a concentration of 2 ppm (Bakerspigel & Miller, 1953; Naseri, 2006) were first selected to test for efficacy in isolating *Xtp* from wood exposed to soil. Tween C medium was selected for its reported efficacy in isolating *X. campestris* pv. *vesicatoria* from soil (McGuire *et al.*, 1986), and *X. campestris* pv. *citri* from infected citrus leaves and infested soil (Graham *et al.*, 1987). NA-CV was selected based on observations from a study on wood-associated soil microbiota in this project that few soil bacteria grew on this medium.

Bacterial suspensions were prepared by incubating infected wood tissue in sterile phosphate buffer 1 (Graham *et al.*, 1987) containing 5.8 g L^{-1} Na_2HPO_4, 3.5 g L^{-1} KH_2PO_4 and 0.1% peptone (Oxoid) or phosphate buffer 2 (McGuire *et al.*, 1986) containing 8.6 g L^{-1} Na_2HPO_4, 5.3 g L^{-1} KH_2PO_4 and 1 g L^{-1} Bacto peptone (Oxoid) and shaking for an hour. The resulting suspensions were serially diluted to 10^{-2} and CFU were enumerated in duplicate on BSPA, Tween C medium and NA-CV. Single *Xtp*-like colonies were streaked on SPA to observe for the typical yellow and mucoid appearance. Suspensions, undiluted to 10^{-2} dilution, of pure culture of *Xtp* were spread on the test media as controls.

Colonies of *Xtp* did not grow on BSPA, Tween C medium or NA-CV from suspensions prepared from wood tissue, regardless of the dilution. Numerous white colonies were observed spreading on the plates. There were also *Xtp*-like colonies, but they did not have the mucoid appearance typical of *Xtp* when subcultured onto SPA. Suspensions prepared from pure cultures of *Xtp* (controls) gave rise to colonies on all the test media, which suggested that either the population of *Xtp* in the samples was too small to allow detection by the cultural method or the presence of fast growing bacteria inhibited the growth of *Xtp*.

3.2.2 BSPA, Tween C medium, NA-CV and ABSPA

To examine if the presence of other bacteria impeded the recovery of *Xtp* on media tested in 3.2.1 and if incubation of woody tissues in antibiotics prior to spreading suspensions on the test media could reduce the growth of such bacteria, another experiment was conducted. As well as BSPA, Tween C medium and NA-CV, ABSPA, used initially in attempts to isolate *Xtp* from wood buried in or on soil in the first 3 months, was also included for comparison and to re-examine its efficacy in the isolation of *Xtp* from wood. Suspensions were prepared by incubating infected wood tissue overnight at room

temperature in either SDW alone, SDW amended with the three antibiotics used in ABSPA (10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 1.4 mg L^{-1} gentamycin) at half strength, or in phosphate buffer 1 and 2, with shaking for 1 h. The resulting suspensions were diluted as above and 100-µL aliquots of each of the dilutions were spread separately in duplicate on ABSPA, Tween C medium and NA-CV for the suspensions in SDW and phosphate buffers 1 and 2, and on BSPA and NA-CV for suspensions in SDW with antibiotics. Single *Xtp*-like colonies were subcultured onto SPA and the resulting colonies subjected to PCR with strain-specific and genus-specific primers to confirm whether they were *Xtp*. Suspensions, undiluted to 10^{-2} dilution, prepared from pure cultures of *Xtp*, prepared as for suspensions from wood but without antibiotics, were spread on the test media as controls.

Suspensions of *Xtp* (controls) spread on each test medium gave rise to colonies of *Xtp* on BSPA, Tween C medium and NA-CV but only a few colonies developed on ABSPA. Suspensions prepared from wood and spread on BSPA, ABSPA, Tween C medium or NA-CV failed to give rise to colonies of *Xtp*. On some ABSPA plates, numerous non-*Xtp* bacterial colonies were observed. *Xtp*-like colonies were observed on Tween C medium and NA-CV, but none gave rise to colonies typical of *Xtp* when subcultured onto SPA nor did they give positive reaction in the PCR with either set of primers. Soaking wood in SDW amended with antibiotics did not reduce the growth of other bacteria on plates.

These results indicated that the media tested were not suitable for studying survival of *Xtp* in infected wood that had been in contact with soil. Thus, there remained the need to develop an alternative medium for this purpose. The poor ability of ABSPA to allow recovery of *Xtp* from suspensions prepared from pure cultures suggested that the antibiotics or the concentrations in the medium inhibited or prevented the growth of *Xtp.*

3.2.3 BSPA supplemented with antibiotics

Pathovars of *X. campestris* have been reported to respond differently to gentamycin. While gentamycin at less than 1 µg mL^{-1} and at 4 µg mL^{-1} inhibited the growth of *X. campestris* pv. *campestris* (Randhawa & Schaad, 1984) and *X. campestris* pv. *phaseoli* (Claflin *et al.*, 1987), respectively, it did not inhibit the growth of *X. campestris* pv. *translucens* at 8 - 10 µg mL^{-1} (Schaad & Forster, 1985). As suspension of pure culture of *Xtp* grew poorly on ABSPA, as described in section 3.2.2, the effect of gentamycin on growth of *Xtp* was assessed using BSPA amended with (a) 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and gentamycin at 1.4, 1.0, 0.7 or 0.5 mg L^{-1} and (b) each of the four concentrations of gentamycin alone. First, growth of *Xtp* on each modified BSPA medium was tested by inoculation with pure cultures of *Xtp* prepared from two sources. Culture 1 was obtained by streaking on a BSPA plate a loopfull of bacteria that had been subcultured weekly on SPA and maintained at 28°C in darkness. Culture 2 was prepared by streaking on another BSPA plate a loopfull of stock suspension stored at -80°C. The plates were then incubated at 28°C for 2 days in darkness. Two loopsfull of bacteria of each culture were then incubated in SDW, sterile nutrient broth (NB, Oxoid) or phosphate buffer 1 or 2, diluted to 10^{-4} and 100-µL aliquots of the dilutions were spread in duplicate on each of the test media, or on BSPA as a control. The plates were assessed from 2 to 14 days after inoculation.

Xtp from suspensions prepared from the two sources of culture grew well on BSPA. From suspensions of *Xtp* culture 1 spread on each medium, growth was observed only on BSPA amended with gentamycin at 0.5 mg L^{-1}, but only one colony was observed per plate. Growth of *Xtp* from the suspension of culture 2 was observed on BSPA plates amended with gentamycin at 0.5 and 0.7 mg L^{-1}, ranging from 3 to more than 30 colonies per replicate plate. Colonies were not observed on BSPA plates with gentamycin at 1 and

1.4 mg L^{-1}. While an effect of the age of culture on the recovery of *Xtp* was not obvious, gentamycin seemed to inhibit the recovery of *Xtp*, especially at concentrations of 1 and 1.4 mg L^{-1}.

3.2.4 BSPA with antibiotics, D5 and Tween C medium

BSPA with antibiotics was tested for use in isolating *Xtp* from wood retrieved from the burial experiment by spreading aliquots of bacterial suspensions resulting from infected wood on BSPA amended with each of the following: 0.5 or 0.7 mg L^{-1} gentamycin, 10 mg L^{-1} cephalexin or 1 mg L^{-1} ampicillin. Growth on these media was compared with that on BSPA amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin, D5 for *Xanthomonas* (Kado & Heskett, 1970) and Tween medium C. Suspensions of 3-day-old pure culture of *Xtp* were spread on the test media and on BSPA as controls.

On control plates, undiluted suspensions of *Xtp* prepared in SDW gave rise to colonies on BSPA plates, regardless of whether the antibacterial antibiotics were absent, added together or alone into the medium. Lower concentrations of *Xtp* (10^{-1} and 10^{-2} dilutions) failed to give rise to colonies on any plates with antibiotics. Suspensions of *Xtp* prepared in SDW, undiluted or diluted to 10^{-1}, spread on Tween C plates resulted in colonies but the 10^{-2} dilution did not. All three dilutions resulted in colonies on BSPA with antibiotics and Tween C medium when the suspensions were prepared in NB. Suspensions prepared in SDW or NB failed to give rise to colonies on D5 medium. Suspensions from wood spread on the test media failed to give rise to colonies of *Xtp*, but growth of other bacteria was observed.

The results indicated that D5 was not suitable for the growth of *Xtp* and, as such, the isolation of *Xtp* from woody materials. Although BSPA amended with antibiotics and

Tween C allowed recovery of *Xtp*, their inability to recover the pathogen when at lower concentrations and to suppress the growth of other bacteria from wood that had been in contact with soil made them unsuitable for isolating *Xtp* from environmental samples.

3.2.5 XTS, yeast-extract dextrose calcium carbonate (YDC), NA and NA amended with antibiotics

XTS (Schaad & Forster, 1985) and YDC (Lelliott & Stead, 1987), reported suitable for isolation of xanthomonads, were compared with NA amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin (NA+A) and NA without the antibiotics. Wood pieces were incubated in 9 mL of SDW or Luria-Bertani (LB) (Sambrook & Russell, 2001) (Appendix A) overnight at room temperature, the resulting suspensions were serially diluted to 10^{-2} and 100-µL aliquots of each dilution were spread on each of these media. Suspensions of 3-day-old pure cultures of *Xtp* in SDW and LB medium were used as controls. Plates were assessed as described above. The experiment was repeated for NA, NA+A and XTS with wood pieces incubated only in SDW overnight. Resulting suspensions were serially diluted to 10^{-3} and 100-µL aliquots of each dilution were spread on NA, NA+A and XTS.

Recovery of *Xtp* from wood and pure culture suspensions in SDW and LB broth is summarised in Table 3.2. Observation and identification of *Xtp* on the YDC plates was not possible due to the opalescence of the medium, so this medium was not considered any further. In general, *Xtp* grew better on NA+A than XTS, even at the dilution of 10^{-2}, regardless of whether it originated from wood or pure culture. In addition, single colonies on NA+A were more defined in shape, as they grew separately, while colonies on XTS were more convex, mucoid and sometimes several merged. Colonies from NA, NA+A and XTS were confirmed to be *Xtp* by subculture on SPA and by PCR. Colonies of other

microorganisms were still observed on all three media, more so on NA than the other media, but in all cases fewer than on the media described in sections 3.2.1-3.2.4. From the 10^{-2} dilution spread on NA+A and XTS, only a few other bacterial colonies were observed on the plates while *Xtp* was abundant.

In the repeated experiment, *Xtp* was recovered on all control plates of all three media. Suspension from infected wood tissue did not give rise to colonies of *Xtp* on XTS, but yielded colonies on NA and NA+A plates, although fewer than in the first experiment. *Xtp* was not recovered on NA and NA+A inoculated with dilution 10^{-3} of the suspension prepared from woody tissue.

Plating efficiency of NA+A was determined by comparison with nutrient agar (NA) as a standard medium and was assessed by CFU counts of 10-fold serial dilutions from three replicate plates. Cell suspensions were prepared by incubating two loopsfull of 3-day-old culture of *Xtp* in SDW overnight and resulting suspensions of *ca.* 10^8 CFU mL^{-1} were then diluted to 10^{-7}. Aliquots of 100 µL of each dilution were spread in triplicate on NA+A and NA. The plates were incubated at 28°C and CFU were enumerated from 3 to 14 days. Dilutions yielding more than 30 CFU per plate were counted. The plating efficiency was expressed as percentage of number of CFU recovered on NA+A over number of CFU recovered on NA (Klement *et al.*, 1990). The experiment was repeated once.

Xtp was recovered from all dilutions on both NA+A and NA (Fig 3.1), and the plating efficiency of NA+A ranged from 90 to 100%.

Table 3.2 Recovery of *Xanthomonas translucens* pv. *pistaciae* from suspensions prepared from 3-day-old pure culture and from woody tissues on: nutrient agar (NA); nutrient agar amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin (NA+A); XTS; and yeast extract dextrose carbonate (YDC) medium.

Medium	Diluent[a]	Undiluted		10^{-1}		10^{-2}	
		Wood[a]	Control	Wood	Control	Wood	Control
NA	SDW	++++[b]	+++++	+++	++++	++	+++
	LB	++	+++++	+	++++	+	+++
NA+A	SDW	++++	+++++	+++	++++	++	+++
	LB	++	+++++	+	++++	+	+++
XTS	SDW	+++	++++	++	+++	+	++
	LB	-	++++	-	+++	-	++
YDC	SDW	n/a[c]	n/a	n/a	n/a	n/a	n/a
	LB						

[a]Diluents: SDW = sterile distilled water; LB = Luria-Bertani broth.

[b]Colonies per plate: +++++ = colonies merging and too many to count; ++++ = >2000 colonies per plate; +++ = ≥1000-2000; ++ = ≥100-<1000; + = ≥30-<100; and - = no growth of *Xtp*. Data are means of three replicate plates.

[c]n/a: colonies not discernable due to opalescence of medium or plates contaminated.

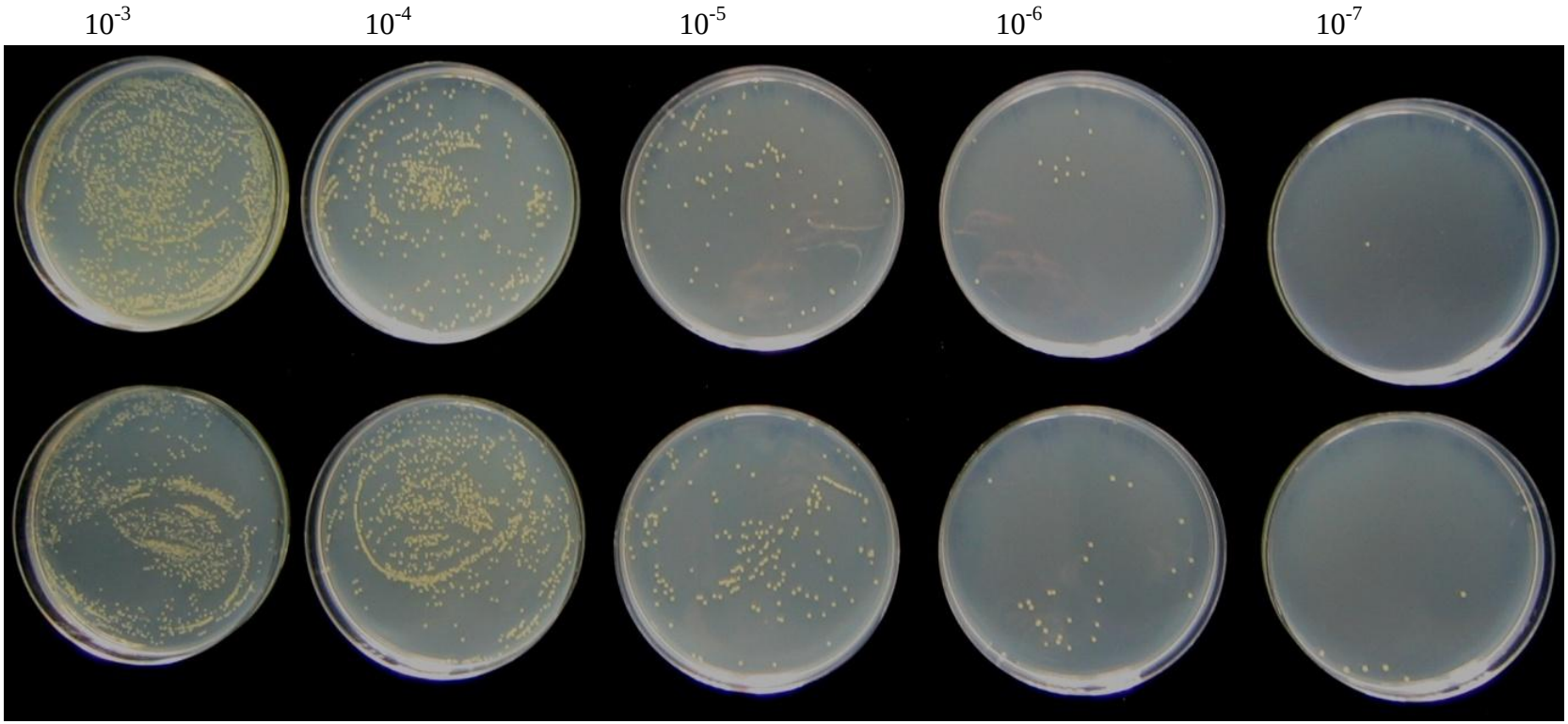

Figure 3.1 Colonies of *Xanthomonas translucens* pv. *pistaciae* on nutrient agar (NA) amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin (NA+A, top row) and on NA (bottom row). From left to right, five sequential ten-fold dilutions of a suspension prepared from a pure culture.

3.3 Discussion

NA+A was determined to be a suitable semi-selective medium for the isolation of *Xtp* from wood, on the basis of its selectivity and high plating efficiency (90-100%) to recover *Xtp* from infected wood materials that had been in contact with soil. The antibiotics used in NA+A were those in ABSPA, which was developed by Sedgley *et al.* (2004) from XTS (Schaad & Forster, 1985), for the isolation of *Xtp* when fast-growing bacteria might be present. NA+A differed from ABSPA and XTS in that it did not contain sucrose, whereas ABSPA and XTS contained sucrose and glucose, respectively. In addition, gentamycin used in NA+A was half of the concentration in ABSPA.

The development of a semi-selective medium for the isolation of *Xtp* from wood in this study was based initially on the evaluation of semi-selective media previously reported by other researchers to be effective in isolating other *Xanthomonas* species from environments such as soil and plant materials in contact with soil. Although suspensions prepared from pure cultures of *Xtp* gave rise to colonies on all the media tested, except for D5, none of them proved to be effective in isolating *Xtp* from pistachio wood buried in or placed on the soil surface.

Development of NA+A was based on the observations that when subculturing bacteria isolated from soil or wood on NA to obtain pure cultures for testing antagonism, many of them grew slowly and colonies were much smaller than if they were plated out on (B)SPA. The addition of 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin appeared to promote the growth of *Xtp* and assist in suppressing growth of other microorganisms. When compared with XTS, another selective medium for *Xanthomonas*, NA+A proved to be more consistent in isolating *Xtp* from wood and yielding more colonies on plates. Furthermore, NA+A allowed recovery of *Xtp* at

concentrations as small as 1 to 10 CFU mL^{-1}, indicating the efficacy of the medium in isolating the pathogen. Although subculture of colonies isolated on NA+A onto SPA, the specific medium for *Xtp*, was generally required to confirm identification, NA+A was selected as a medium to assess wood from burial experiments.

Chapter 4 Effect of burning and high temperature on survival of *Xanthomonas translucens* pv. *pistaciae* in infected pistachio branches and twigs

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Short title: Survival of *Xanthomonas* in heat-treated wood

Effect of burning and high temperature on survival of *Xanthomonas translucens* pv. *pistaciae* in infected pistachio branches and twigs

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Abstract

This paper reports the efficacy of burning and heat-treating pistachio branches and twigs as a means of disposing of prunings from trees infected with *Xanthomonas translucens* pv. *pistaciae (Xtp)*. Burning of pistachio wood, naturally infected with *Xtp*, was conducted twice under field conditions. Viable *Xtp* was detected in some non-burned wood, but not in charcoal, ash or partially burned wood. Controlled laboratory experiments were conducted with pure cultures of *Xtp* and naturally and artificially infected pistachio wood. In liquid culture, 65°C was lethal to *Xtp*, whereas survival at 60°C or less varied with culture medium and duration of exposure. *Xtp* survived in infected wood exposed to 40-55°C for at least 60 min but was killed by exposure to 60°C for 15 min or more. Overall, the results of burning and heat-treatment were consistent, and confirmed that burning was a reliable eradication technique to dispose of infected wood, such as prunings, providing the pathogen was exposed to a temperature of 60°C or greater for at least 15 min.

Introduction

Eradication of plant pathogens following an incursion is essential to safeguard the profitability and sustainability of plant industries in particular, and the socioeconomy and environment in general. Standard methods for eradication of plant pathogens include burning or burial of infected materials. Burning is preferred as it is considered to eliminate the affected materials, killing any pathogen it may contain (Ebbels, 2003). Although there have been reports where burning only reduced the incidence of, or contained, pathogens (Hardison, 1976; Pereira *et al.*, 1996), there are numerous examples of successful eradication of plant pathogens by burning alone or where burning was a part of an integrated management programme. For example, fire blight was sucessfully eradicated from Australia (Rodoni *et al.*, 2002) and Sweden (Gråberg, 1993) and citrus canker was eradicated from Thursday Island (Jones, 1991), Lambell's Lagoon near Darwin (Broadbent *et al.*, 1995) and the Emerald district of Queensland (Gambley *et al.*, 2009). Despite the apparent success of burning infected or exposed trees as an eradication strategy for bacterial pathogens, it is possible that pathogens may survive in ash or non-burned plant debris, particularly if material is partially embedded in soil. The temperature and duration of burns are also important factors in pathogen mortality, which determine the success of the burning. However, such information has rarely been provided (Sosnowski *et al.*, 2009; 2012).

Pistachio dieback is a bacterial disease of woody tissue that provides a local model with which to assess the effectiveness of existing strategies to eradicate exotic, systemic, bacterial pathogens of woody perennials that threaten Australian agriculture. The disease was first observed in Australian pistachio orchards in 1989 (Facelli *et al.*, 2001) and now occurs in the main pistachio growing regions of Australia. It has caused the death of more than 10% of trees in some areas (Edwards & Taylor, 1998), and impedes the expansion of

the Australian pistachio industry. The causal agent, *Xanthomonas translucens* pv. *pistaciae* (*Xtp*) (Giblot-Ducray *et al.*, 2009), occurs mainly in woody tissue of the scion, *Pistacia vera*, where it usually induces staining of the xylem. The bacterium has been isolated less frequently from the rootstock, generally *Pistacia integerrima* (Facelli *et al.*, 2009). The distribution of *Xtp* in pistachio wood is often discontinuous, while the bacterium has been isolated from leaves only rarely and not from roots or associated soil samples (Facelli *et al.*, 2009). Although the means of survival and dissemination are not known, transmission of *Xtp* via pruning has been demonstrated (Taylor *et al.*, 2005).

The aim of this study was to evaluate the efficacy of burning as a means of disposal of pruned branches and woody tissues infected with *Xtp*, and subsequently, the response of the pathogen to heat treatment. The experiments were intended to provide data on which to base future eradication strategies for controlling newly emerging bacterial pathogens of woody tissues.

Materials and methods

Field burning experiments

The effect of exposure to flames and heat in a bonfire on the survival of *Xtp* in naturally infected pistachio wood was assessed in two field experiments. Both were conducted at the Department of Primary Industries Victoria, Irymple, in winter, to comply with fire bans imposed in other seasons in bushfire-prone areas. One was done in 2008 (34°15'.14"S, 142°12'.41"E, elevation 63 m a. s. l.) and the second one in 2009 (34°13'.06"S, 142°11'.16"E, elevation 58 m a. s. l.). To minimise the likelihood of false negative results arising from the discontinuous distribution of *Xtp*, small pieces of wood confirmed to be infected were enclosed in mesh bags and attached with wire to metal poles, then retrieved for assessment after the bonfire.

Branches and twigs with stained xylem typical of dieback were taken in 2008 from a pistachio tree known to be infected in the Waite Campus orchard, University of Adelaide, South Australia (34°58'.18"S, 138°38'.00"E, elevation 127 m a. s. l.) (E. Facelli, The University of Adelaide, Australia, personal communication) and in 2009 from infected trees in a commercial orchard in Robinvale, Victoria (34°34'.46"S, 142°46'.47"E, elevation 61 m a. s. l.) (Facelli *et al.*, 2005). The presence of *Xtp* was confirmed by culturing as described by Facelli *et al.* (2005) with slight modifications and by PCR as described by Marefat *et al.* (2006). Briefly, small segments of branches or twigs were surface-disinfested by dipping in 95% ethanol and flamed. After cutting the ends and removing the bark with a sterile scalpel, fragments of 0.5-1 mm thick and 5-10 mm in length were excised and soaked in 9 mL sterile distilled water (SDW) overnight at room temperature (approximately 22°C). Aliquots (100 µL) of the resulting suspension were spread onto Petri plates of sucrose peptone agar (SPA) (Moffett & Croft, 1983) amended with 150 mg L^{-1} Benlate® (BSPA) (Facelli *et al.*, 2005) in 2008 or onto nutrient agar (NA, Oxoid) supplemented with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin (NA+A) in 2009. NA+A was modified from XTS, a medium semi-selective for *X. campestris* pv. *translucens* (Schaad & Forster, 1985); the plating efficiency of *Xtp* on NA+A was 90-100% in two replicates over time. Aliquots (1.5 mL) of the suspensions were also centrifuged, the pellets resuspended in 15 µL SDW and 1 µL used as template in PCR with primers specific for the pathogen (Marefat *et al.*, 2006). Branches and twigs which were PCR-positive and resulted in isolation of *Xtp*-like colonies were cut into pieces of 1 cm long (for branches of 3-5 cm diameter) or 5-7 cm long (for twigs of 1.5-2 cm diameter). Five to 12 such pieces of woody material (total *c.* 50 g), cut the day before the experiments, were enclosed in stainless steel mesh (0.9 mm aperture, 0.37 mm wire diameter, 50% open area) (Sefar Metal Mesh Pty Ltd) folded to form bags, to investigate the effect of direct exposure to the flames on survival of *Xtp.* At the same time, another set

111 of pieces of wood was placed in glass Petri dishes (100 mm in diameter) before enclosing
112 in wire mesh, to examine the effect of the heat of the fire on pathogen survival.

113 In 2008, six steel poles were placed upright at random within a 5- (length) x 3.5-
114 (width) x 0.5-(depth) m pit, dug into soil (sandy clay loam) approximately 10 m from a
115 grape vineyard, 35 days before igniting the bonfire. The soil was dry to the touch. One
116 mesh bag containing exposed infected wood was attached to each of four poles at 20 cm
117 and three poles at 50 cm above the pit floor and another bag was buried 5 cm below the pit
118 floor at pole 6. One mesh bag containing infected wood in a Petri dish was attached to each
119 of two poles at 20 cm and two poles at 50 cm above the pit floor. The pit was then filled
120 with 32-day-old dried grapevine canes as fuel (Sosnowski *et al.*, 2012). Another mesh bag
121 containing infected wood was kept in the laboratory as a control.

122 In 2009, three replicate pits, 5 m apart, were dug at 5-15 m from a small grove of
123 pistachio trees. Mesh bags containing infected wood exposed or inside Petri dishes (*c.* 20
124 of each) were attached to steel poles with wire at four heights in relation to the pit floor (0,
125 20, 50 cm above and 5 cm below) and the poles were placed at five positions within each
126 pit. The pits were then filled with branches, without leaves, cut from pistachio trees. Half
127 of these branches had been pruned from trees in winter 2008 and left in the open, and the
128 remainder had been cut and dried for about 7 weeks before the experiment. These materials
129 were distributed evenly among the three pits. Five mesh bags, each containing 110-120 g
130 of infected wood, were kept in the laboratory as controls.

131 The temperature of the fire above and below the pit floor in which the infected
132 wood was burned was estimated using methods modified from Lanoiselet *et al.* (2005). In
133 brief, Tempilstik crayons (Tinco Ltd) with melting points of 50, 60, 70, 80, 90, 110, 130,
134 150, 170, 190, 210, 220, 230, 240 and 250°C and 40, 50, 60, 70, 80, 200, 400, 600, 788,
135 982 and 1200°C were used in 2008 and 2009, respectively. Pieces of crayon (one specific

temperature per tube) were inserted into glass test tubes (60 x 5 mm), which were sealed with aluminium foil. The tubes were then placed into 100-mm-diameter glass Petri dishes, enclosed in mesh bags and attached to the poles at 0, 20, 50 cm above and 5 cm below the pit floor. Additionally, one bag was buried 10 cm below the pit floor at the base of each pole. After the bonfire, the maximum temperature, as indicated by the Tempilstik crayons, was recorded for each position.

Bonfires were lit using a flame-thrower on 12 August 2008, a dry sunny day, and in 2009 in the afternoon of 16 July for pits 1 and 2 and the morning of 17 July for pit 3, when conditions were damp (see Table 1).

Immediately after cooling, all mesh bags were retrieved and the contents weighed. The weight of the contents in mesh bags or Petri dishes before and after the bonfire was compared using a paired t-test with 95% confidence intervals. The contents of each mesh bag or Petri dish were assessed for colour, dryness and cracking compared with the control. Remains were then categorised as non-burned, partially burned, charcoal and ash according to degree of charring. Non-burned material was defined as comprising pieces of wood that remained natural in colour with slightly moist bark. Partially burned material comprised pieces of wood that changed colour from light to dark brown or black, dried out, cracked on the surface. Charcoal comprised wood that had turned black, brittle and porous, and ash was mainly fine, grey powder. The proportions of non-burned and/or partially burned, or charcoal and/or ash in mesh bags or Petri dishes were expressed as a percentage of the total number. Following assessment, three samples were taken at random from each mesh bag or Petri dish from each of the positions to assess survival of *Xtp* by culturing and PCR as described above. Where the remains were charcoal, they were first ground using a sterile mortar and pestle.

Survival of Xtp

In 2008, charcoal or ash was mixed thoroughly with a sterile tea-spoon and samples of 0.1 and 0.5 g from each batch of charcoal or ash were incubated separately in 9 ml SDW overnight at room temperature (*c.* 22°C). Where sufficient remains were available, an additional portion of 1 g charcoal or ash was also assessed. Also, small amounts of charcoal or ash were placed directly on BSPA amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 1.4 mg L^{-1} gentamycin (ABSPA, E. Facelli, The University of Adelaide, Australia, personal communication). Tissues (0.5-0.7 g) from non-burned and control wood were soaked in 9 ml SDW overnight at room temperature. Aliquots (100 µL) from all resulting suspensions were spread onto each of two plates of ABSPA. The suspensions were also used for pathogen detection by PCR (Marefat *et al.*, 2006). In 2009, samples were prepared for assessment of survival and PCR in the same manner, except that each sample was standardised to 0.5-0.6 g. As the plating efficiency of ABSPA proved variable in 2008, NA+A was used to isolate *Xtp* from the remains of the bonfires in 2009.

***In vitro* temperature experiments**

The effect of temperature on survival of *Xtp* in liquid culture media and in artificially and naturally infected wood was examined to establish the critical time-temperature relationship lethal to the pathogen. *Xtp* isolate DAR 75532, obtained from a diseased pistachio tree in a commercial orchard at Kyalite, New South Wales (Facelli *et al.*, 2005) was used where a pure culture was required.

Thermal death time

The method of Brown (2009) was used. Cultures of *Xtp* were grown in sucrose peptone broth (SPB) and nutrient broth (NB) overnight at 28°C. From each suspension, 10 mL, with 10^9 colony forming units (CFU) mL^{-1}, were transferred into a McCartney bottle then

immersed in a water bath adjusted sequentially to 40, 45 then 50°C. These temperatures were chosen based on previous observations that the pathogen appeared to grow slowly at 40 and 50°C (data not shown). Mercury-filled thermometers were inserted into bottles of sterile SPB and NB to monitor the temperature of the broth cultures. Aliquots from each temperature treatment were removed aseptically every 10 min for up to 60 min, serially diluted 10-fold to 10^{-6}, then 10 µL of each dilution was pipetted in triplicate onto SPA and NA. Controls comprised aliquots removed before the McCartney bottles were placed in the water bath. The plates were incubated inverted at 28°C in the dark for up to 14 days and CFU were enumerated. The experiment consisted of two replications over time. Data were subjected to analysis of variance (ANOVA) using the statistical software GENSTAT version 11.1 (Lawes Agricultural Trust). Treatment means were compared by the least significant difference (LSD) procedure at the 5% significance level.

Thermal death point

The above procedure was used to determine thermal death point except that the overnight suspensions of *Xtp* in SPB and NB were exposed to 50, 55, 60, 65 and 70°C for only 10 min (Brown, 2009). Aliquots from each temperature treatment were then diluted 10-fold to 10^{-6} and three replicate drops plated onto SPA and NA to determine the temperature at which *Xtp* was killed in 10 min. The plates were incubated inverted at 28°C and CFU enumerated as above. The experiment was conducted once with three replicate bottles for each temperature treatment. CFU counts before and after the temperature treatment were compared using a paired t-test with 95% confidence intervals.

Survival in infected twigs and wood

Based on the results of experiments to examine the effect of burning and high temperature in culture, the effect of incubation at 40-60°C on survival of *Xtp* in inoculated twigs and naturally infected branch segments was assessed. Twigs were inoculated by a vacuum infiltration method modified from Salowi (2010). Twigs (0.7-1.2 cm in diameter and 7 cm long) were collected from confirmed pathogen-free 4-year-old *P. vera* cv. Sirora that had been maintained in pots in a shade-house located at the Waite Campus or from confirmed disease-free trees (cv. Sirora, 33 years old) in a dieback-free commercial orchard at Saddleworth, South Australia (34°05'.02"S, 138°46'.70"E, elevation 324 m a. s. l.). The twigs were vacuum-infiltrated with suspensions of *Xtp* (250 μL, 10^8 CFU mL^{-1}) then placed in Petri dishes and sealed with Parafilm. After 10 days at 28°C, one-third of each twig was processed to confirm the presence of the pathogen and determine the population prior to heat treatment. The remaining two-thirds of those twigs that yielded an initial population of 10^4 CFU mL^{-1} or greater were used for the temperature experiments.

For naturally infected wood, twigs from branches with excessive resinous exudate were collected from the aforementioned pistachio tree in the Waite Campus orchard to confirm infection status. Branches bearing twigs confirmed to contain viable pathogen were collected and cut into segments 2-3 cm long for small branches (1-2 cm diameter) and 1-3 cm long for larger branches (>2 cm diameter). These segments were then bisected longitudially through visibly stained xylem so that each half was likely to contain bacteria. One section was processed immediately to determine the initial population of the bacteria and the other section was exposed to the designated temperature prior to counting bacterial.

Before exposing the twigs or branch segments to each temperature treatment, they were enclosed in Petri dishes, with a moist Whatman filter paper (grade 41, 90 mm diameter) wedged into the lid to prevent drying out. The Petri dishes were then sealed with

plastic film (GLAD® Products Australia). Exposure time was recorded from the time that the incubator reached the designated temperature, generally 15-20 min after the plates of twig or wood had been placed inside the incubator. All experiments were set up as a completely randomised design. The time required for internal tissues of a twig (*c.* 1 cm diameter) or wood piece (*c.* 1 cm thick) to reach 50, 55 and 60°C was determined using a Hastings Data Logger (Gemini Data Loggers (UK) Ltd). The pith temperature was measured independently three times by inserting the probe of the logger into the pith of three additional twigs or pieces of wood in a Petri dish. The area surrounding the point of entry of the probe was covered with heat-resistant plasticine. Data were recorded every 10 min for 4 h.

In a preliminary experiment with artificially infected twigs (experiment 1), a single plate containing five twigs was exposed to 50°C for 60, 120, 150 and 180 min or to 55 and 60°C for 60, 120 and 180 min. In a second experiment (experiment 2) with a new batch of inoculated twigs, three replicate plates, each comprising two twigs per plate, were exposed to 50, 55 and 60°C for the same durations as in experiment 1. Subsequently, new batches of twigs were exposed to 40°C for 30 and 60 min (experiment 3), or to 55 (experiment 4) and 60°C (experiment 5) for 15, 30, 45 and 60 min. Each temperature treatment, comprising three replicate plates with two twigs, was conducted twice over time.

In the experiment with naturally infected wood, there were five replicate plates, each containing three branch segments. The plates were placed in an incubator at 50°C for 60, 120 and 150 min.

After treatment, the twigs or branch segments were surfaced sterilised, cut into pieces (0.5- 1mm) with secateurs that had been dipped in ethanol and flamed, then soaked in SDW overnight at room temperature. The viability of *Xtp* was assessed by transferring,

in triplicate, 10 µL aliquots of serial dilutions to 10^{-5} of the resulting suspensions onto NA for the twigs or spreading of 100-µL aliquots of serial dilutions to 10^{-2} on NA+A for the branch segments. The plates were incubated at 28°C and CFU enumerated as described previously. Where the temperature treatment did not kill all the pathogen in the exposed twigs or branch segments, the bacterial counts after heating were compared with the initial population of the same twig or branch and the reduction in the number of viable bacteria was expressed as the geometric mean (Keck *et al.*, 1995). The geometric mean is defined as "the *n*th root of the product of the data" (Crawley, 2007). It is used to measure the central tendency of processes that change multiplicatively rather than additively (Crawley, 2007) and for data that have a logarithmic pattern (Lanley, 1979).

Results

Burning experiments

The fire in 2008 lasted for 30 min. Remains in mesh bags after the bonfire were mainly ash and a small amount of charcoal, while Petri dishes contained mainly charcoal and a few pieces of partially burned wood. The wood buried below the pit floor was not burned. The temperature crayons indicated that the fire exceeded 250°C at all locations where the samples were attached to poles above the pit floor, while temperatures 5 cm below the pit floor reached between 50 and 60°C. No viable *Xtp* was isolated from the remains, even from the non-burned wood, nor was the pathogen detected by PCR. Viable *Xtp*, *c.* 10^2 CFU mL^{-1}, was isolated from all control wood pieces.

Total combustion time in 2009 varied between pits (Table 1). In general, after the fire was ignited, intense flames lasted for 20 min in all three pits. Small flames lingered for another 20 min in pit 1. Two corners (poles 4 and 5) in this pit furthest from the flame-thrower were exposed to little or no fire, so that the fuelwood and samples in mesh bags

were not burned. In pit 2, after the intense flames subsided, small flames remained at two corners (poles 1 and 2) of the pit and the last flame died at pole 2, 105 min after ignition. The samples under the pit floor at pole 1 were partially burned and those at pole 2 were blackened on the surface. Dew was observed on the woody material in pit 3, delaying the start of the fire. The flames stayed mainly in the central part of this pit, but died off quickly after ignition. As considerable fuel wood at the four corners was not burned, the branches were piled up again at each corner close to the lingering flames until most were burned. Isolated flames continued for another 70 min in three corners (poles 1, 2 and 5) and 85 min in the other (pole 4).

Material retrieved from the 2009 burning experiment comprised non-burned and partially burned wood, charcoal and ash. Thirty-five, 40 and 45% of the mesh bags contained wood pieces burned to charcoal and/or ash in pits 1, 2 and 3, respectively. More mesh bags attached to poles 1, 2 and 3 (58%, 42% and 42%, respectively) contained wood pieces that were burned to charcoal and/or ash than those attached to poles 4 and 5 (33% and 25%, respectively). Approximately 87% of the mesh bags with wood pieces placed on the pit floor and 60% of those suspended 20 cm above the pit floor were burned to charcoal and/or ash, compared with 0% and 13% of the samples placed 5 cm below and suspended 50 cm above the pit floor, respectively. For wood pieces in Petri dishes, 25%, 30% and 35% of the dishes contained material that was burned to charcoal and/or ash in pits 1, 2 and 3, respectively. For all pits combined, 60%, 53%, 7% and 0% of Petri dishes contained wood burned to charcoal and/or ash on the pit floor, at 20 and 50 cm above the pit floor and 5 cm below the pit floor, respectively. Those wood pieces that were burned to charcoal and/or ash were generally in Petri dishes placed on the pit floor that had broken as a result of the heat. For wood in mesh bags and Petri dishes, weight after the bonfire was significantly less than prior to burning (Table 2).

307 Of 360 samples of wood, charcoal and ash from mesh bags and Petri dishes (180
308 each) processed in the survival assay, viable pathogen was isolated from 13 pieces of
309 wood. Six of these wood pieces (two in two mesh bags and four in two Petri dishes) were
310 buried 5 cm below the surface and three pieces (two in a Petri dish and one in a mesh bag)
311 were suspended 20 cm above the pit floor on poles 4 and 5 in pit 1 (Fig. 1a, b). The other
312 four were from three mesh bags, two suspended 50 cm above the pit floor on poles 2 and 4
313 in pit 1 and one on pole 4 in pit 3. All samples which yielded viable *Xtp*, except one from
314 the west side of pit 1, were on the east side of pits 1 and 3 furthest from the flame-thrower.
315 Viable pathogen was not isolated from any samples from pit 2. The population of viable
316 pathogen recovered from samples suspended 20 cm and 50 cm above the pit floor ranged
317 from 10^1 to 10^3 CFU mL^{-1}, while that from samples buried 5 cm below the pit floor from
318 10^2 to 10^4 CFU mL^{-1}. The population of viable pathogen for wood pieces placed away
319 from the fire as controls ranged from 10^2 to more than 10^4 CFU mL^{-1}. Most wood that
320 yielded viable *Xtp* was not burned and had slightly moist bark.

321 *Xtp* was detected by PCR in all wood that yielded viable pathogen, including
322 controls, mainly in non-burned and partially burned wood samples, except for one ash
323 sample, for which a faint amplicon was detected.

324 The maximum temperature recorded by melted crayons was equal to or >200°C but
325 <400°C for most of the locations above the pit floor, and ranged from < 40°C to > 80°C at
326 5 cm below the pit floor (Fig. 1c). The heat penetrated 10 cm below the pit floor, and
327 exceeded 80°C at some positions in pit 3.

328

329

In vitro **temperature experiments**

Thermal death time

The trend of pathogen response to 40, 45 and 50°C in both replications was similar. However, because of differences between replications in time required to kill the pathogen at 50°C, each data set was analysed separately and the mean population (CFU mL^{-1}) of suspensions exposed to the three temperatures for one replication are presented (Fig. 2). The population did not change ($P > 0.05$) when the suspensions of *Xtp* cells in NB and SPB were exposed to 40°C for up to 60 min. The population decreased when suspensions in NB were exposed to 45°C for 10 min and this continued ($P < 0.001$) as exposure time increased, whereas the population in SPB did not change over time. There was a significant reduction ($P < 0.001$) of population when suspensions in both NB and SPB were exposed to 50°C for 10 min and viable pathogen was not recovered from NB suspensions after 60 min, whereas the pathogen continued to survive in SPB at 50°C for 60 min (Fig. 2). However, no viable pathogen was observed on NA and SPA in the other replicate of the suspensions exposed to 50°C for 30 and 40 min, respectively (data not shown).

Thermal death point

Exposure to 50 or 55°C for 10 min significantly reduced ($P < 0.001$) the population of *Xtp* in both NB and SPB suspensions but did not completely kill the pathogen (Table 3). Exposure to 60°C in NB completely killed the pathogen in 10 min, and significantly reduced the population in SPB. No viable pathogen was detected after exposure to 65 or 70°C for 10 min.

351 *Survival in infected twigs and wood*

352 Pith temperature increased rapidly in the first 10 and 20 min of incubation, from ambient
353 condition of about 22°C to approximately 4°C less than the designated temperatures, then
354 slowly reached the designated temperatures in another 30-40 min (Fig. 3). Once the
355 designated temperatures were reached, the pith temperature stayed constant for the
356 remaining incubation period. In the first experiment with artificially infected twigs, viable
357 *Xtp* was isolated from one, four and three twigs of five that had been exposed to 50°C for
358 60, 120 and 150 min, respectively (Table 4). In the second experiment, viable *Xtp* was
359 isolated from two of six twigs after exposure for 60 and 120 min, but not from twigs
360 exposed for 150 min. However, in twigs that yielded viable pathogen, the number of viable
361 bacteria after heat treatment decreased by at least 97% (from 9.3 to 3 x 10^3) compared with
362 that before treatment. On average, only 1.702, 0.048-0.489 and 0.018% of the bacteria
363 remained viable in the twigs exposed to 50°C for 60, 120 and 150 min, respectively (Table
364 4). Viable pathogen was not recovered from any twigs exposed to 50°C for 180 min, nor
365 from twigs exposed to 55 or 60°C for 60 min or longer. In the third experiment, when
366 artificially infected twigs were exposed to 40°C, the pathogen was isolated from the
367 majority of twigs exposed for 30-60 min although CFU varied (Table 5). The pathogen
368 was isolated from artificially infected twigs that had been exposed to 55°C in experiment
369 4-1 (i.e. experiment 4, replicate 1), from a decreasing number of twigs with increasing time
370 of exposure, but not in experiment 4-2 (experiment 4, replicate 2) with a new batch of
371 twigs. The temperature recorded in the incubator for experiment 4-1 fluctuated between 51
372 and 52°C in the first 20 minutes. Nevertheless, the population of the bacteria after exposure
373 to 55°C was 80 to almost 100% less than the initial population in the same twigs (Table 5).
374 The pathogen was not isolated from twigs exposed to 60°C for 15 min or longer
375 (Experiment 5-1 and 5-2; Table 5).

Exposure of naturally infected pistachio wood to 50°C for 60-180 min did not completely kill the pathogen (Table 6). The number of viable bacteria recovered from branch segments heated for 60 or 180 min varied greatly, although the population of viable *Xtp* in the majority of branch segments decreased by more than 98% compared with that before exposure to this temperature. Although there was no clear trend in the number of viable bacteria in naturally infected wood with duration of exposure to 50°C, overall, pathogen survival was poor, as indicated by geometric means of 0.366-3.253 (Table 6).

Discussion

Burning in an open pit was an effective means of eradicating *Xtp* from pistachio wood providing that all the wood was burned completely or reached the lethal temperature for sufficient time to kill the pathogen. The lethal temperature for *Xtp in vitro* was 60-65°C, depending on the culture medium, and the pathogen was not recovered from twigs heated in oven at 60°C for 15 min or more. Recovery of viable bacteria from wood that was incompletely burned or that penetrated the soil and escaped exposure to lethal temperature illustrated the importance of ensuring that all wood is burned or heated sufficiently.

In the field experiments, no viable pathogen was isolated from any of the samples after the bonfire when the infected wood was placed on the pit floor, where the temperature reached between 200 and 400°C at most locations, which suggested that eradication was achieved. Incomplete combustion at some locations furthest from the flame-thrower, for example poles 4 and 5 in pit 1, allowed the pathogen to survive. The majority of the wood pieces that yielded viable pathogen from these two locations were buried below the pit floor, where the temperature remained below 40°C, indicating that any pathogen in debris that penetrates into the soil might not be eradicated by burning, particularly if the pieces of wood are large and heat penetration is poor. Sosnowski *et al.* (2012) also reported that the

fungus *Elsinoe ampelina* was eradicated from grapevine canes by burning, providing that infected canes did not penetrate the soil below the fire pit. Viable cells of the pathogen were also isolated from non-burned wood suspended above the pit floor: however, had these materials not been attached to poles, they are likely to have fallen into the flames and been incinerated. The relatively small number of bacteria isolated from the control pieces of wood in 2008 compared with 2009 may reflect the use of ABSPA rather than NA+A in the first year. Overall, the data suggest that buried or non-burned debris could act as a source of inoculum for subsequent disease if the pathogen survived in buried wood was transported via human or other vectors to areas with susceptible hosts.

As the use of thermal crayons in the field experiments did not allow the establishment of time-temperature relationships nor the measurement of temperature reached inside woody tissues, *in vitro* experiments were conducted to elucidate the effect of exposure to heat over time. *Xtp* survived in liquid SPB and NB at 40°C, and the pathogen was not eradicated when exposed to 45 and 50°C for 60 min or to 55 or 60°C for 10 min. Furthermore, heat tolerance was greater in SPB than in NB, which may be related to the production of extracellular polysaccharide in the presence of sucrose (Souw & Demain, 1979) that could protect the cells from heat. This phenomenon was reported by Leach *et al.* (1957), in that the thermal death point for *Xanthomonas phaseoli* was 2-4°C higher when grown on glucose-casein-hydrolysate agar, on which polysaccharide was produced, than in nutrient broth. Thermal death times reported for other xanthomonads have varied. For example, Keck *et al.* (1992) reported 50°C for 40 min to be lethal to *Xanthomonas campestris* pv. *pelargonii*, whereas cells of *X. fragariae* were killed at 56°C for 15 min or 52°C for 60 min (Turechek & Peres, 2009). Although 55°C was not lethal to *Xtp*, the population decreased significantly. The lethal threshold for the pathogen appeared to be 60°C in NB and 65°C in SPB, suggesting that the thermal death point lies between 60 and 65°C. In comparison, the thermal death point of *Xanthomonas oryzae* pv. *oryzae*

was 53 (Stall *et al.*, 1993; Sharma, 2006), although the influence of culture medium on survival was not specified.

Survival of *Xtp* was prolonged in pistachio wood, the pathogen being eradicated from inoculated twigs after exposure to 50°C for 180 min, but not from naturally infected branch segments. The time (50-60 min) required for internal tissues to reach designated temperatures in this study suggests that viable pathogen isolated from wood which had been incubated at 50°C for 60-180 min or more might have resided in the innermost tissues. Although the population was greatly reduced, the failure to kill all bacteria means that heating at 50°C would be unsuitable as a means of eradication. *Xtp* produces polysaccharide slime *in planta* (E. Facelli, The University of Adelaide, Australia, personal communication), which might protect the pathogen from damage due to heat (Leach *et al.*, 1957). Treating at 55ºC for 60 min failed in some cases to eradicate *Xtp* from artificially infected tissue, but it was always eradicated when the exposure was increased to 120 min, suggesting that any protective effect of polysaccharide slime was temporary.

The pathogen survived in liquid culture and artificially infected twigs at 40°C for 60 min, 50°C for 60-150 min, or in naturally infected wood at 50°C for more than 180 min. In contrast, in the field experiments it was not isolated from wood pieces at the few locations below the pit floor where crayons indicated a temperature of 40°C or more. It may be that the pathogen in these wood pieces was reduced to below the detection limit of the method used for isolation. In addition, the observation that the wood was dry, cracked or turned brown to black indicates that it might have been exposed to the heat for a long duration. Desiccation as a result of heat might also affect the survival of the pathogen in the wood tissues, and may merit further research. The results of the burning experiment where viable pathogen was not isolated from any samples that reached 60°C were in agreement with the *in vitro* experiments, both in liquid medium culture and twigs, and once again, confirmed the lethal effect of this temperature on the pathogen's survival.

In summary, *Xtp* was eradicated when infected wood was burned to charcoal or ash. This could be achieved by providing adequate penetration of flames and duration of heat. Failure to do this allowed the pathogen to survive in some cases. Incomplete eradication of this or a similar pathogen might result in reintroduction of the disease to areas where susceptible hosts are present and, consequently cause eradication programmes to fail. Deep burial of the remains, which usually follows burning in eradication programmes, might help to prevent the escape of any pathogen surviving in incompletely burned wood.

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Figure 1 Isolation of viable *Xanthomonas translucens* pv. *pistaciae* (*Xtp*) on nutrient agar with antibiotics from the remains of (a) infected wood in mesh bags and (b) infected wood in Petri dishes along with (c) indicative temperature (°C) based on Tempilstik crayons, after bonfires in 2009. Each pit was 3 x 5 m and 0.5 m deep. Locations from which viable *Xtp* was isolated are denoted with (⊕) and those from which viable *Xtp* not isolated with (▭). A flame-thrower was applied to each pit following the direction of the prevailing wind; east - southeast for pit 1, south for pit 2 and east-northeast for pit 3.

Figure 2 Mean number of colony forming units (CFU; log transformation) of *Xanthomonas translucens* pv. *pistaciae* after exposure to 40 (diamond), 45 (square) and 50°C (triangle); (a) incubated in sucrose peptone broth and enumerated on sucrose peptone agar, (b) incubated in nutrient broth and enumerated on nutrient agar.

Figure 3 Increase in pith temperature of pistachio twigs (*c.* 1 cm diameter) or wood pieces (*c.* 1cm thick) incubated at 50, 55 and 60°C for 60 min. Temperature was recorded by inserting a probe connected to a data logger into the pith region of the twigs or wood pieces. The mean of three replicates for each temperature is presented.

Table 1 Weather conditions and characteristics of the fires in three pits in which pistachio wood was burned in 2009. Fires in pits 1 and 2 were lit in the afternoon of 16 July and in pit 3 in the morning of 17 July. Weather data were based on data provided by http://www.eldersweather.com.au

Characteristics	Pit 1	Pit 2	Pit 3
Weather conditions			
- Air temperature (°C)	12.6	13.5	4.3
- Relative humidity (%)	70	48	97
- Wind speed (km h^{-1})	7 (gusts 11)	13 (gusts 20)	2 (gusts 5)
- Prevailing wind direction	East-southeast	South	East- northeast
Characteristics of fire			
- Starting time	12:10 h	16:15 h	8:55 h
- Accelerant time (min)	4	4.5	9.5
- Intense burn duration (min)	20	20	20
- Total burn time (min)	40	40-105	20-105

Table 2 Mean weight (g) of pistachio wood pieces, before and after burning, in mesh bag or Petri dish attached to five poles at various positions above the floor in three pits in 2009. Weight of wood in each mesh bag or Petri dish before and after the bonfire was compared using a paired *t*-test with 95% confidence interval. Means were computed from weight data for 15 mesh bags or Petri dishes, from one position above the pit floor on each pole in each pit

	Sample height in relation to pit floor	Mean weight per bag or Petri dish (g)		Paired *t*-test P values
		Before bonfire	After bonfire	
Mesh bags	-5 cm	118.23	86.05	< 0.0001
	0 cm	117.57	19.84	< 0.0001
	20 cm	118.52	37.57	< 0.0001
	50 cm	118.26	73.73	< 0.0001
Petri dishes	-5 cm	55.59	44.57	0.0019
	0 cm	55.71	22.93	< 0.0001
	20 cm	56.26	24.66	< 0.0001
	50 cm	55.53	41.36	< 0.0001

Table 3 Thermal death point for *Xanthomonas translucens* pv. *pistaciae* in suspension in nutrient broth (NB) or sucrose peptone broth (SPB) exposed for 10 min at a range of temperatures, then enumerated (CFU mL^{-1}) on nutrient agar (NA) or sucrose peptone agar (SPA), respectively. The experiment was conducted with three replicates.

	NB/NA		SPB/SPA	
Temperature (°C)	Treatment	Control[a]	Treatment	Control
50	4.2×10^{5} [b]	2.1×10^{9}	3.4×10^{5}	2.1×10^{9}
55	1.9×10^{3}	1.7×10^{9}	7.2×10^{5}	2.0×10^{9}
60	0	1.2×10^{9}	8.8×10^{2}	2.5×10^{9}
65	0	1.9×10^{9}	0	2.1×10^{9}
70	0	2.1×10^{9}	0	2.5×10^{9}

[a] Controls comprised aliquots from each of the three replicate liquid culture bottles, which were enumerated on SPA and NA before the bottles were immersed in water baths at 50-70°C.

[b] Populations of viable bacteria before and after treatment were estimated using the method of Miles and Misra (1938) and compared using a paired *t*-test with 95% confidence intervals.

Table 4 Recovery of viable *Xanthomonas translucens* pv. *pistaciae* from artificially infected pistachio twigs before (one-third of each twig) and after (remaining two-thirds of each twig) exposure to 50°C for various durations in two experiments. The percentage of viable bacteria remaining following heat treatment is given along with the geometric mean

Exposure duration (min)	Experiment	Number of twigs which yielded pathogen	Number of CFU [a] mL^{-1}		Viable bacteria (%)	Geometric mean[b] (%)
			Before treatment	After treatment		
60	1	1/5	3.0×10^6	4.7×10^2	0.016	N/A
	2	2/6	2.9×10^5	2.4×10^3	0.828	
			1.2×10^5	4.2×10^3	3.500	1.702
120	1	4/5	3.8×10^7	2.0×10^5	0.526	
			2.4×10^7	1.8×10^3	0.008	
			2.1×10^7	1.9×10^3	0.009	
			8.0×10^6	1.2×10^4	0.150	0.048
	2	2/6	3.2×10^5	2.6×10^3	0.813	
			1.7×10^5	5.0×10^2	0.294	0.489
150	1	3/5	2.3×10^7	1.4×10^4	0.061	
			1.5×10^7	5.3×10^3	0.035	
			9.7×10^6	2.7×10^2	0.003	0.018
	2	0/6	-	-	-	N/A
180	1	0/5	-	-	-	N/A
	2	0/6	-	-	-	N/A

CFU:- Colony forming units. N/A: not applicable

[a] Geometric mean is a type of mean or average defined as 'n^{th} root of the product of the data' (Crawley, 2007).

Table 5 Recovery of viable *Xanthomonas translucens* pv. *pistaciae* from artificially infected pistachio twigs before (one-third of each twig) and after (remaining two-thirds of each twig) exposure to 40-60°C for various durations. Each temperature treatment comprised three replicate plates, each containing two twigs and was repeated once. The percentage of viable bacteria remaining following heat treatment is given along with the geometric mean

Temperature (°C)	Exposure duration (min)	Experiment-Replication	Number of twigs, of 6, which yielded pathogen	Number of CFU mL^{-1}		Viable bacteria remained (%)	Geometric mean[a] (%)
				Before treatment	After treatment		
40	30	3-1	3	4.4×10^6	1.4×10^6	31.818	
				3.5×10^7	3.3×10^7	94.286	
				1.3×10^8	2.2×10^6	1.692	17.187
		3-2	5	1.9×10^8	1.9×10^7	10.000	
				4.0×10^6	2.5×10^4	0.625	
				6.3×10^7	1.3×10^7	20.635	
				1.1×10^7	3.3×10^6	30.000	
				2.5×10^7	2.7×10^4	0.108	3.344
	60	3-1	3	1.3×10^6	2.5×10^5	19.231	
				2.3×10^7	2.6×10^7	113.043	
				2.0×10^8	1.5×10^7	7.500	25.357
		3-2	6	1.9×10^7	1.5×10^7	78.947	
				2.5×10^7	2.2×10^7	88.000	
				1.0×10^7	1.5×10^5	1.500	
				6.3×10^6	1.6×10^5	2.540	
				2.1×10^7	3.0×10^7	142.857	
				1.2×10^7	3.2×10^7	266.667	31.666

55	15	4-1[b]	6	2.0×10^7	1.4×10^5	7.000	
				2.2×10^6	5.5×10^3	0.250	
				2.7×10^7	5.3×10^6	19.630	
				4.5×10^7	3.4×10^5	0.756	
				1.5×10^7	2.5×10^4	0.167	
				5.3×10^6	2.9×10^3	0.055	0.786
		4-2	0	-	-	-	N/A
	30	4-1	3	1.6×10^7	5.3×10^2	0.003	
				2.9×10^6	3.3×10	0.001	
				1.1×10^6	3.3×10^2	0.030	0.005
		4-2	0	-	-	-	N/A
	45	4-1	1	2.7×10^5	1.3×10^2	0.048	N/A
		4-2	0	-	-	-	N/A
	60	4-1	1	3.6×10^6	8.7×10^2	0.024	N/A
		4-2	0	-	-	-	N/A
60	15	5-1 & -2	0	-	-	-	N/A
	30	5-1 &- 2	0	-	-	-	N/A
	45	5-1 &- 2	0	-	-	-	N/A
	60	5-1 &- 2	0	-	-	-	N/A

CFU:- Colony forming units. N/A: not applicable.

[a] Geometric mean is a type of mean or average defined as 'n^{th} root of the product of the data' (Crawley, 2007).

[b] The temperature recorded in the incubator for experiment 4-1 fluctuated between 51 and 52 in the first 20 min of incubation of twigs.

Table 6 Recovery of viable *Xanthomonas translucens* pv. *pistaciae* from naturally infected pistachio wood before (half of each branch segment) and after (other half) exposure to 50°C for various durations. Each temperature treatment comprised five replicate plates, each containing three pieces of wood. The percentage of viable bacteria remaining following heat treatment is given along with the geometric mean

Exposure duration	Number of branch segments which yielded bacteria /number of branch segments tested[a]	Number of CFU mL^{-1}		Viable bacterium remained (%)	Geometric mean (%)[b]
		Before exposure	After exposure		
60 min	8/14	7.3×10^3	6.3×10^3	86.364	
		2.9×10^4	4.0×10^1	0.140	
		3.5×10^4	3.0×10^1	0.085	
		3.6×10^4	2.0×10^1	0.559	
		2.2×10^4	8.0×10^1	0.369	
		1.7×10^4	5.7×10^3	33.333	
		3.1×10^3	2.7×10^3	85.761	
		6.4×10^3	1.0×10^1	0.156	1.764
120 min	3/14	7.7×10^3	6.0×10^1	0.783	
		4.2×10^3	2.0×10^1	0.482	
		7.7×10^3	1.0×10^1	0.130	0.366
150 min	1/15	9.3×10^3	3.0×10^3	32.143	N/A
180 min	3/15	2.8×10^3	1.0×10^1	0.353	
		4.0×10^3	2.4×10^3	60.401	
		6.2×10^2	1.0×10^1	1.613	3.253

CFU: Colony forming units. N/A: not applicable.

[a]One branch segment from each of 60 and 120 min durations was excluded as it yielded no colonies from either section before and after treatment.

[b]Geometric mean is a type of mean or average defined as 'n^{th} root of the product of the data' (Crawley, 2007).

Figure 1

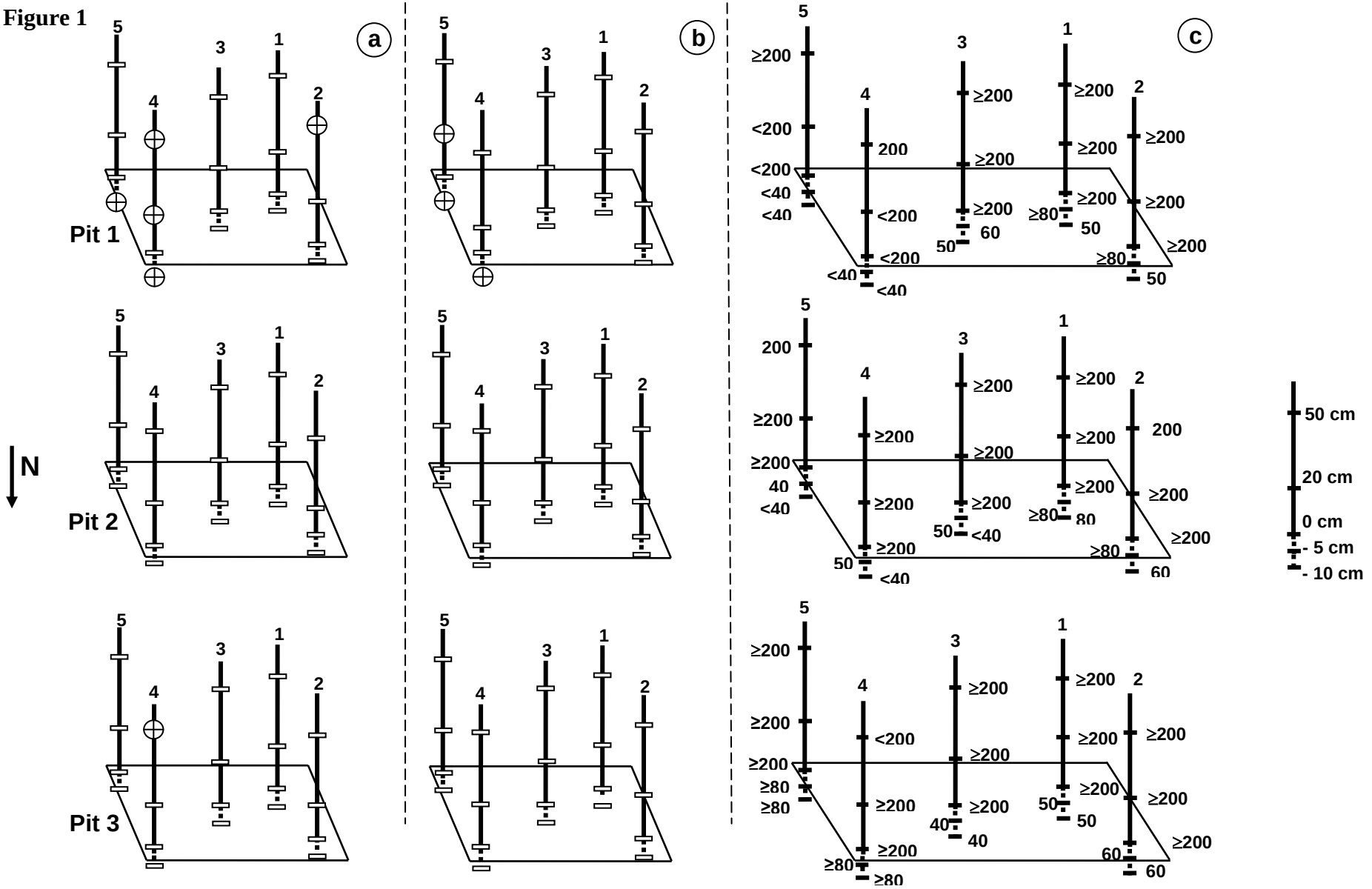

a
b
c
Pit 1
Pit 2
Pit 3
N
5
4
3
1
2
≥200
<200
200
<40
≥80
50
60
50 cm
20 cm
0 cm
- 5 cm
- 10 cm

Figure 2

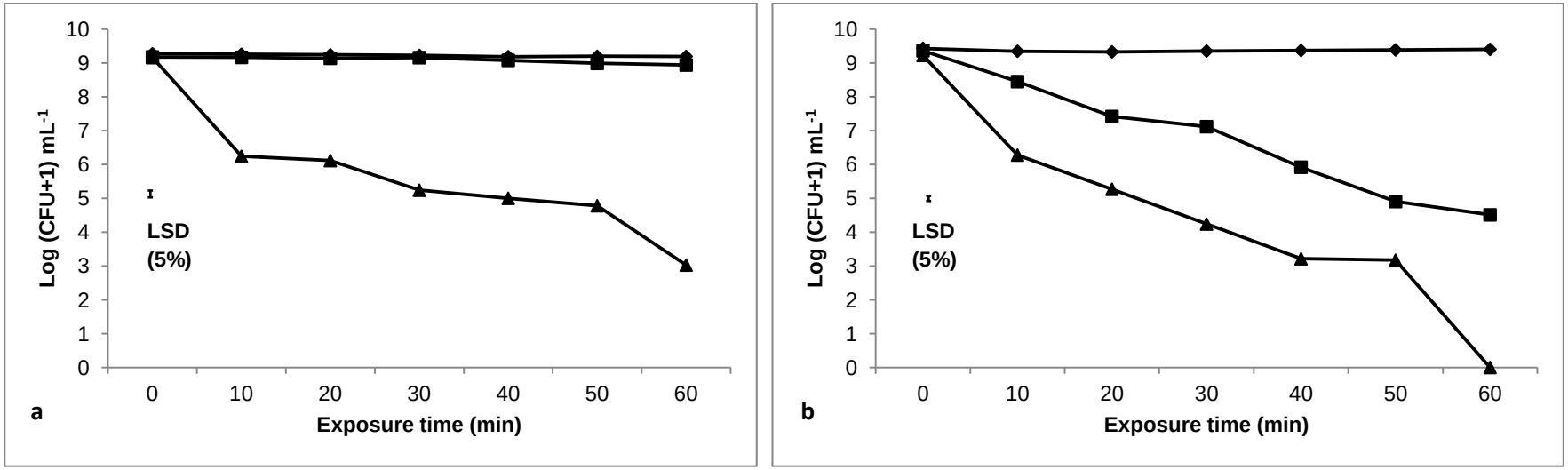
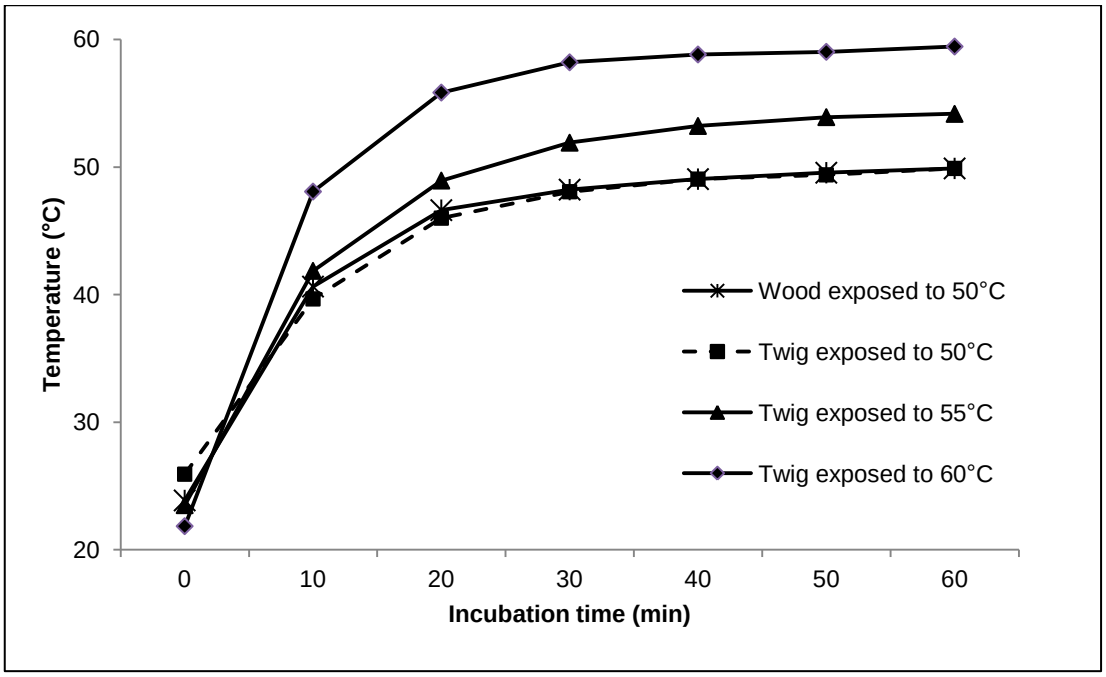


Figure 3


60
50
40
30
20
Temperature (°C)
0
10
20
30
40
50
60
Incubation time (min)
Wood exposed to 50°C
Twig exposed to 50°C
Twig exposed to 55°C
Twig exposed to 60°C

Chapter 5 Persistence of *X. translucens* pv. *pistaciae* in infected pistachio wood buried in, or placed on, the soil

5.1 Introduction

Deep burial of infected plant materials is one of the two standard methods recommended by the Australian Quarantine and Inspection Services (http://www.iwgq.com.au, 04/2007) for eradication of plant pathogens (see Section 1.4.2), particularly for the disposal of large volumes of materials such as potatoes, root vegetables or other juicy or fleshy produce (Ebbels, 2003). Successful eradication of bacterial pathogens, such as *Erwinia amylovora*, by burial of host material was reported in Australia (Daly & Rodoni, 2007). In addition, burial was a part of an integrated approach for prevention and control of citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (syn. *X. citri* and *X. campestris* pv. *citri*) in Florida (Graham *et al*., 2004). Graham *et al*. (1987) studied survival of the citrus canker pathogen in infected leaves buried in soil or placed on the soil surface and found that the pathogen survived 120 days in leaves left on the soil surface but only 85 days in leaves buried in moist soil. However, no information is available on duration of survival of *E. amylovora* or other systemic bacteria of perennial crops in infected materials buried in soil. In a review of eradication strategies, Sosnowski *et al.* (2009) concluded that more scientific evidence is required to substantiate the methods leading to successful campaigns.

As Australia is currently free from fire blight and other systemic bacterial diseases, such as Pierce's disease of grapes, their causal pathogens are considered high-priority EPPs in Australia. Thus, information on effect of burial on survival of pathogens is of importance to the country as a basis for developing eradication strategies, should an incursion of these pathogens occur. *Xtp*, as a wood-inhabiting bacterium endemic to

Australia, was chosen as a model to assess the effectiveness of burial in eradicating systemic bacterial pathogens of woody perennials.

There is extensive literature on survival of *Xanthomonas* pathogens in debris of annual crops plants and foliage of perennial crops, buried in or placed on the soil surface (see Section 1.4.2). It has been reported that *Xanthomonas* survived longer in diseased leaves of grapefruit (Graham *et al.*, 1987), cowpea (Sikirou & Wydra, 2004) and common bean (Torres *et al.*, 2009) placed on the soil surface than when buried from 10 to 20 cm deep in soil. Gent *et al.* (2005) observed that *X. axonopodis* pv. *allii* survived in infested onion leaves buried in soil for as long as in those placed on the soil surface. However, populations of the pathogen were greater in the leaves on the soil surface than in buried leaves. In contrast, Duffy (2000) observed no clear effect of burying debris on the decline of *X. axonopodis* pv. *diffenbachiae*, the causal agent of anthurium blight.

As discussed in section 1.4.2, *Xanthomonas* pathogens could survive a long time in soil when protected in plant materials (Schaad & White, 1974; Schultz & Gabrielson, 1986), but lost viability when the host tissues have decomposed (Brinkerhoff & Fink, 1964). They are generally considered not to survive or multiply in soil in a free state (Schroth *et al.*, 1979; Lee, 1920). Persistence of xanthomonads in crop residues varies depending on weather conditions (Brinkerhoff & Fink, 1964; Kocks *et al.*, 1998; Torres *et al.*, 2009) and soil moisture (Sabet & Ishag, 1969; Graham *et al.*, 1987; Sikirou & Wydra, 2004).

Although xanthomonads do not survive when infected tissues of annual crops or foliage of perennial crop decompose (Brinkerhoff and Fink, 1964; Schultz and Gabrielson, 1986; Graham and McGuire, 1987), there is no information on survival of vascular bacterial pathogens in woody debris of perennial crops. Therefore, this study was conducted to assess survival of *Xtp,* a wood-inhabiting bacterium, in infected pistachio

wood buried in orchard soil in comparison with wood left on the soil surface, which is common practice following pruning in commercial orchards. Rainfall and air and soil temperature at the experimental site were recorded to examine possible associations between environment and pathogen survival.

5.2 Materials and methods

Burial experiments were conducted in an open environment in the University of Adelaide, Waite Campus orchard, South Australia (34°58'.18"S, 138°38'.00"E, elevation 127 m). Each experiment comprised two treatments (buried *vs.* surface placement) arranged in a completely randomised design. Segments or mulch of naturally infected pistachio branches were used in 2008 and artificially infected twigs were used in addition in 2010. Survival of *Xtp* in treated materials was assessed for up to 31 months. Microclimatic conditions at the experimental site were monitored from August 2008 to March 2011.

5.2.1 Plant materials

5.2.1.1 Naturally infected wood

For burial in 2008, branches of 3 - 4 cm in diameter with staining of the xylem typical of dieback were collected from a tree confirmed to be infected with *Xtp* in the Waite Campus orchard (Chapter 4). Pieces of wood from each branch were processed to confirm the presence of the pathogen by culturing on BSPA and by PCR with *Xtp*-specific primers as described in section 2.4.1. Branches which gave rise to colonies typical of *Xtp* on BSPA and confirmed positive with PCR were then used for the experiment.

In 2010, twigs from branches with staining of the xylem typical of dieback were collected from trees confirmed to be infected with *Xtp* in a commercial orchard in

Robinvale, Victoria (34°34'.46" S, 142°46'.47" E, elevation 61 m) (Facelli *et al.*, 2005). The presence of *Xtp* was confirmed by culturing on NA and by PCR as described above. Branches that bore twigs which yielded colonies and the amplicon expected for *Xtp* were used for the experiment.

5.2.1.2 Artificially infected twigs

In 2010, twigs artificially infected with *Xtp*, prepared as described in section 2.5, were used in addition to naturally infected wood. Prior to burial, one-third of each twig was processed as described in section 2.3.2 to confirm the presence of the pathogen and determine the initial population. The remaining part of each twig that yielded an initial population from 10^6 - 10^8 CFU mL^{-1} was subsequently used in the experiment.

5.2.2 Soil

Soil used for the experiment was collected from the Waite Campus orchard. Before potting, six soil samples were taken at random from the heap to determine pH, electrical conductivity (EC) and texture. To measure the pH and EC, each soil sample was first diluted by mixing 10 g of air-dried soil (passed through a 2-mm sieve) with 50 mL of reverse osmosis (RO) water. The suspensions were then shaken for 1 h at room temperature and allowed to settle for 20 min before taking the measurements of pH and EC using a pH and conductivity meter, respectively (Rayment & Higginson, 1992).

The texture of the soil was determined using a method modified from Smith and Tiller (1977) as follows: approximately 15 g of sieved air-dried soil of each sample was dispersed in a 250-mL screw-top bottle by adding 10% Calgon (sodium metahexaphosphate) solution, 0.5 mL 0.6 M NaOH and 150 mL RO water. The suspensions were vigorously shaken on a rotary wheel for 24 h. Afterwards, the

suspensions were further diluted with RO water in a measuring cylinder to ensure the mixture contained less than 1% of suspended material, stirred and allowed to settle for 3 h. Twenty five millilitres of each suspension at the depth of 3 cm was then transferred into a pre-weighed heat resistant plastic container with a lid and placed in an oven set at 105°C for 24 h. After cooling in a desiccator, the samples were re-weighed to obtain an apparent weight of clay, which was then corrected for dispersants added (M_{clay}). The suspensions remaining after taking out the clay samples were carefully poured off to remove only clay and silt layers. RO water was then added to the remaining layers in the measuring cylinders and the contents were stirred before leaving the suspensions undisturbed for about 5 min to allow all particles greater than 20 µm to settle at the bottom. The supernatants were then poured off. This process was repeated until the supernatants were clear. The remaining layers in the cylinders were then collected and oven-dried to obtain the mass of sand ($M_{sand)}$. The percentages of clay, sand and silt were calculated as follows:

$$\%T = \frac{\frac{M_{clay}}{V_s} \times V_t}{M_{OD\ soil}} \times 100$$

$$\%S_{tot} = \frac{M_{sand}}{M_{OD\ soil}} \times 100$$

$$\%Silt = 100 - (\%T - \%S_{tot})$$

%T = clay content; M_{clay} = corrected mass of clay; V_s = sample volume; V_t = total volume; M_{ODsoil} = total mass of oven dry soil sample S_{tot} = total content of sand; M_{sand} = mass of sand

The soil was categorised as having a sandy-clay-loam, clay-loam to sandy-clay texture, pH of 8.7 - 9 and electrical conductivity (EC) of 0.07 - 0.1 dS m^{-1}.

5.2.3 Burial of infected wood

5.2.3.1 Experiment 1 (August 2008 to March 2011)

Branches confirmed to have viable *Xtp* were first cut into discs of 1 cm thick. Branch segments with a diameter greater than 2 cm were cut into half (Fig. 5.1a) transversely

through visibly stained xylem to increase the likelihood that the pathogen was present in each half. Methods adapted from those described by Naseri *et al*. (2008) were used, with modification, as follows. All branch segments were pooled, then three or four segments (*ca*. 20 g total) were randomly selected and enclosed in a plastic mesh bag (10 x 10 cm; aperture 1 mm^2) (Fig. 5.1b). The mesh bags were individually buried 10 cm deep (Fig. 5.1c) in pots (20 cm diameter) filled with orchard soil or placed on the soil surface (Fig. 5.1d). The pots were placed in the south west corner of the Waite Campus orchard in August 2008 (Fig. 5.2b). A weather station (Model T MetStation, Western Electronic Design, Loxton, South Australia) (Fig. 5.2) was placed close by to record weather conditions during the experiment. A Hastings Data Logger (Gemini Data Loggers (UK) Ltd) was set on the right of the plot of wood segments with its probe inserted into soil at the same depth as the wood sample in one representative pot to record the soil temperature at hourly intervals.

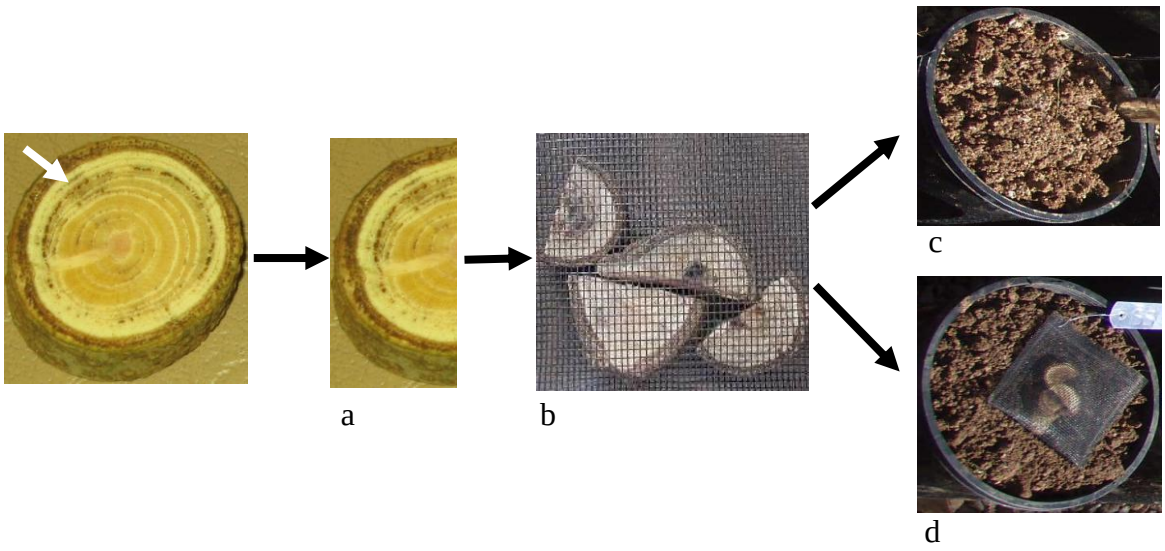


Figure 5.1 Preparation of infected branch segments for the burial experiments in 2008 and 2010. A branch segment with discolouration typical of pistachio dieback (arrow) was cut in half (a) and three-four such segments enclosed in each plastic mesh bag (b) prior to burying in pots filled with orchard soil (c) or placement on the soil surface (d).

Figure 5.2 Pots containing mesh bags of infected mulched wood (a) or branch segments (b) buried 10 cm deep in orchard soil or placed on the soil surface, were arranged at random in the south west corner of the Waite Campus orchard, South Australia in August 2008. A weather station (red arrow) was set up aside to record weather conditions during the experiment. A Hastings Data Logger was set on the right of the plot of wood segments (white oval) with its probe inserted into soil at the same depth as wood in one representative pot to record soil temperature at hourly intervals. Wire mesh nets were used to exclude birds and other animals.

Four replicate mesh bags of wood segments were retrieved from each treatment monthly to assess survival of *Xtp* over 24 months. Two additional retrievals were made at 28 and 31 months after burial. No wood was assessed at month 4, due to the need to develop a selective medium for isolation of the pathogen (see Chapter 3). At each retrieval, the appearance of the wood in terms of decomposition, based on integrity of the epidermal layer and friability, was assessed. The branch segments from each mesh bag were then rinsed in running tap water to remove excess soil, dried separately on sterile filter paper discs or towel paper for 2 h in a laminar air flow and weighed. The difference in weight of wood in each mesh bag before and after being buried in the orchard soil or placed on the soil surface was expressed as a percentage. The data for percent weight reduction of wood over 31 months were subjected to analysis of variance (ANOVA) using GenStat version 11.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2011). The rate of percent decrease of wood weight was calculated as follows:

$$\text{Rate} = \frac{\text{Percent wood weight decrease}}{\text{Number of months buried or on soil surface}}$$

From months 1 to 24 and at month 28, two branch segments were randomly selected from each mesh bag and each segment was further cut into half (Fig. 5.3). One half was used to assay for survival of *Xtp* as described in section 5.2.4 and the other half was assessed for wood-associated microbiota and antagonism of *Xtp* as described in section 5.2.5. For the last retrieval, 31 months after the beginning of the experiment, all remaining wood segments were assessed for survival of *Xtp* and wood-associated bacteria.

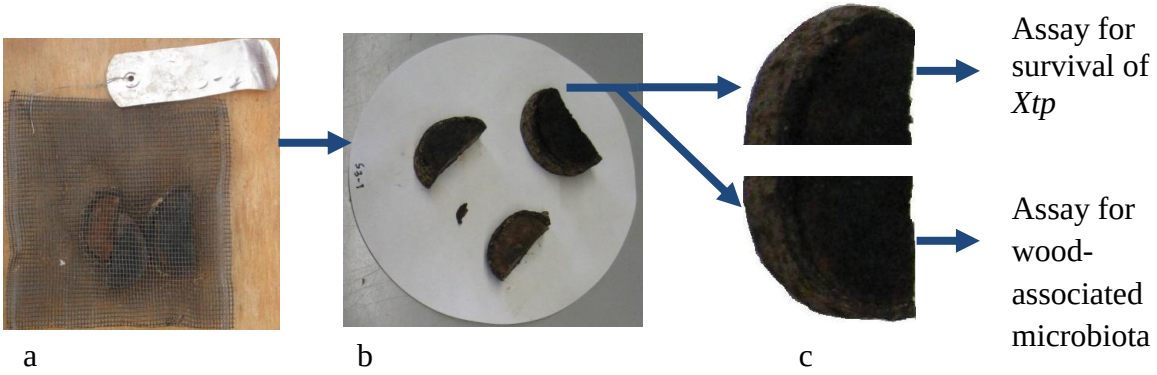


Figure 5.3 Process used for monthly retrieval of mesh bags and contents to prepare for assays on survival of *Xanthomonas translucens* pv. *pistaciae* and wood-associated microbiota, (a) a mesh bag containing branch segments, (b) segments after rinsing in running tap water and draining on a filter paper disc in a laminar air flow and (c) a segment was cut in half for survival assay (top part) and wood-associated microbiota assay (bottom part).

In addition to branch segments, mulched wood was also used for the burial experiment in 2008. Branches from pistachio trees in a commercial orchard known to harbour *Xtp* (Facelli *et al.*, 2005) were first subjected to mulching using a commercial mulching machine in the orchard (Fig. 5.4a-c), then pieces of mulched wood (5 - 10 cm long; *ca.* 20 g total) were collected immediately and transported to Adelaide. They were enclosed in plastic mesh bags, which were then individually buried in pots filled with orchard soil or placed on the soil surface (Fig. 5.4d-f). In August 2008, the pots were placed randomly in a plot in the south west corner of the Waite Campus orchard (Fig. 5.2a). Four replicate bags from each treatment were destructively sampled 7 and 8 months later. Subsequently, two replicate bags from each treatment were sampled every 3 months for up to 23 months. Survival of *Xtp* was assessed using two segments of mulched wood

randomly selected from each of the four mesh bags from each treatment for the first 2 months and using four segments from each of the two mesh bags from each placement depth for the subsequent retrievals.

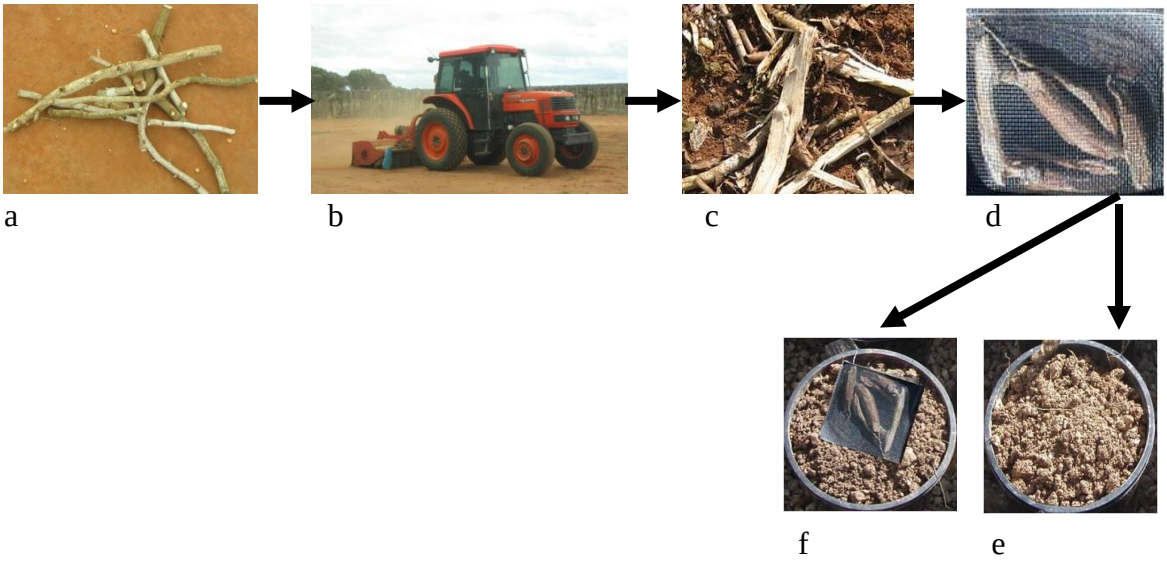


Figure 5.4 Preparation of mulched wood for the burial experiment in 2008. Pistachio branches were mulched (a-c) and then pieces of mulched wood (5 - 10 cm long, total of *ca.* 20 g) were enclosed in plastic mesh bags (d) prior to burying in pots filled with orchard soil (e) or placement on the soil surface (f).

5.2.3.2 Experiment 2 (August 2010 to January 2011)

As it was not possible to isolate viable *Xtp* from naturally infected wood for the first 4 months of the first burial experiment due to growth of other microorganisms, the experiment was partially repeated in 2010 for 5 months. Naturally infected branch

segments were prepared as for the first burial experiment and artificially infected twigs were prepared as described in section 5.2.1.2. Forty plastic mesh bags, each containing *ca.* 20 g of branch segments, were then buried 10 cm deep in separate pots (20 cm diameter) filled with orchard soil or placed on the soil surface (20 of each). Another 40 mesh bags, each containing 8 - 11 g of artificially infected twigs, were similarly buried in pots or placed on the soil surface. In August 2010, the pots were randomly arranged in two separate plots, one for naturally infected wood and the other for artificially infected twigs, adjacent to the first burial experiment. Each month, for 5 months, four replicate mesh bags of naturally infected wood and four mesh bags of artificially infected twigs from each treatment were retrieved to assess survival of *Xtp* in wood and twigs as described in section 5.2.4 and by PCR as described in section 2.4. Upon retrieval, the wood in each mesh bag was weighed. The weight loss was expressed as a percentage of the original weight and the data were subjected to ANOVA as described previously.

5.2.4 Survival and detection of *Xtp* in wood buried in soil or placed on the surface

In the first 5 months of Experiment 1, branch segments were first sterilised in 1% sodium hypochlorite solution for 1 min, rinsed three times in SDW and dried on sterile paper towels in a laminar air flow. Prior to cutting the wood into pieces of 0.5 - 1 mm thick, bark was removed by a sterile scalpel and surface wood tissues which had been in contact with soil were removed using sterile secateurs to minimise contamination by soil microorganisms. From month 6, when bark of the wood segments on the soil surface had detached from the wood and buried segments became darker, bark was removed before sterilisation. Prior to cutting the wood into pieces, surface tissues were removed with sterile secateurs. In all cases, wood pieces (0.5 - 0.7 g) were incubated in SDW overnight at room temperature and CFU in the resulting suspension were enumerated using the SPC method (see Section 2.3) in duplicate. In the first 3 months of retrieval in Experiment 1,

ABSPA was used and for all subsequent retrievals NA+A was used (see Section 3.2.5). For each retrieval, PCR was used in addition to detect the pathogen in resulting suspensions as described in section 2.4. *Xtp*-like colonies on NA+A were confirmed by streaking on SPA and observing plates for appearance of typical yellow mucoid colonies and by subjecting to PCR. A similar procedure was used for mulched wood retrieved from Experiment 1 and for wood and twigs retrieved from Experiment 2. Detection of *Xtp* in mulched wood by PCR was conducted for months 20 and 23 after burial or placement of wood and at all times for wood segments and twigs in Experiment 2.

5.2.5 Microbiota associated with wood in contact with soil and antagonism of *Xtp*

Retrieved branch segments were first sliced into 1 - 1.5 mm thick pieces, surface sterilised in 0.5% sodium hypochlorite solution for 30 s, rinsed three times in SDW and blotted dry on sterile towel papers in a laminar air flow. Three pieces of wood were then placed directly on NA amended with 150 mg L^{-1} Benlate® (NA+B) and another three pieces were placed on dilute V-8 agar amended with streptomycin at 30 mg L^{-1} (Erwin *et al.*, 1987) (Appendix A). Plates were incubated in darkness at 25°C for 3 - 5 days. Fungal colonies from the wood pieces were subcultured onto potato dextrose agar (PDA) (Appendix A). Bacterial colonies, selected based on their cultural characteristics on NA (colony shape, size, colour, elevation and edge), were subcultured onto NA several times to obtain pure cultures for antagonism tests.

To screen for antagonism, a suspension of *Xtp*, 10^5 CFU mL^{-1}, was spread on SPA plates an hour before applying with a loop to the centre of each plate (in a circle of *ca.* 3 mm diameter) a small amount of a pure culture of bacteria isolated from the wood. Plates were incubated at 28°C in darkness and any inhibition zone in the lawn of *Xtp* was measured 2 - 3 days later. Four measurements from the edge of the bacterial colony to the

edge of the inhibition zone were taken perpendicular to each other, then averaged to give a measurement of the inhibition zone. The bacteria were not considered to be antagonistic to *Xtp* when they produced no inhibition zone. The antagonism was considered to be weak when mean inhibition zones were 0.1 to less than 0.5 cm, moderate when mean inhibition zones were more than 0.5 to less than 1 cm, and strong when mean inhibition zones were 1 cm or greater. Antagonists, inclusive of weak, moderate and strong types, were expressed as a percentage of the total number of bacteria tested. Changes in the percentage of antagonists in relation to sampling time were examined for independence using the G-test (Sokal & Rohlf, 2000).

5.2.6 Environmental factors

The temperature of the soil inside one pot at the same depth as buried wood was recorded using a Hastings Data Logger from August 2008 to March 2011 (see Section 5.2.3.1). The data were recorded hourly then converted to mean daily temperature. The mean monthly air temperature and rainfall were obtained from daily records by the weather station from August 2008 to March 2011 (see Section 5.2.3.1). For the 2010 burial experiment, an additional Hasting Data Logger was set up to record the temperature at the soil surface.

5.3 Results

5.3.1 Appearance of wood

After the first month in Experiment 1, the bark of most branch segments retrieved from the soil surface was readily detached or showed signs of separation from the wood while there was little or no sign of detachment of bark from wood buried 10 cm deep in the orchard soil. Cracks were observed on the surface of a few wood segments placed on the soil

surface and these increased over time. The side of the branch segments that had faced upwards on the soil surface became faded from the second month and turned grey in the following months, while the side which had been in contact with the soil first turned brown then black. Both sides of branch segments buried in the soil turned brown or black. Visually, there was no sign of disintegration of woody tissues, even 31 months after the wood segments had been buried in or placed on the soil surface (Fig. 5.5). However, branch segments buried in soil became increasingly soft and easier to cut from about 1 year after burial until the end of the burial period. Wood on the surface became more brittle due to cracks, and was easier to cut. Observations for the branch segments buried in or placed on the soil in Experiment 2 were similar.

Figure 5.5 Appearance of pistachio wood, naturally infected with *Xanthomonas translucens* pv. *pistaciae*, 31 months after (a) burying 10 cm deep in pots filled with orchard soil or (b) placing on the soil surface.

In Experiment 1, the weight of the branch segments retrieved from soil decreased by 6% in the first month of burial and by approximately 40% after 6 months (Table 5.1). The weight fluctuated between the retrievals and tended to decrease more in the summer months. The rate of decrease in weight of wood segments placed on the soil surface slowed down from

Table 5.1 Percent decrease in weight of pistachio wood segments buried in orchard soil or placed on the soil surface over 31 months in Experiment 1 relative to fresh weight prior to burial or placement on the soil surface.

Treatment	Sampling time[1]												
	Sept 08	Oct 08	Nov 08	Jan 09	Feb 09	Mar 09	Apr 09	May 09	Jun 09	Jul 09	Aug 09	Sept 09	Oct 09
Buried	6.11[2]	18.27	34.62	42.50	40.54	31.31	38.92	20.83	23.36	29.73	32.58	34.32	33.10
Surface	30.09	31.95	35.04	37.95	35.65	36.04	33.57	28.59	30.80	37.41	41.54	40.62	45.72
Difference of mean[3]	*S	S	NS	S	S	S	S	S	S	S	*S	NS	*S

Treatment	Sampling time[1]											
	Nov 09	Dec 09	Jan 10	Feb 10	Mar 10	Apr 10	May 10	Jun 10	Jul 10	Aug 10	Dec 10	Mar 11
Buried	54.96	49.16	51.10	51.12	46.68	45.32	40.08	32.68	38.18	39.95	50.47	40.36
Surface	48.94	48.27	48.93	45.55	45.54	43.89	45.18	35.26	37.48	40.49	54.22	41.35
Difference of mean	S	NS	NS	NS	NS	NS	S	NS	NS	NS	NS	NS

[1] No wood was retrieved in December 2008, September to November 2010 and January to February 2011.

[2] Figures are the mean of percent decrease of weight of wood in four replicate bags; LSD = 5.728; $P < 0.001$.

[3] Paired comparison of means of weight of wood (buried *vs.* surface). *S = Significant at $P < 0.001$; S = Significant at $P < 0.05$; NS = Non-significant at $P > 0.05$.

the second month while that of those buried in soil increased up to the third month, then decelerated (Fig. 5.6). From the fifth to the 31st month, rate of decrease of wood weight buried in soil or placed on the soil surface was similar and very slow (Fig. 5.6). Observations on the rate of decrease in weight of wood segments and twigs in Experiment 2 were similar (Fig. 5.7a and b).

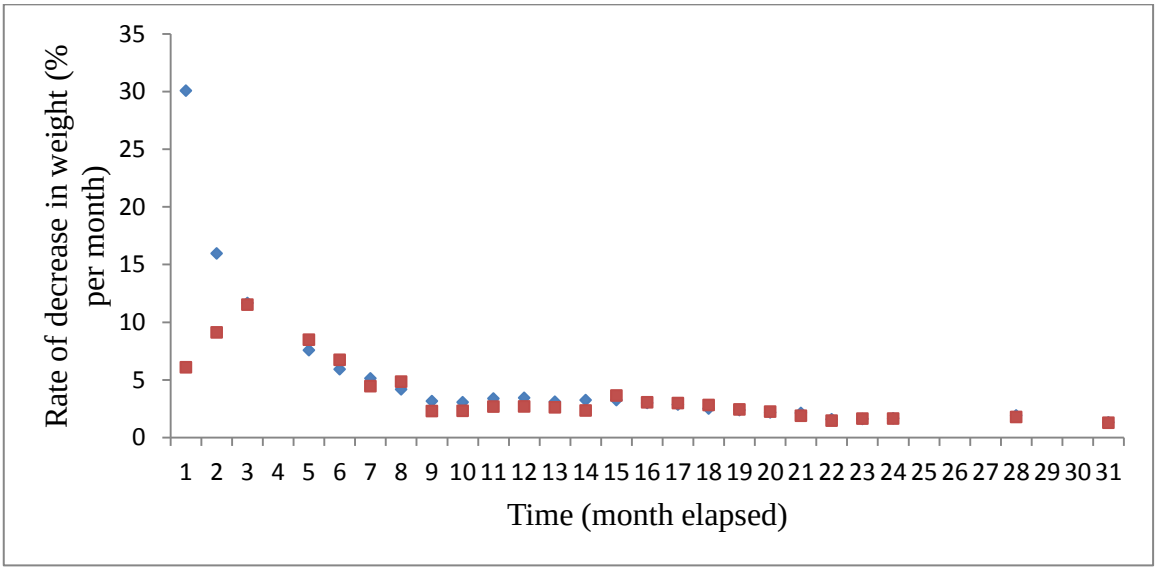


Figure 5.6 The rate of percent decrease in weight of pistachio wood, naturally infected with *Xanthomonas translucens* pv. *pistaciae*, buried in pots filled with orchard soil (blue diamond) or placed on the soil surface (red square) in Experiment 1 over 31 months; no wood sample was assessed at 4, 25 - 27 and 29 - 30 months after wood was buried or placed on the soil surface. LSD = 0.5882, $P < 0.001$.

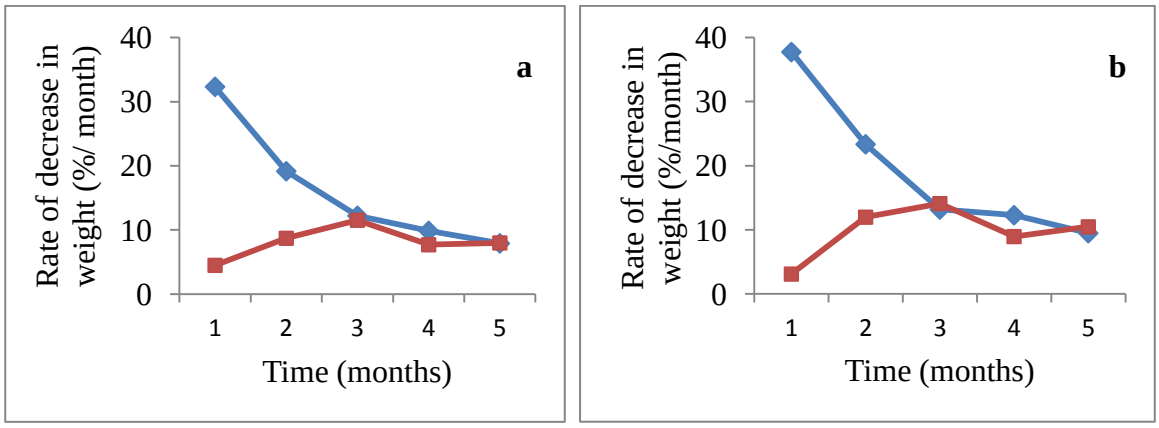


Figure 5.7 The rate of decrease in weight of pistachio, (a) wood naturally infected with *Xanthomonas translucens* pv. *pistaciae* and (b) twigs artificially infected with *Xtp*, buried in pots filled with orchard soil (blue diamond) or placed on the soil surface (red square) in Experiment 2 over 5 months.

5.3.2 Survival and detection of *Xtp* in wood buried in or on the soil surface

5.3.2.1 Experiment 1

Viable *Xtp* was not detected by culturing on ABSPA in the first 3 months after the wood segments were buried in soil or placed on the soil surface, due to growth of other microorganisms associated with wood or soil (Table 5.2). From January 2009, NA+A was used instead of ABSPA. Viable *Xtp* was isolated from wood buried in soil, 5 months after burial, and continued to be isolated, although inconsistently, until 31 months after burial. Generally, viable *Xtp* was isolated from wood samples from one or two pots, except for the retrieval in month 15 after burial when viable *Xtp* was isolated from wood segments from all four pots. Viable *Xtp* was not isolated from wood segments placed on the soil surface at any time.

Following amplification by PCR, an amplicon of 331 bp, expected for *Xtp*, was observed for most samples buried in soil throughout the experiment, including all samples from which viable *Xtp* was isolated, and less frequently in the samples placed on the soil surface (Table 5.2). At the end of Experiment 1, *Xtp* was still detected by PCR in seven of 18 wood samples buried in soil whereas it was not detected in any of the wood samples placed on the soil surface for 28 and 31 months (Table 5.2; Fig. 5.8).

Viable *Xtp* was isolated from mulched wood samples retrieved from soil 7 and 8 months and again 20 and 23 months after burial (Table 5.3). Viable *Xtp* was detected in one mulched wood sample placed on the soil surface of one pot for 20 months but not from the other pot nor in any other mulched samples placed on the soil surface at any other time. *Xtp* was detected by PCR in mulched wood samples buried in soil for 20 and 23 months and in mulched wood placed on the soil surface at 23 months, but not at month 20 when viable *Xtp* was isolated from the mulched wood sample placed on the soil surface.

5.3.2.2 Experiment 2

Viable *Xtp* was isolated from naturally infected wood segments buried in soil only at month 2 and from artificially infected twigs at months 2 and 3 after burial (Table 5.4). While viable *Xtp* was not isolated from naturally infected wood segments placed on the soil surface at any time, it was detected in artificially infected twigs from months 1 to 3 after the twigs were placed on the soil surface. *Xtp* was detected by PCR in most samples, including some that failed to yield viable *Xtp*.

Table 5.2 Viability and presence of *Xanthomonas translucens* pv. *pistaciae* (*Xtp*) in naturally infected pistachio wood buried in orchard soil in pots or placed on the soil surface over 31 months in Experiment 1.

Time	Months elapsed	Samples, of four pots per treatment, that yielded viable *Xtp* on NA+A[a]		Samples, of four pots per treatment, that were positive by PCR	
		Buried	Surface	Buried	Surface
Sep 08	1	_[b]	_	3	3
Oct 08	2	_	_	4	4
Nov 08	3	_	_	4	2
Dec 08	4	n/a[c]	n/a	n/a	n/a
Jan 09	5	2	0	3	2
Feb 09	6	1	0	4	4
Mar 09	7	0	0	4	2
Apr 09	8	2	0	4	3
May 09	9	2	0	4	2
Jun 09	10	2	0	4	3
Jul 09	11	0	0	4	2
Aug 09	12	0	0	4	2
Sep 09	13	1	0	4	0
Oct 09	14	0	0	4	4
Nov 09	15	4	0	4	2
Dec 09	16	0	0	4	0
Jan 10	17	1	0	4	3
Feb 10	18	0	0	4	4
Mar 10	19	2	0	4	0
Apr 10	20	1	0	4	1
May 10	21	1	0	4	4

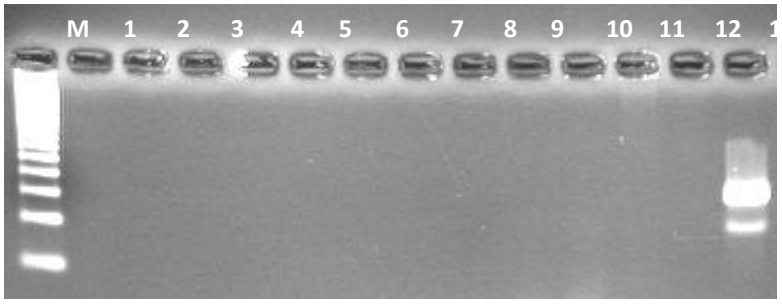
Jun 10	22	2	0	3	4
Jul 10	23	1	0	4	4
Aug 10	24	1	0	4	4
Dec 10	28	2	0	3	0
Mar 11	31	1	0	4	0

[a]NA+A: Nutrient agar amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin.

[b]"_": Viable *Xtp* was not detected by culturing on sucrose peptone agar amended with 150 mg L^{-1} Benlate®, 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 1.4 mg L^{-1} gentamycin (ABSPA) in the first 3 months due to overgrowth by other microorganisms associated with wood or soil.

[c]n/a: Not applicable, wood was not retrieved in the fourth month (December 2008) whilst an alternative to ABSPA for the isolation of *Xtp* from wood buried in soil or placed on the soil surface was developed.

a



b

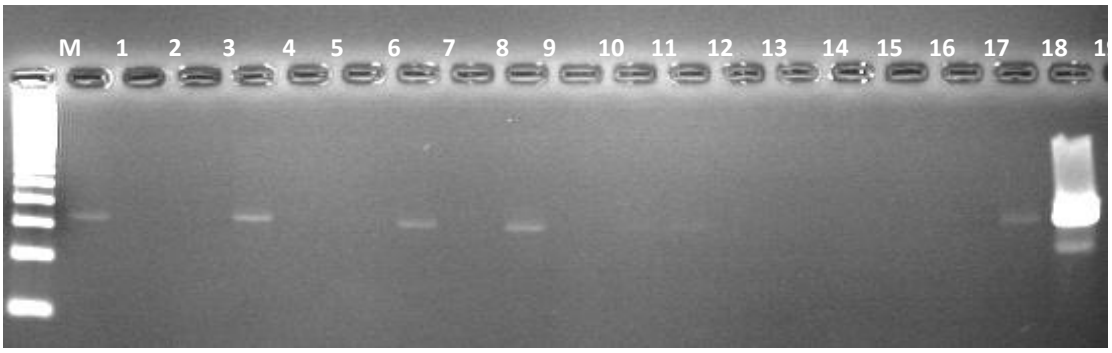


Figure 5.8 Detection by PCR of *Xanthomonas translucens* pv. *pistaciae* (*Xtp)* in naturally infected pistachio wood 31 months after (a) placement on the soil surface and (b) being buried 10 cm deep in soil in Experiment 1. Samples prepared by incubating woody tissue in sterile distilled water overnight, centrifuging the resulting suspension at 10,000 rpm for 10 min and resuspending the pellet in 15 µL sterile distilled water, then amplification using *Xtp*-specific primers as described by Marefat *et al.* (2006a).

(a) Lanes: M, molecular size marker (100 bp ladder, Invitrogen, Australia); 1-3a, samples prepared from pot 1; 4-7a, samples from pot 2; 8-10a, samples from pot 3; and 11-12a, samples from pot 4; 13a, positive control (pure culture of *Xtp*).

(b) Lanes: M, molecular size marker (100 bp ladder, Invitrogen, Australia); 1-3b, samples prepared from pot 1; 4-8b, samples from pot 2; 9-14b, samples from pot 3; and 15-18b, samples from pot 4; 19b, positive control (pure culture of *Xtp*).

Table 5.3 Viability and presence of *Xanthomonas translucens* pv. *pistaciae* in naturally infected mulched pistachio wood buried in orchard soil in pots or placed on the soil surface over 23 months from August 2008 to July 2010 in Experiment 1.

Time	Months elapsed	Samples, of four pots per treatment, that yielded viable *Xtp* on NA+A[a]		Samples, of four pots per treatment, that were positive by PCR	
		Buried	Surface	Buried	Surface
Mar 09	7	2/4	0/4	n/a[b]	n/a
Apr 09	8	4/4	0/4	n/a	n/a
Jul 09	11	0/2	0/2	n/a	n/a
Oct 09	14	0/2	0/2	n/a	n/a
Jan 10	17	0/2	0/2	n/a	n/a
Apr 10	20	2/2	1/2	2/2	0/2
Jul 10	23	2/2	0/2	2/2	2/2

[a]NA+A: Nutrient agar amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin.

[b]n/a: Not applicable, PCR was not conducted for mulched wood samples at months 7, 8, 11, 14 and 17.

Table 5.4 Viability and presence of *Xanthomonas translucens* pv. *pistaciae* in naturally infected wood and artificially infected twigs of pistachio buried in orchard soil or placed on the soil surface over 5 months from August 2010 to January 2011 in Experiment 2.

Month	Months elapsed	Materials	Samples, of four pots per treatment, that yielded viable *Xtp* on NA+A[a]		Samples, of four pots per treatment, that were positive by PCR	
			Buried	Surface	Buried	Surface
Sep 10	1	Wood	0	0	4	4
		Twigs	0	3	4	4
Oct 10	2	Wood	3	0	4	4
		Twigs	4	1	4	4
Nov 10	3	Wood	0	0	3	4
		Twigs	2	1	2	4
Dec 10	4	Wood	0	0	4	2
		Twigs	0	0	3	4
Jan 11	5	Wood	0	0	3	2
		Twigs	0	0	4	4

[a] NA+A: Nutrient agar amended with 10 mg L^{-1} cephalexin, 1 mg L^{-1} ampicillin and 0.7 mg L^{-1} gentamycin.

5.3.3 Microbiota associated with wood and their antagonism of *Xtp*

A number of fungi with different cultural characteristics were observed and subcultured on PDA. The isolation of wood-associated fungi was terminated in June 2009 due to time constraints. However, white and grey mycelium was often observed on the surface of wood buried in soil and placed on the soil surface.

Based on the colony characteristics, a diverse group of bacteria was isolated from naturally infected wood segments buried in soil or placed on the soil surface over 17 months, from January 2009 to May 2010. The largest number of bacteria in terms of variety isolated from wood segments placed on the soil surface was in August 2009 and from those buried in soil was in March 2010. When established in pure culture, the bacteria produced different degrees of inhibition of the growth of *Xtp* on SPA (Fig. 5.9), with mean radius of inhibition zones ranging from nil to greater than 1 cm. Even though some bacteria did not produce any inhibition zones in the lawn of *Xtp*, their colonies grew relatively large and, occasionally, covered almost the whole plate so that no or little growth of *Xtp* was observed.

In general, among the wood-associated bacteria, more bacteria antagonistic to *Xtp* than non-antagonistic ones were isolated from wood buried in soil or placed on the soil surface, except in a few months where the number of non-antagonistic bacteria was equal or nearly equal to the number of antagonistic bacteria (Table 5.5). Over 14 sampling months, strong antagonists were isolated in 10 of the months from the wood buried in soil and in 7 of the months from the wood placed on the soil surface, whereas moderate and weak antagonists were present in almost all months. Isolation of bacteria that were antagonistic to *Xtp* varied amongst the retrievals, starting with only a few isolates in January 2009 but increasing over time. However, changes in the percentage of bacterial isolates that were antagonistic to *Xtp* were not related to sampling month ($P > 0.05$).

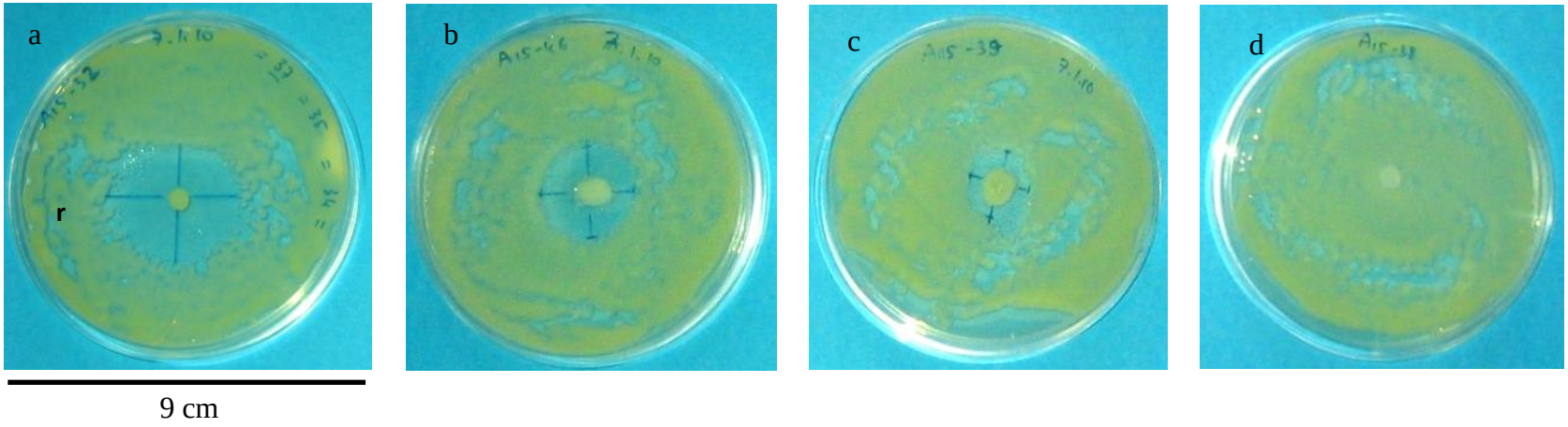


Figure 5.9 Representative *in-vitro* antagonism of *Xanthomonas translucens* pv. *pistaciae* by bacteria isolated from wood buried in soil and placed on the soil surface. The bacteria were categorised based on average of four radii of inhibition zones (r) as (a) strong ($r \geq 1$ cm), (b) moderate (0.5 cm $\leq r <$ 1 cm), (c) weak antagonist ($0 < r < 0.5$ cm) and (d) non-antagonist ($r = 0$ cm).

Table 5.5 Number of bacteria isolated from naturally infected pistachio wood segments retrieved from Experiment 1 from January 2009 to May 2010 that were antagonistic to *Xanthomonas translucens* pv. *pistaciae* (*Xtp*), and degree of antagonism on sucrose peptone agar.

Month	Months elapsed	Bacteria isolated from wood segments buried in soil						Bacteria isolated from wood segments on soil surface					
		Strong[a]	Moderate[b]	Weak[c]	Non-antagonist[d]	Total	Percentage of isolates antagonistic[e]	Strong	Moderate	Weak	Non-antagonist	Total	Percentage of isolates antagonistic
Jan 09	5	1	2	4	0	7	100	0	5	4	0	9	100
Feb 09	6	0	0	8	2	10	80	0	2	6	2	10	80
Mar 09	7	0	3	5	0	8	100	3	4	3	2	12	83
Apr 09	8	2	7	1	0	10	100	7	11	0	0	18	100
May 09	9	1	12	6	5	24	79	1	6	11	4	22	82
Jul 09	11	2	3	2	6	13	54	2	10	11	8	31	74
Aug 09	12	1	6	8	15	30	50	4	6	5	19	34	44
Sep 09	13	5	7	6	9	27	67	2	4	4	6	16	63
Oct 09	14	3	10	4	3	20	85	0	6	2	9	17	47
Nov 09	15	5	6	3	11	25	56	2	5	3	15	25	40
Jan 10	17	1	13	4	14	32	56	0	13	4	9	26	65
Mar 10	19	2	10	11	11	34	68	0	5	6	11	22	50
Apr 10	20	0	13	9	6	28	79	0	11	13	6	30	80
May 10	21	0	8	14	11	33	67	0	2	16	13	31	58

[a]Strong (mean inhibition zone r ≥ 1 cm), [b]moderate (0.5 cm ≤ r < 1 cm), [c]weak antagonist (0 < r <0.5 cm) and [d]non-antagonist (r = 0 cm). [e]The percentage of bacterial isolates that were antagonistic to *Xtp* at different sampling times was examined for independence using the G-test (Sokal & Rohlf, 2000) at $P > 0.05$.

5.3.4 Environmental factors

5.3.4.1 Experiment 1 (August 2008 to March 2011)

Seasonal changes in mean daily air and soil temperature followed the same pattern over the experimental period although air temperature was generally lower than soil temperature (Fig. 5.10). Mean daily air temperature was generally below 15°C between May and mid-October, as was soil temperature between May and September. The coolest days in terms of air temperature were 6 July 2009 (7.3°C) and 6 August 2010 (7.1°C) and of soil temperature were 7 July 2009 (8°C) and 6 August 2010 (7.9°C). Warm temperature (above 20°C) occurred mainly from November to March and peaked in January, ranging from 34.7 to 38.7°C for air and 37.5 to 38.5°C for soil.

Both soil and air diurnal temperature fluctuated greatly. Although minimum mean daily temperature for air and soil was around 7 and 8°C, respectively, minimum hourly temperature for air and soil during cold days dropped to 2.5 and 2.7°C, respectively. On an hourly basis, the highest temperature generally occurred in January, with a peak at 56.4°C on 31 January 2011 for soil temperature and at 46.8°C on 28 January 2009 for air temperature.

There was little or no rain from August 2008 to June 2009 (Fig. 5.10). From July 2009, rain fell every month until the end of the experiment. The greatest monthly rainfall of 128 mm was recorded in July 2009. The maximum daily rainfall was 35.4 mm in March 2011, the last month of the experiment. Due to technical problems, rainfall data were not recorded by the MetStation from 2 to 15 September 2008, 20 October to 22 November 2009, 9 December 2009 to 15 February 2010 and 28 January to 20 February 2011 and a few days in some other months. The rainfall data recorded by the Australian Bureau of

Meteorology at Beaumont weather station (34°57'15"S, 138°39'31"E and 225 m elevation) (http://www.bom.gov.au/climate/data/), *ca.* 2.5 km from the Waite Orchard, were used for these periods.

5.3.4.2 Experiment 2 (August 2010 to January 2011)

By placing an additional Hasting Data Logger on the surface of a pot in which wood segments had been placed, it was observed that while mean daily air temperature for most of the days was lower than mean daily soil temperature, mean daily soil surface temperature was often higher than soil temperature at 10 cm deep in the pot (Fig. 5.11). Lowest mean daily soil and surface temperature was 10 and 10.8°C and highest was 35.3 and 36.2°C, respectively. In terms of hourly temperature, when the air temperature was below 15°C, soil at 10 cm below the surface was often warmer than the surface temperature, but when it reached 20°C and above, surface temperature was often higher than soil 10 cm below the surface. The minimum hourly soil surface temperature was 2.6°C and maximum was 60.6°C while hourly minimum 10 cm below the surface was 4.6°C and the maximum was 51.4°C. On some days between November and January, the temperature of soil at 10 cm deep and on the surface exceeded 40°C for a few hours from mid-morning to mid-afternoon.

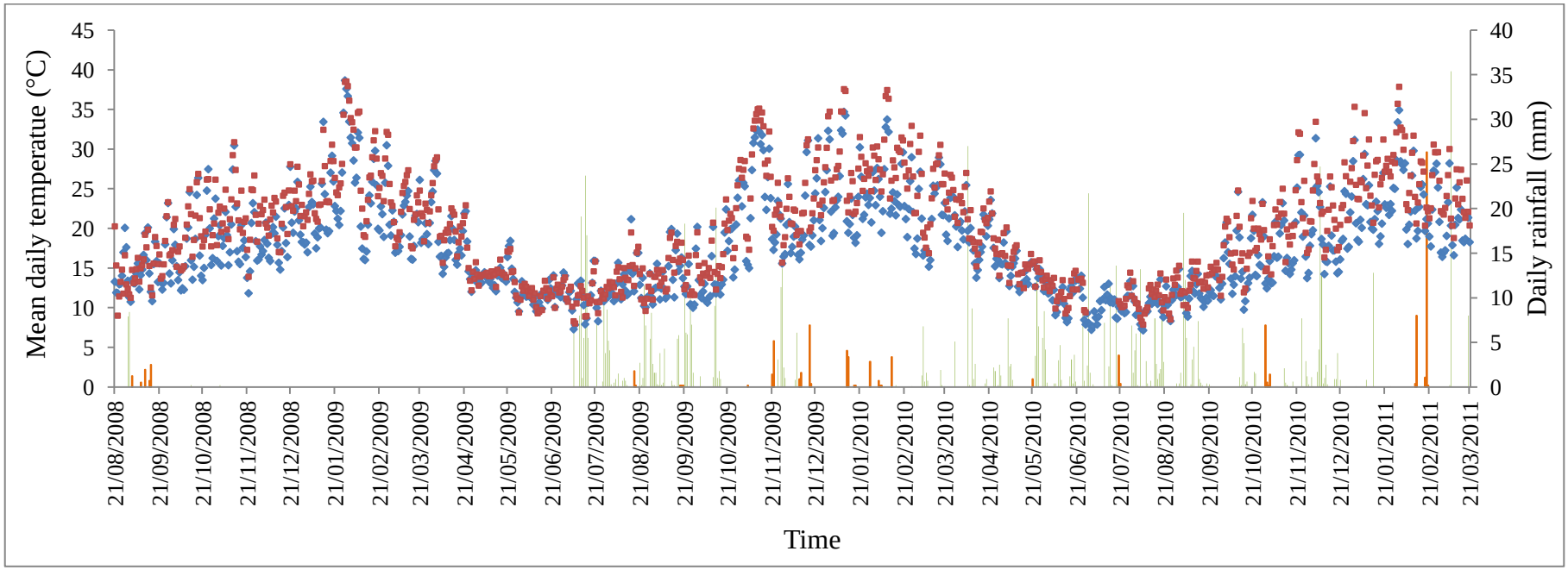


Figure 5.10 Mean daily soil temperature at 10 cm deep in pots recorded by a Hastings Data Logger (red square), mean daily air temperature (blue diamond) and daily rainfall (green column) recorded by a weather station set in the Waite Campus orchard from 21 August 2008 to 21 March 2011 or recorded at the BOM Beaumont weather station (orange column) for Experiment 1.

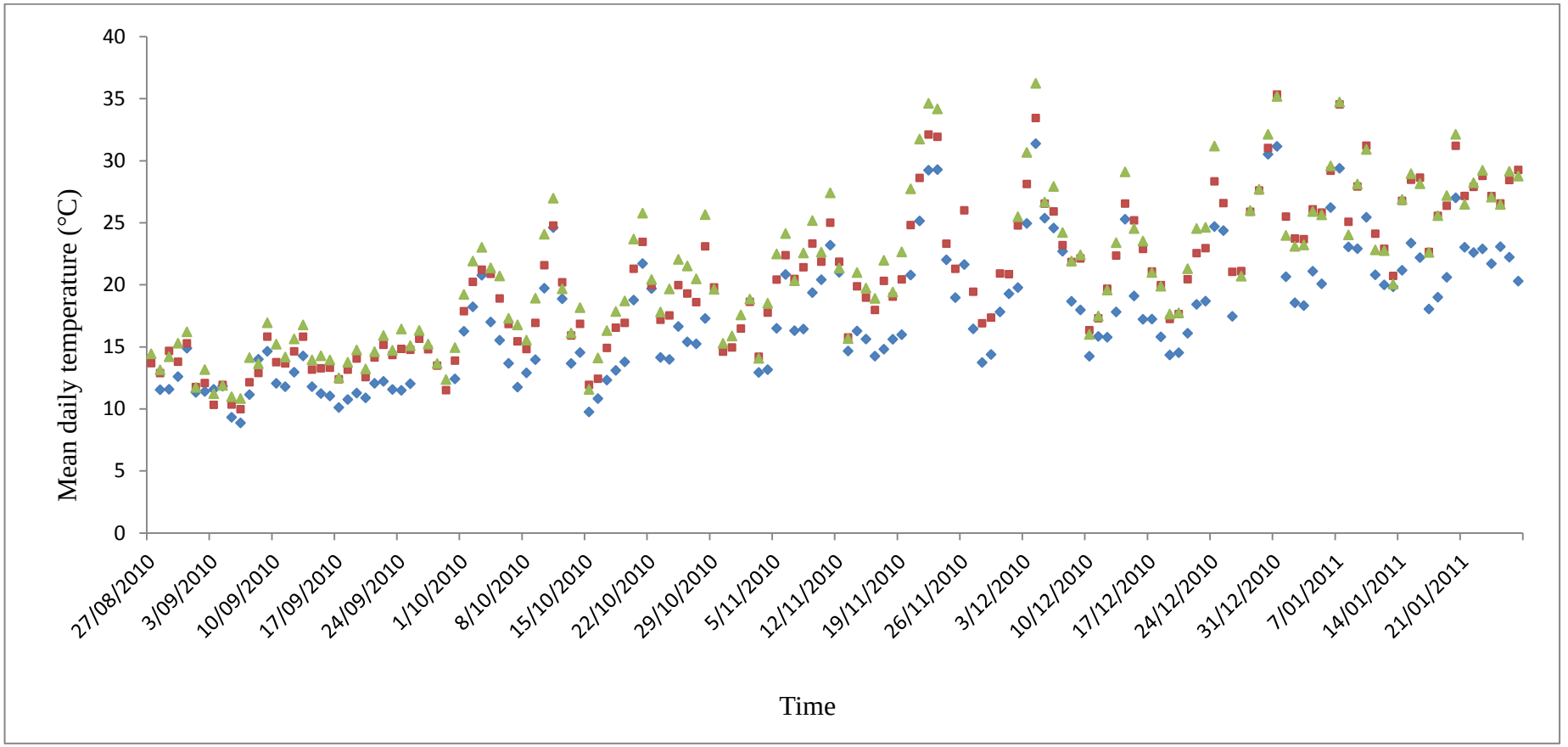


Figure 5.11 Mean daily temperature for the air (blue diamond), the soil at 10 cm deep in pots (red square) and the soil surface (green triangle) in Experiment 2, recorded from August 2010 to January 2011 by Hastings Data Loggers.

5.4 Discussion

This study demonstrated substantial long-term survival of *Xanthomonas translucens* pv. *pistaciae*, a wood-inhabiting bacterial pathogen, in naturally infected wood segments (at least 31 months) and in mulched wood (at least 23 months) buried in soil. Even though *Xtp* was not recovered from naturally infected wood segments on the soil surface as early as one month after placement, it remained viable in one mulched wood sample for 20 months and in artificially infected twigs for 3 months after placement on the soil surface.

Although the semi-selective medium NA+A reduced substantially the growth of other soil- or wood-associated bacteria, and so allowed the isolation and identification of *Xtp*, on some occasions other bacteria produced colonies with pigment and size similar to those of *Xtp*, making it difficult to identify the pathogen. As a result, the *Xtp* population could not be quantified in this study. Further research to improve this semi-selective medium is warranted to optimise the isolation and enumeration of *Xtp* from infected pistachio in contact with soil, to permit quantification of the population of *Xtp* over time.

The results of this study differed from those obtained in research on other xanthomonads (Graham *et al.*, 1987; Sikirou & Wydra, 2004; Torres *et al.*, 2009; Arnaud-Santana *et al.*, 1991; Jones *et al.*, 1986), in that *Xtp* survived longer in host tissue buried in soil than that on the soil surface. Differences in environmental conditions, nature of buried plant material, saprophytic microbiota or pathogen may have contributed to this difference in survival. In particular, burial has often been reported to hasten the decomposition of plant debris and, consequently, expose pathogens to soil, an environment that is often unfavourable for the majority of plant pathogenic bacteria, including xanthomonads (Patrick, 1954; Kocks *et al.*, 1998). In this study, pistachio wood appeared to be so resistant to decomposition that the woody tissues, even though they became softer over

time, remained intact for at least 31 months, which in turn might have protected *Xtp* from loss of viability. A number of factors might have contributed to this slow rate of decomposition. First, woody tissues have high content of lignin and contain resin (Sedgley *et al.*, 2004), known to be particularly resistant to decomposition (Brady & Weil, 2002). Second, the alkaline soil (pH of 8.7 - 9) used in this experiment might have contributed to the delayed decomposition of pistachio wood, as soil with near-neutral pH, which allows a diverse microbial population to be active, is considered to favour rapid decomposition (Brady & Weil, 2002). In addition, enclosure of the infected pistachio wood in mesh bags might have slowed down the decomposition due to limited intimate soil-residue contact and exclusion of decomposition activities by large arthropods, earthworms and invertebrates. Finally, in this study, warm temperatures (25 to 45°C) considered to be conducive to rapid decomposition (Brady & Weil, 2002; Sylvia *et al.*, 2005) tended to occur from late spring (November) to the end of summer (February) when rainfall was very scant.

Soil factors such as temperature, moisture and pH have been described as influencing survival of *Xanthomonas* species in buried crop residues. Mean daily temperature of the soil in the burial experiment ranged from 7.9 to 38.5°C, which mirrors the range of temperature for the growth of *Xanthomonas* (7 - 35°C) (Stall *et al.*, 1993). Vasudeva (1963) reported that *X. campestris* pv. *citri* survived considerably longer in diseased leaves of citrus in moist soil kept at 5 - 15°C (150 days) than at 30°C (52 days) and was killed at 40°C. Other xanthomonads, such as *X. oryzae*, were reported to survive longer at low than at high relatively humidity and temperature (Hsieh & Buddenhagen, 1975). In flooded soil and in soil at 40% moisture content, *X. oryzae* could survive in diseased rice leaves for only 12 - 20 days at 30 and 20°C, while in soil with water content close to 0%, the pathogen could survive for 180 and 360 days at 30 and 20°C, respectively.

Similarly, *X. axonopodis* pv. *phaseoli* var. *fuscans* survived longer in bean leaflets during the dry than the wet season (Torres *et al.*, 2009). Soil moisture was not recorded in the experiment involving burial of *Xtp*-infected pistachio wood. However, with a maximum monthly rainfall of 128 mm, soil in pots containing pistachio wood was not likely to become saturated, a condition that hastens the loss of infectivity by xanthomonads (Arias *et al.*, 2000; Sabet & Ishag, 1969; Graham *et al.*, 1987). This may help to explain the persistence of *Xtp* in the pistachio wood buried in soil. In addition, the alkaline soil used in the present study might allow longer survival of *Xtp,* as it was reported by Vasudera (1963) that *X. citri* could survive longer at pH 9 than at acid pH. However, the effect of pH on survival of *Xtp* has yet to be studied.

Dry conditions are considered favourable for survival of bacteria in plant residues (Schuster & Coyne, 1974), especially in those on the soil surface as they are protected from antagonistic microflora (Patrick, 1954; Stout & Heal, 1967). In addition, plant pathogenic bacteria might enter hypobiosis, a state of reduced metabolism, but increased resistance to harmful conditions such as desiccation and unfavourable temperature (Leben, 1981). Furthermore, xanthomonads produce an exopolyssacharide (EPS) slime in the infected host plant which facilitates survival in plant tissue in unfavourable conditions (Stall *et al.*, 1993). Enclosure of cells of *X. campestris* pv. *phaseoli* in EPS resulted in prolonged survival and protected the bacteria against ultraviolet radiation (Leach *et al.*, 1957) and extreme desiccation (Wilson *et al.*, 1965). Nevertheless, *Xtp* in the present study lost viability relatively quickly when naturally infected wood was placed on the soil surface. This loss of viability might be associated with the rapid desiccation of tissue during the first months of placement on surface, as shown by more than 30% decrease in wood weight during that period of time. Dry weights were not measured in this study. However, as no sign of disintegration of tissue was observed at this time, decrease in wood weight was likely due to water loss through evaporation caused by wind and prevailing dry

weather. Continued loss of water in subsequent months, even though at a slower rate, may have caused *Xtp* to become more susceptible to other unfavourable conditions, such as high temperature and intensive sunlight during late spring and summer. In particular, high temperature (above 30°C), which occurred occasionally from October 2008, 2 months after the beginning of the experiment, and continued until March 2009, might have caused the loss of viability of *Xtp* in wood on the surface. In addition, a negative reaction in PCR for extracts of all wood samples at 28 and 31 months on the soil surface indicates that the pathogen DNA may have been degraded.

Results of Experiment 2 provided information on survival of *Xtp* in wood for the first 5 months of burial in soil or placement on the soil surface. Loss of viability of *Xtp* in naturally infected wood was confirmed to occur as soon as the first month after placement of wood on the soil surface. Even though mean daily air temperature was not high during the experimental period, wood on the surface was exposed quite often to 40°C or more from the end of October 2010 and, during hot days, surface temperature exceeded 50°C or even more than 55°C for a few consecutive hours. These temperatures reduced recovery of *Xtp* from infected wood (50°C) or killed it (55°C), as presented in Chapter 4. The isolation of viable *Xtp* from artificially infected twigs, however, might have been due to higher initial population of *Xtp* in these twigs. In addition, the patchy distribution of *Xtp* in naturally infected wood (Facelli *et al.*, 2009) might have resulted in 'false negative' results.

While infrequent isolation of *Xtp* from buried wood might have been related to uneven distribution of the bacteria in naturally infected wood (Facelli *et al.*, 2009), it is possible that *Xtp* entered the viable but nonculturable state (see 1.5.2.2). The possibility that *Xtp* may enter this state as a survival mechanism in response to environmental conditions during burial will be considered in the next chapter.

There was no clear trend in frequency of isolation of viable *Xtp* from wood buried in soil and, although it was not possible to draw a conclusion on population decrease of *Xtp* in buried wood over time, it was often observed that few *Xtp* colonies grew on culture plates. The small number of viable *Xtp* might have been related to the presence of other bacteria associated with wood, which showed various levels of inhibition of the growth of *Xtp in vitro*. Some bacterial isolates produced an antibiotic effect on solid culture media, with inhibition zones ranging from 0.5 to 1.2 cm. Furthermore, growth of some bacteria over medium spread with a lawn of *Xtp* exhibited a competition phenomenon which may occur in the soil and in turn reduced the number of viable *Xtp*. However, despite their potential in inhibiting the growth of *Xtp in vitro*, it was obvious that these bacteria were not able to eliminate *Xtp* from the buried wood, probably due to other limiting factors in a complex environment like soil. For example, Brian (1957) highlighted a number of factors that affect the production of antibiotics in soil, including availability of carbon source, presence of other soil microflora which might be competitive or antagonistic to the antibiotic-producing bacteria, adsorption of antibiotic by soil colloids and chemical and biological breakdown of antibiotics in soil.

In summary, *Xtp* was not eliminated from infected pistachio wood by burial in this study. Prevailing dry conditions during the study might have contributed to the slow loss of viability of *Xtp.* Future research is recommended to clarify the effect of soil moisture on the survival of *Xtp* in buried wood. In addition, the role of microbiota associated with wood buried in or placed on the soil merits further research should burial be employed as a means of disposal of infected woody materials. Finally, survival of other systemic bacteria, such as *E. amylovora*, could be studied in a similar manner.

Chapter 6 Potential of *Xanthomonas translucens* pv. *pistaciae* to enter the viable but nonculturable state

6.1 Introduction

When entering an environment different from their optimal growth conditions, all kinds of living organisms may need to adopt one or more survival strategies. Bacteria are no exception. Vegetative cells of some Gram-positive bacteria form endospores, a highly resistant survival structure (Nicholson *et al.*, 2002; Madigan *et al.*, 2006) which allows them to persist in unfavourable environments (Piggot & Hilbert, 2004). In contrast, some non-spore-forming Gram-negative bacteria, including *Xanthomonas*, produce polysaccharide exudates in infected host plants which allows them to survive in a range of conditions (Schuster & Coyne, 1974; Stall *et al.*, 1993). In addition, in response to various environmental conditions, plant pathogenic bacteria might slow their metabolism and, according to Leben (1981), cells may enter hypobiosis, a state that permits them to persist better in unfavourable conditions than do active growing cells. A similar phenomenon of reduced metabolic activity in bacteria, referred to as viable but nonculturable state (VBNC), has been reported for many plant pathogenic including *Xanthomonas* (Ghezzi & Steck, 1999; Del Campo *et al.*, 2009) and is considered to be a long-term survival mechanism employed primarily by Gram-negative bacteria (Gauthier, 2000) (see Section 1.5.2.2).

VBNC is defined as a state in which bacterial cells are not able to develop into colonies on solid growth medium, but remain intact and alive (Roszak & Cowell, 1987). By definition, the primary characteristic of VBNC cells is the loss of culturability. As noted in section 1.5.2.2.1, several factors induce this state in bacteria, such as nutrient

starvation (Cook & Bolster, 2007), incubation outside of the normal temperature range for growth (Maalej *et al.*, 2004; Wong & Wang, 2004) and heavy metals (Ghezzi & Steck, 1999; Alexander *et al.*, 1999). Copper was the first metal reported to induce the VBNC state in plant pathogenic bacteria, such as *Agrobacterium tumefaciens* (Alexander *et al.*, 1999), *Erwinia amylovora* (Ordax *et al.*, 2006), *Ralstonia solanacearum* (Grey & Steck, 2001), *Xanthomonas axonopodis* pv. *citri* (Del Campo *et al.*, 2009), *X. campestris* pv. *campestris* (*Xcc*) (Ghezzi & Steck, 1999) and *X. campestris* pv. *vesicatoria* (Pernezny & Collins, 1997). *Xcc* entered the VBNC condition in sterile soil and liquid cultures and, in the presence of copper, the time for entry into this condition was hastened (Ghezzi & Steck, 1999).

The time required to enter the VBNC state varies from hours (Grimes & Colwel, 1986) to days (Wolf & Oliver, 1992; Ghezzi & Steck, 1999; Alexander *et al.*, 1999; Ordax *et al.*, 2006). Response of the VBNC cells to a reversal of inducing factors, referred to as "resuscitation", generally requires days (Nilsson *et al.*, 1991; Ordax *et al.*, 2006). As discussed in section 1.5.2.2.3, VBNC state can be determined by direct viable count (Kogure *et al.*, 1979), through measurement of respiration (Zimmerman *et al.*, 1978), by using fluorescently labelled monoclonal antibodies in combination with direct viable count (Xu *et al.*, 1982; Grimes & Colwel, 1986), or flow cytometry (Morgan *et al.*, 1991). The LIVE/DEAD® *Bac*light™ Bacterial Viability Kit (http://probes.invitrogen.com) has been used to estimate both total and viable counts of bacteria, including *X. campestris* pv. *campestris* (Ghezzi & Steck, 1999). The kit comprises two nucleic acid binding stains: SYTO™ 9, which penetrates all bacterial membranes and propidium iodide, which penetrates only cells with damaged membranes. When the two stains are combined, bacteria with intact cell membranes, including VBNC, fluoresce green, while bacteria with damaged membranes fluoresce red.

During the burial experiment (Chapter 5), it was not always possible to isolate *Xtp* on culture plates from infected pistachio wood buried in or placed on the soil. It is likely that the pathogen was exposed to unfavourable conditions during burial, such as nutrient depletion, desiccation, fluctuations in temperature or exposure to heavy metals, which hindered the culturability of the cells. As the soil used for the experiment was collected from the orchard where copper has been used for years in the control or prevention of diseases of horticultural crops in the orchard (B. Pike, pers. comm., 2008) and copper has been reported to induce the VBNC state in other *Xanthomonas* pathogens, the aim of this study was to investigate if copper could induce the VBNC condition in *Xtp*.

6.2 Materials and methods

6.2.1 Development of a protocol for examining VBNC state in *Xtp* and resuscitating putative VBNC cells

6.2.1.1 Effect of cupric sulphate on growth of *Xtp*

Prior to the investigation into the potential of copper to induce the VBNC state in *Xtp*, an experiment was conducted to determine the lethal effect of copper on growth of *Xtp*. Aliquots of 100 µL of overnight *Xtp* suspensions, 10^7 CFU mL^{-1}, in SPB and NB, prepared as described in section 2.3.1, were spread on SPA and NA supplemented with copper at 0.005, 0.01, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 1.5, 2, 2.5 and 3 mM. Suspensions of *Xtp* were also plated on SPA and NA without cupric sulphate as controls. The growth of *Xtp* was evaluated after incubation for 3 to 14 days at 28°C in darkness.

6.2.1.2 VBNC - Experiment 1 (January 2009 to February 2011)

6.2.1.2.1 Preparation of bacterial suspension and microcosms

The method of Ghezzi and Steck (1999) was used with modifications. *Xtp* was revived from stock culture stored at -80°C on SPA as described in section 2.1. The bacterial suspensions were prepared by inoculating two loopsfull of colonies of 3-day-old culture of *Xtp* into Luria-Bertani broth (LB) and incubating overnight with shaking at 28°C (LB-28) or at room temperature (LB-R) (*ca.* 22°C). Cells were harvested from each suspension the following day by centrifugation at 10,000 rpm for 10 min, washed twice in sterile mineral salts solution (1 g L^{-1} NH_4Cl, 0.3 g L^{-1} $MgSO_4$, 0.15 g L^{-1} KCL, 0.01 g L^{-1} $CaCl_2$ and 2.5 mg L^{-1} $FeSO_4$), then resuspended in mineral salts solution to establish two microcosms (LB-28 and LB-R) of an approximate concentration of 10^8 *Xtp* cells mL^{-1}, estimated using a haemocytometer (Weber, England).

6.2.1.2.2 Supplementing *Xtp* microcosms with copper

Prior to the addition of cupric sulphate, each microcosm was diluted 10-fold to a concentration of approximately 10^7 *Xtp* cells mL^{-1}. Aliquots of each microcosm were aseptically distributed into each of four 50-mL centrifuge tubes. Cupric sulphate stock solution (25 mM) was added separately into three of the tubes of LB-R microcosm to a final concentration of 0.005, 0.01 and 0.05 mM, respectively. The fourth tube contained copper-free bacterial microcosm as a negative control. LB-28 microcosms were prepared in the same way. These microcosms will be referred to from now on as "original" microcosms. The tubes containing the original microcosms were then incubated at 28°C to assay for culturability of *Xtp* over time.

6.2.1.2.3 Counts of culturable cells

Culturability of *Xtp* in each original microcosm was examined on day 0, within an hour of adding copper, and after 3, 62, 90, 153, 306, 334, 451, 499 and 780 days of incubation with cupric sulphate. From day 0 to day 306, undiluted and 10^{-4}-diluted 100-µL aliquots of LB-28 and LB-R microcosms at each concentration of copper were spread on SPA in duplicate to examine culturability of *Xtp* in each microcosm. After day 334, aliquots of each microcosm were first serially diluted to 10^{-4} before spreading 100-µL aliquots of undiluted, 10^{-2}- and 10^{-4}-diluted microcosms on SPA. From day 451 to 780, the microcosms were serially diluted 10-fold to 10^{-5} and all dilutions were plated out on SPA and NA using the Miles and Misra method (1938).

6.2.1.2.4 Resuscitation of nonculturable cells

To determine if the nonculturable cells of *Xtp* could be resuscitated, the resuscitation method described by Ordax *et al.* (2006) was used with modifications. Four liquid media, namely King's B (KB) broth, sucrose peptone broth (SPB), autoclaved "juice" (AJ) and filter sterilised "juice" (FJ) of young pistachio leaves and shoots, were assessed for their ability to facilitate resuscitation of *Xtp* cells in microcosms containing cupric sulphate. AJ and FJ were prepared by blending 100 g of young pistachio leaves and shoots in 400 mL of RO water. The suspension was cleared by filtration through single then double layers of cheese cloth. Part of the resulting suspension was autoclaved to make AJ and the other part was filter sterilised using a Millipore disposable filter, 0.45 µm pore size, to obtain FJ. Undiluted or 10-fold-diluted aliquots of each liquid medium were separately added to an equal volume of 10^{-3}- and 10^{-4}-diluted aliquots of the 120-day-old original microcosms in 0.01 mM cupric sulphate (Assay 1). The dilution 10^{-4} was selected for the resuscitation assays based on the result of the previous plating that this dilution did not often give rise to

colonies on SPA. The dilution 10^{-3} was used, in addition, to examine if the nonculturable cells were also present at this concentration and were able to be resuscitated. The SPB-, AJ- and FJ-supplemented bacterial microcosms, referred to as "resuscitated" microcosms from now on, were then incubated at 28°C with shaking for 24 and 48 h, prior to spreading on SPA and KB medium. Two liquid media and one incubation duration were then chosen to assay the resuscitation of the bacterial cells in 153-day-old microcosms in 0.01 and 0.05 mM cupric sulphate (Assay 2). The original microcosms were diluted to 10^{-4} before adding the liquid media for resuscitation as the 10^{-3} dilution still gave rise to a few colonies on the culture media. One liquid medium was selected for resuscitation assays for all copper concentrations at days 306, 334, 451, 499 and 780 (Assays 3, 4, 5, 6 and 7, respectively). The resumption of culturability at each copper concentration was assayed on SPA and NA using the Miles and Misra method (1938). For all assays (1 - 7), aliquots from the original VBNC microcosms at corresponding dilutions were plated out on culture media as controls.

To examine whether colonies arising following resuscitation were, in fact, regrowth of a few undetected culturable cells, dilution studies, as described by Whitesides and Oliver (1997), were conducted on days 451, 499 and 780 for all original microcosms (regrowth assay). Briefly, aliquots of each original microcosm were serially diluted 10-fold to 10^{-7} to reduce the possibility of any culturable cells being present and growing. Ten-fold diluted SPB was then added separately to each dilution and the suspensions incubated at 28°C for 48 h, and culturability of cells in the resuscitated microcosms at each dilution was assayed on SPA and NA.

6.2.1.2.5 Validation of LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit

To determine the viability of nonculturable cells, the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen, USA) was used. The kit was first validated with a pure culture of *Xtp*, not exposed to copper, following the manufacturer's instructions with slight modifications. Two loopsfull of *Xtp* were grown in 20 mL of nutrient broth with shaking at 28°C overnight. The resulting bacterial suspension was centrifuged at 10,000 rpm for 10 min and the supernatant was removed before resuspending the pellet in 2 mL of 0.85% sodium chloride (NaCl). One millilitre of the suspension was added to a centrifuge tube containing 20 mL of 0.85% NaCl (for live bacteria) and 1 mL was added to another tube containing 20 mL of 70% isopropyl alcohol (for dead bacteria). Samples were incubated at room temperature for 1 h with mixing every 15 min, then centrifuged at 10,000 rpm for 10 min. The pellets were resuspended in 20 mL of 0.85% NaCl and the suspensions were centrifuged again in the same conditions before resuspending each pellet in 10 mL of 0.85% NaCl. Two stains, SYTO® 9 and propidium iodide, were mixed at three ratios: 1:1 as per manufacturer's instructions, 1:2 and 2:1, respectively. Mixtures of the two stains were added separately to each suspension of live or dead bacteria, at a ratio of 3 µL of the stain mixture to 1 mL of bacterial suspension, and incubated for 15 (manufacturer's instruction), 20 or 30 min in the dark at room temperature. Cells were then collected by vacuum filtration onto 0.4 or 0.2 µm black polycarbonate filters (Millipore, Australia) and examined with an Axiophot epifluorescence microscope (Zeiss, Germany) with a filter set consisting of an excitation filter (BP 450 - 490), emission filter (LP 520) and dichroic beam splitter (FT 510). Viable cells were expected to fluoresce green while nonviable cells would fluoresce red.

Cells in the 0.005 mM copper original microcosm of Experiment 1 were assayed for viability after 17 months. Cells were harvested by centrifuging an aliquot of 500 µL of

the original microcosm at 10,000 rpm for 10 min and the pellet washed twice in NaCl before resuspending in 500 µL of NaCl for staining. In addition, direct staining of cells by adding mixtures of the stains to aliquots of the 0.005 mM copper original microcosm at the ratio stated above was also conducted.

6.2.2 VBNC - Experiment 2 (September 2010 to February 2011): Application of the protocol

The protocol developed in Experiment 1 was used with slight modifications. The preparation of bacterial microcosms and addition of cupric sulphate (original microcosms) are illustrated in Fig. 6.1. The experiment comprised two replicates and culturability of *Xtp* in the microcosms was assayed on days 0, 2, 4, 7, 14, 21, 28, 42, 56, 70, 84 and 154 on SPA and NA, using the Miles and Misra method (1938). Viable cell counts were determined from the day 0 microcosm using the bacterial viability kit as described above. The ratio of 1 part of SYTO® 9 to 2 parts of propidium iodide was chosen for the assay of viability. The two other ratios of stains (1:1 and 2:1) were used at some stages. A new batch of black membranes was used. In addition, a confocal microscope (Leica Microsystems, Germany), set up at wavelengths of 480 - 500 nm for SYTO® 9 and 490 - 635 nm for propidium iodide, was also used to examine cells for viability.

Resuscitation assays were conducted at days 2, 28, 42, 56, 84 and 154 days of incubation of the microcosms in cupric sulphate. On each day, the original microcosms were first serially diluted to 10^{-7}, then an equal volume (200 - 500 µL) of 10-fold-diluted SPB was added separately to each 2-mL eppendorf tube containing undiluted or diluted aliquots of the original microcosms and mixed well before incubating at 28°C with shaking for 48 h. Resuscitated aliquots of dilutions 10^{-1} to 10^{-7} were plated out directly on SPA and NA using the method of Miles and Misra (1938) while resuscitated aliquots of undiluted

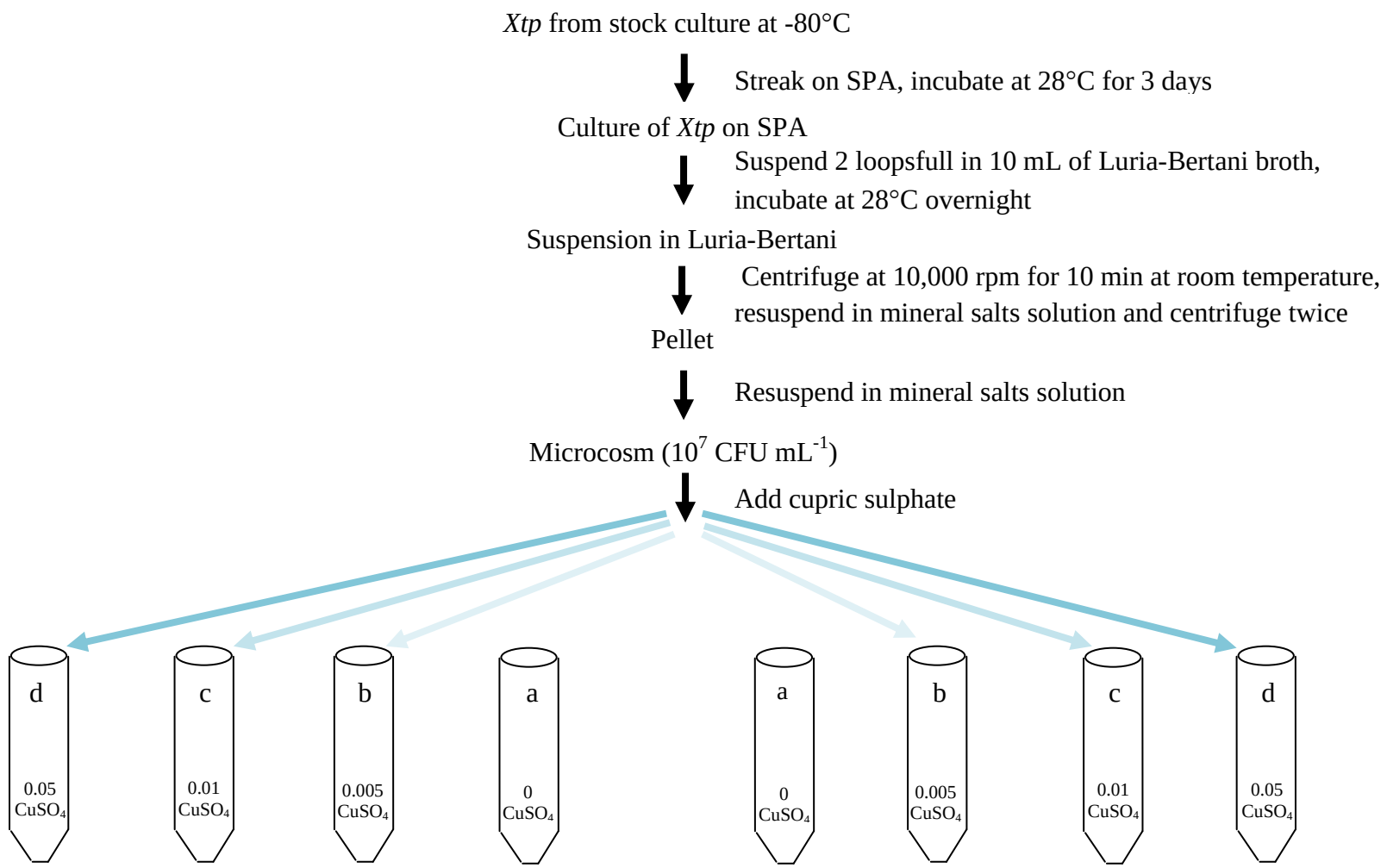


Figure 6.1 Preparation of bacterial microcosms for testing the occurrence of VBNC state in *Xanthomonas translucens* pv. *pistaciae* in Experiment 2. Two replicate series of microcosms with cupric sulphate at 0 (a) 0.005 (b), 0.01 (c) and 0.05 (d) mM were prepared.

microcosms were first serially diluted to 10^{-6} or 10^{-7} before plating onto SPA and NA to quantify the culturable cells in the resuscitated undiluted microcosms. For the resuscitation of culturability in the 0.05 mM copper microcosm on day 2, undiluted and diluted aliquots of the original microcosm were incubated in SPB for 48 and 168 h to examine if the culturability of cells could be restored after an extended period of incubation in SPB. The resuscitated aliquots of undiluted microcosms in 0.05 mM copper were then diluted to 10^{-6} and culturability was assayed on SPA and NA using the Miles and Misra method (1938).

6.2.3 Test for copper-resistant mutants

To determine if culturable cells from the original microcosm with 0.05 mM copper in the VBNC Experiment 2 represented the emergence of copper-resistant mutants during incubation, two independent experiments were conducted as follows.

6.2.3.1 Experiment 3

Prior to the experiment, culturable cells from original microcosms with 0.05 mM copper were subjected to a PCR assay, Gram stained for examination under a microscope (Leitz Wetzlar, Germany) and compared with cells from a pure culture of *Xtp*. After the cells were confirmed to be *Xtp*, two replicate microcosms to test for the presence of copper-resistant mutants were established from each of two sources of culture. Culture 1 was started by spreading aliquots of 5 µL of 0.05 mM cupric sulphate microcosm from VBNC Experiment 2 on SPA to obtain single colonies, which were then subcultured periodically on SPA nine times over 5 weeks. Culture 2 was started as for culture 1, but subcultured only once to obtain a pure culture. Microcosms (M-1 and M-2) were then established by incubating a small amount of 3-day-old colonies of cultures 1 and 2, respectively, in mineral salts solutions. The microcosms were gently shaken to allow the colonies to disperse well, then adjusted, if required, to approximately 10^7 CFU mL^{-1} cells using a

haemocytometer (Weber, England). The initial concentration of culturable cells in the microcosms was determined by plating aliquots of each microcosm on NA and SPA using the Miles and Misra method (1938). A microcosm from colonies of a 3-day-old culture of *Xtp* on SPA (M-*Xtp*) was also established as a positive control. Cupric sulphate was added to each microcosm to a final concentration of 0.05 mM. Microcosms established from each culture without cupric sulphate were used as negative controls. Aliquots from these microcosms were then pipetted out on SPA and NA at the time of the addition of cupric sulphate or establishment of negative control microcosms, and after incubation for 2, 4, 7, 14 and 56 days. CFU were counted from day 2 for SPA, and day 3 for NA, to day 14.

6.2.3.2 Experiment 4

The experiment above was repeated with additional assays for culturability of *Xtp* in microcosms at 3, 4, 5 and 6 h after the addition of cupric sulphate or establishment of the control microcosms. CFU were enumerated on SPA and NA as described above.

6.3 Results

6.3.1 Development of a protocol for examining VBNC state in *Xtp* and resuscitating putative VBNC cells

6.3.1.1 Effect of cupric sulphate on growth of *Xtp*

Xtp grew on all control plates as well as on NA plates containing cupric sulphate from 0.005 to 0.5 mM and on SPA plates from 0.005 to 0.4 mM cupric sulphate but did not grow on SPA plates containing cupric sulphate from 0.5 to 3 mM nor on NA plates from 1 to 3 mM. These inhibitory concentrations were 8 and 10 times higher than the highest copper concentration (0.05 mM) used in the VBNC experiments.

6.3.1.2 VBNC- Experiment 1 (January 2009 to February 2011)

6.3.1.2.1 Counts of culturable cells

Culturability of *Xtp* in the original microcosms at all copper concentrations decreased at different rates over the 780 days of the experiment (Table 6.1). The culturability in the copper-free LB-R microcosm decreased over 306 days from 10^7 to 10^3 CFU mL^{-1}, increased slightly to *ca.* 10^4 CFU mL^{-1} at day 334 then was undetectable from day 451 until the end of the experiment. In contrast, culturable cell counts in the copper-free LB-28 microcosm decreased over 306 days to 10^4 CFU mL^{-1} then remained at this level until the end of the experiment. A decrease in the culturability of cells to 10^4 CFU mL^{-1} occurred over 90 days in 0.005 and 0.01 mM copper. This culturability level remained in LB-R until day 499 before dropping to 10^3 CFU mL^{-1} at day 780, whereas the decrease to 10^3 CFU mL^{-1} in LB-28 occurred after 334 days. There was no growth from the aliquots of the 0.05 mM microcosm on SPA plates at day 3 whereas growth was moderate at day 62 before it dropped again below the detection limit at day 306 in LB-28 and day 334 in LB-R. In all cases where culturability was observed, development of colonies from the 0.05 mM microcosms on SPA plates was slower than that from the other three microcosms (Fig. 6.2).

Table 6.1 Culturability of *Xanthomonas translucens* pv. *pistaciae* (*Xtp*) in LB-R[a] and LB-28[b] original microcosms from days 0 to 780, enumerated on sucrose peptone agar, in the absence and presence of copper.

Time	Days elapsed	Concentration of copper (mM) in LB-R microcosm				Concentration of copper (mM) in LB-28 microcosm			
		0	0.005	0.01	0.05	0	0.005	0.01	0.05
Jan 09	0	+++++[c]	+++++	+++++	++++	+++++	+++++	+++++	++++
Jan 09	3	+++++	+++++	++++	-	+++++	+++++	++++	-
Mar 09	62	+++	+++	+++	+++	+++	+++	+++	+++
Apr 09	90	+++	++	++	++	+++	++	++	++
Jun 09	153	+++	++	++	+	+++	++	++	+
Nov 09	306	+	+	++	++	++	++	++	-
Dec 09	334	++	++	++	-	++	+	+	-
Apr 10	451	-	++	++	-	++	+	+	-
Jun 10	499	-	++	++	-	++	+	+	-
Feb 11	780	-	+	+	-	++	+	+	-

[a]LB-R, culture of *Xtp* prepared in Luria-Bertania broth with shaking at room temperature.

[b]LB-28, culture of *Xtp* prepared in Luria-Bertania broth with shaking at 28°C in an incubator.

[c]Culturability of *Xtp* in liquid microcosm: +++++ = *ca.* 10^7 CFU mL^{-1}, ++++ = *ca.*10^6 CFU mL^{-1}, +++ = *ca.* 10^5 CFU mL^{-1}, ++ = *ca.* 10^4 CFU mL^{-1} and + = *ca.* 10^3 CFU mL^{-1}. No growth of *Xtp* is presented by (-).

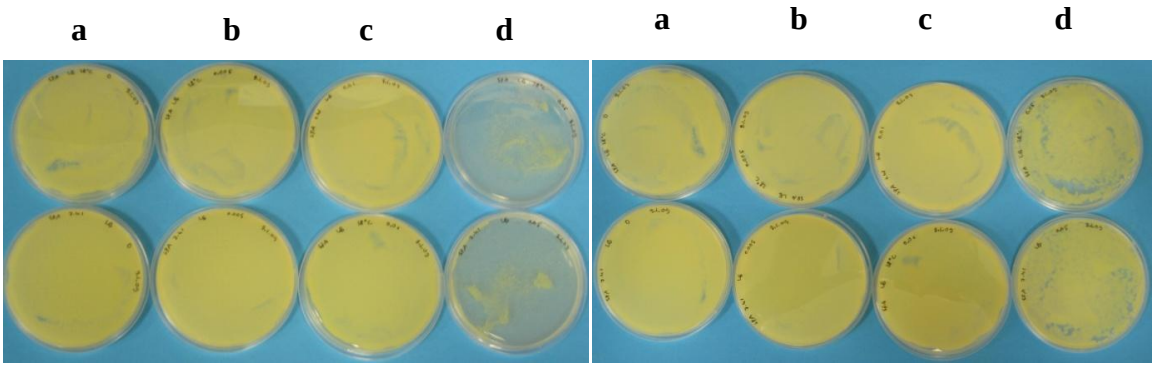


Figure 6.2 Culturability of *Xanthomonas translucens* pv. *pistaciae* in LB-28 (top row) and LB-R (bottom row) original microcosms containing 0 (a), 0.005 (b), 0.01 (c) and 0.05 (d) mM cupric sulphate at day 0. Plates were observed 3 days (left) and 4 days (right) after plating on sucrose peptone agar and incubating at 28°C.

6.3.1.2.2 Resuscitation of nonculturable cells

In Assay 1, few cells from the original microcosm (control) with 0.01 mM copper, dilution 10^{-3}, developed into colonies while no cell at dilution 10^{-4} developed into a colony on culture media. The culturability of cells in both dilutions was not restored when autoclaved juice or filter sterilised juice of young pistachio leaves and shoots was added to the microcosm, regardless of the dilution of the AJ and FJ and duration of incubation in these solutions. The addition of 10-fold-diluted SPB to the 10^{-3}- and 10^{-4}-diluted aliquots of 0.01 mM copper original microcosm produced the most abundant culturable cells on SPA, followed by the addition of undiluted KB, which produced moderate growth of *Xtp* on SPA. Similar results were obtained when resuscitated cells in these two broths were plated out on KB solid medium. Undiluted SPB and 10-fold-diluted KB slightly resuscitated the nonculturable cells from the LB-28 original microcosm, but did not resuscitate the nonculturable cells from the LB-R original microcosm. More abundant growth of *Xtp* on

culture media was observed after 48 h incubation than 24 h. SPB and KB were selected for use in Assay 2 with incubation duration of 48 h.

In Assay 2, no cell from the 0.01 and 0.05 mM copper original microcosms (control) at dilution 10^{-4} developed into a colony on culture media. The addition of 10-fold-diluted SPB again allowed the greatest resuscitation of nonculturable cells (Table 6.2). Undiluted SPB did not restore culturability in either microcosm with 0.01 mM cupric sulphate but moderately resuscitated the cells in the LB-R microcosm with 0.05 mM copper. KB broth gave variable results, from no to moderate resuscitation of the nonculturable cells.

In Assays 3 to 7, culturability of cells in all original microcosms with 0.005 and 0.01 mM copper was restored when 10-fold-diluted SPB was added. The resuscitation of nonculturable cells from 0.05 mM copper was achieved only in Assay 3, on day 306, when culturable cells were present in the undiluted original microcosm. Similarly, resuscitation of culturability of cells in copper-free microcosm LB-R was not achieved when culturable cells in the original microcosm dropped to zero on day 451. An example of resuscitation of cells from all microcosms on day 334 (Assay 4) is presented in Figure 6.3.

In dilution studies, resuscitated copper-free LB-R and 0.05 mM copper microcosms from all dilutions did not give rise to any colonies on SPA and NA. Generally, abundant growth was observed from aliquots of resuscitated LB-28 copper-free, 0.005 and 0.01 mM copper microcosms, with a gradual decrease of abundance from undiluted to dilution 10^{-4}. No growth was observed on plates that received aliquots of dilutions 10^{-5} to 10^{-7}.

Table 6.2 Resuscitation of cells of *Xanthomonas translucens pv. pistaciae* (*Xtp*) in 10^{-4} dilution of original LB-R[a] and or LB-28[b] microcosms 153 days after adding cupric sulphate at 0.01 and 0.05 mM, enumerated on King's B agar medium and sucrose peptone agar, in Assay 2.

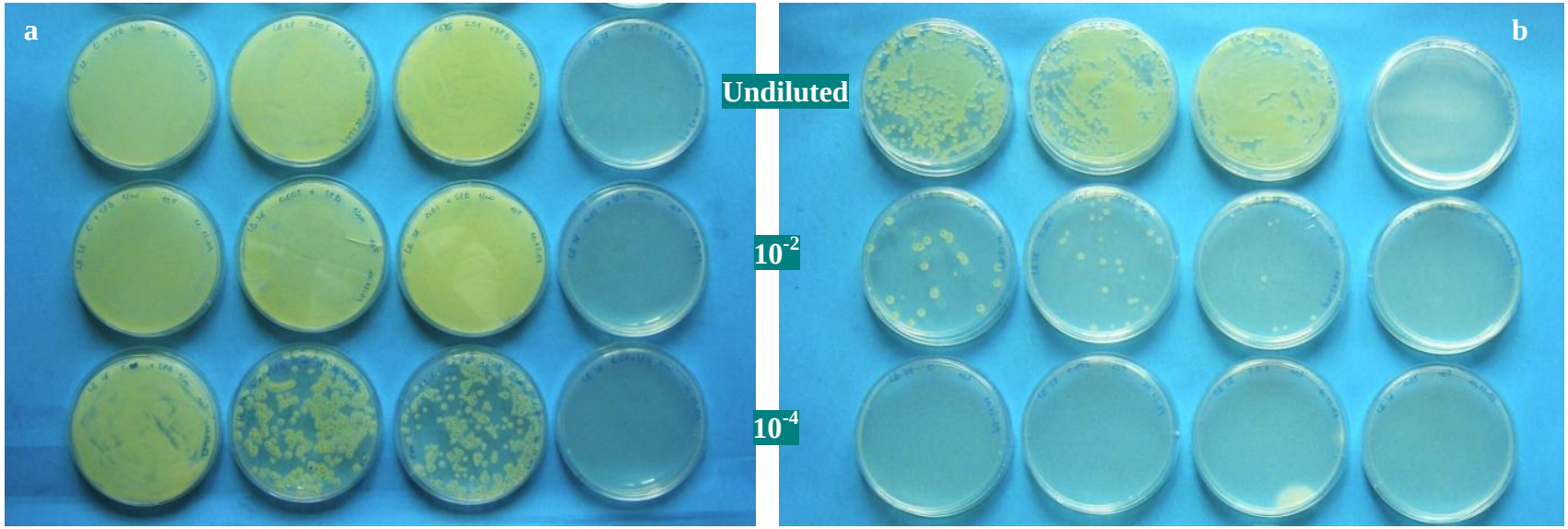
Solid medium for enumeration of colony forming units	Liquid medium for resuscitation	Dilution of liquid medium	Cupric sulphate concentration in microcosms (mM)		
			0.01		0.05
			LB-R	LB-28	LB-R
Sucrose peptone agar	King's B broth	Undiluted	+[c]	++	++
		10^{-1}	-	+	n/a[d]
	Sucrose peptone broth	Undiluted	-	-	++
		10^{-1}	+++	++++	++++
King's B medium	King's B broth	Undiluted	+	+	++
		10^{-1}	-	+	+
	Sucrose peptone broth	Undiluted	-	-	++
		10^{-1}	++++	+++	+++

[a]LB-R, culture of *Xtp* prepared in Luria-Bertania broth with shaking at room temperature.

[b]LB-28, culture of *Xtp* prepared in Luria-Bertania broth with shaking at 28°C in an incubator.

[c]Culturability of resuscitated cells: ++++ = *ca.*10^6 CFU mL^{-1}, +++ = *ca.* 10^5 CFU mL^{-1}, ++ = *ca.* 10^4 CFU mL^{-1} and + = *ca.* 10^3 CFU mL^{-1}. No growth of *Xtp* is presented by (-).

[d]n/a - Non applicable; plates were contaminated.



a1 a2 a3 a4 b1 b2 b3 b4

Figure 6.3 Resuscitation of nonculturable cells of *Xanthomonas translucens* pv. *pistaciae* in 334-day-old original microcosms (a) containing cupric sulphate at 0 (a1), 0.005 (a2), 0.01 (a3) and 0.05 (a4) mM on sucrose peptone agar. Culturability of cells from original microcosms (controls) (b) containing cupric sulphate at 0 (b1), 0.005 (b2), 0.01 (b3) and 0.05 (b4) mM. Culturability and resuscitation of nonculturable cells at three dilutions: undiluted (top row), 10^{-2} (middle row) and 10^{-4} (bottom row) are presented.

6.3.1.2.3 Validation of LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit

In the validation test, stained living bacterial cells fluoresced green and dead bacterial cells fluoresced red (Fig. 6.4). There was no obvious difference between ratios of stains and duration of incubation of the samples in the stains.

The colour of stained cells from the 0.005 mM copper original microcosm varied. Occasionally, the colour of the stained cells was lighter than green or red even when the same ratios of dyes were used or the same duration of incubation was applied. A mixture of SYTO® 9 and propidium iodide at 1:2 ratio appeared to give the clearest view. The stained cells were similar in colour, regardless of whether the cells were first harvested by centrifuging to remove traces of copper or salts, which might interfere with staining, from the bacterial microcosm before staining or the stains were added directly into the aliquot from the microcosm. An example of stained cells from the 0.005 mM cupric sulphate microcosm before and after resuscitation is presented in Figure 6.5. As can be seen, most of the cells taken directly from the copper microcosm were stained red with a few green cells, whereas most of the cells were green following resuscitation.

6.3.2 VBNC - Experiment 2 (September 2010 to February 2011): Application of the protocol

6.3.2.1 VBNC

The culturability of *Xtp* in the original microcosms on NA and SPA differed significantly ($P < 0.001$) over time. On NA, the culturability of *Xtp* in copper-free and 0.005 mM copper original microcosms gradually decreased from 10^7 to 10^5 CFU mL^{-1} from day 0 to day 56 of incubation and remained at this level until the end of the experiment (Fig. 6.6a). On

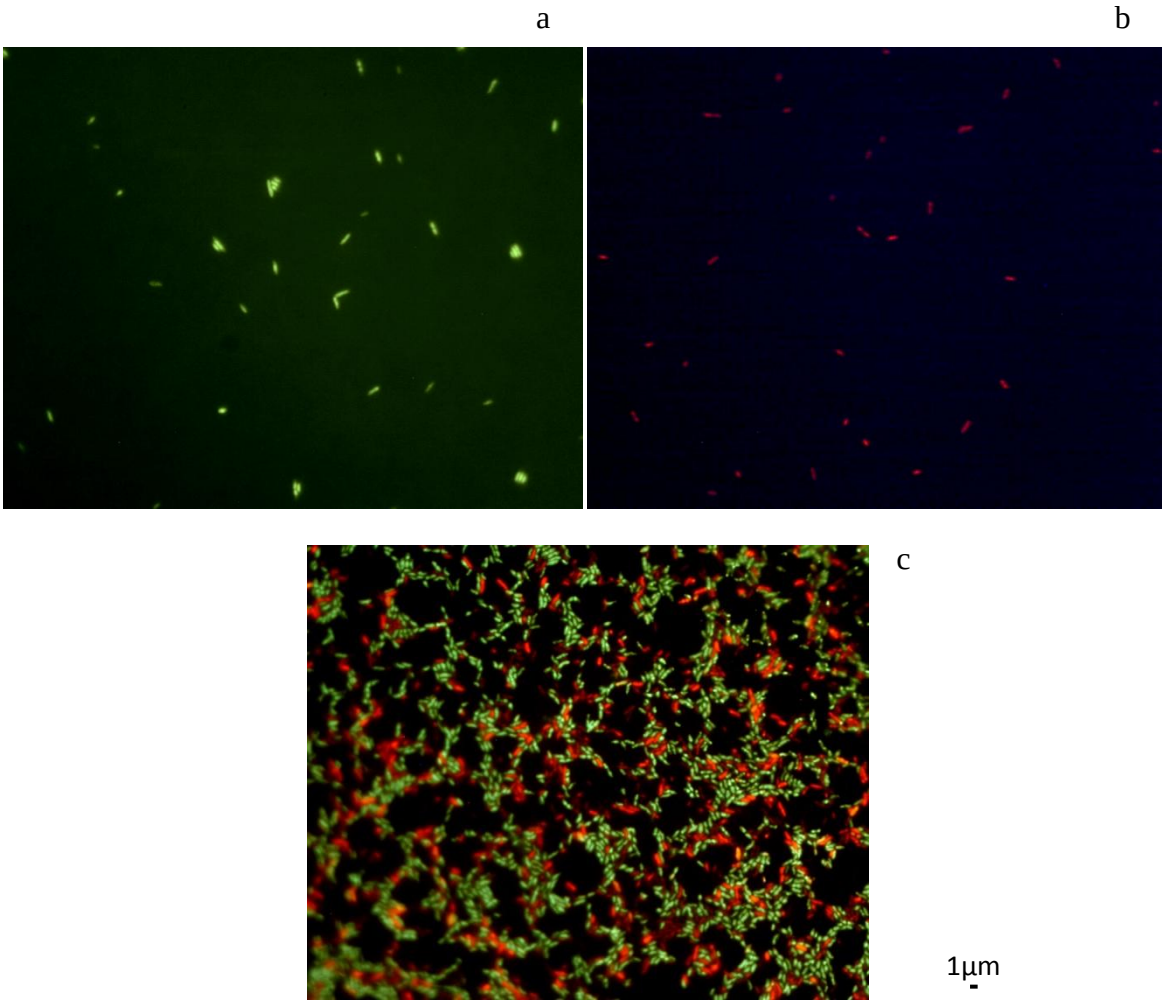


Figure 6.4 Validation of LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. Cells of living (a), dead (b) and mixture of living and dead (c) pure culture of *Xanthomonas translucens* pv. *pistaciae* stained with a mixture of SYTO® 9 and propidium iodide at 1:2 ratio, incubated at room temperature for 15 min and examined using an Axiophot epifluorescence microscope (Zeiss, Germany).

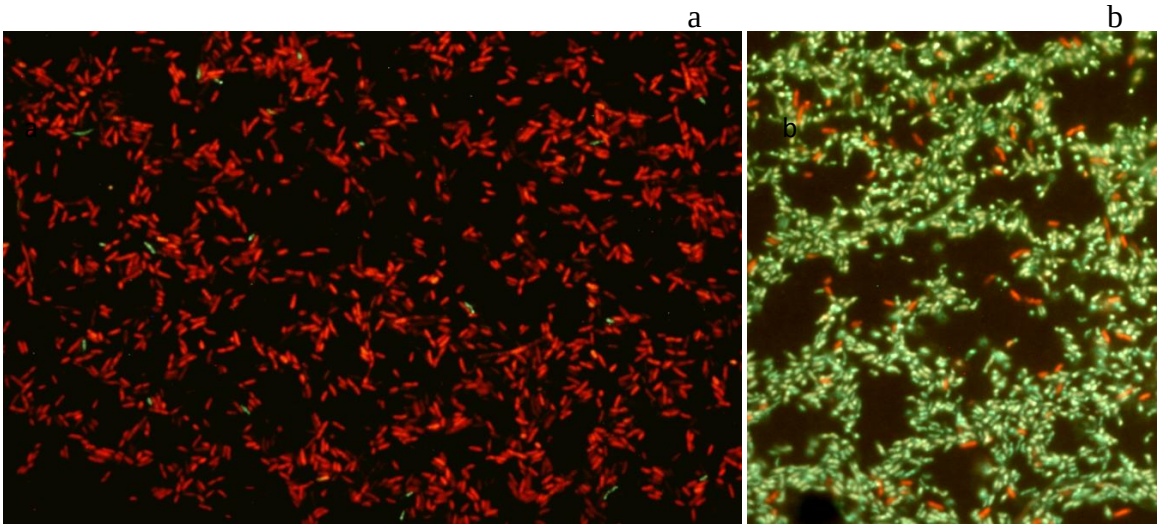
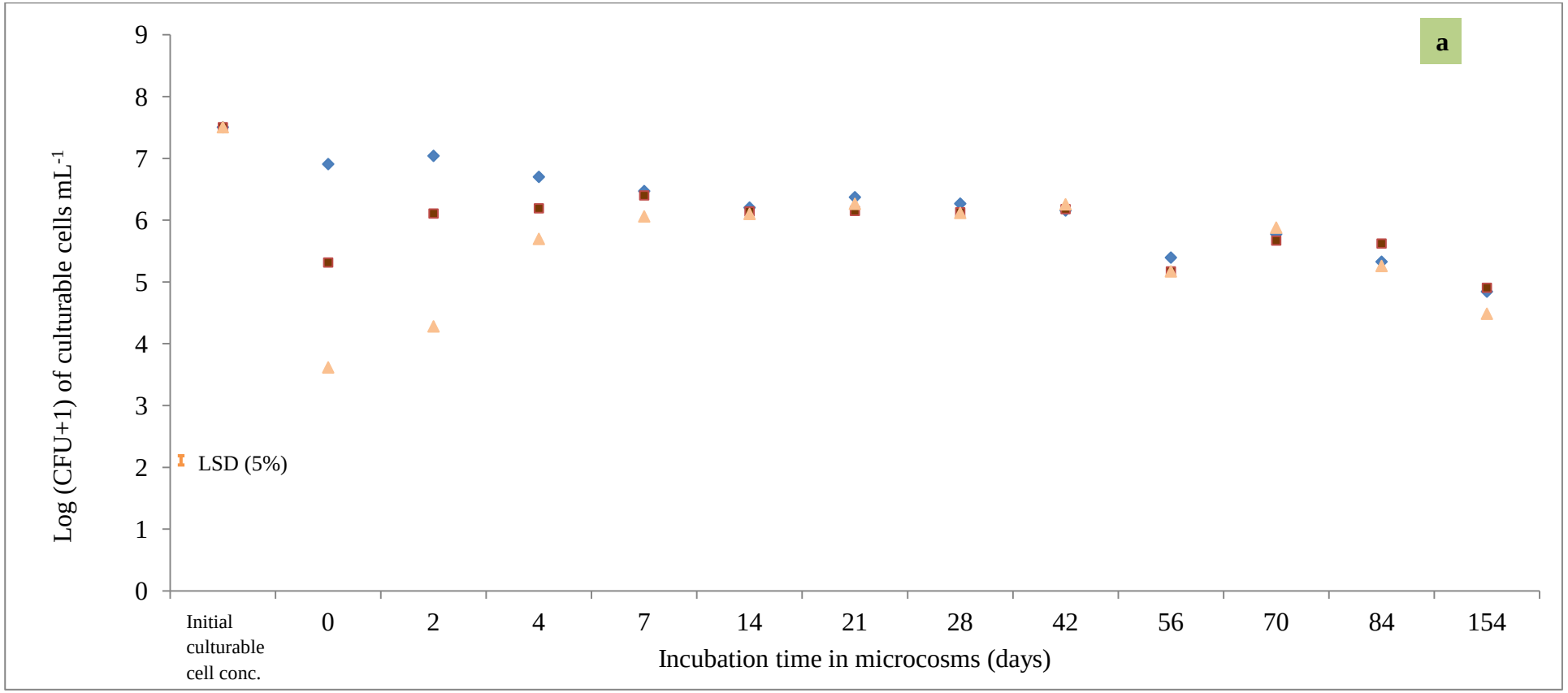


Figure 6.5 Cells of *Xanthomonas translucens* pv. *pistaciae* from 0.005 mM original cupric sulphate microcosm (Experiment 1) after 522 days (a) and resuscitated in 10-fold-diluted sucrose peptone broth from 0.005 mM cupric sulphate microcosm (b), stained in a mixture of SYTO® 9 and propidium iodide at 1:2 ratio, incubated at room temperature for 15 min and examined using an Axiophot epifluorescence microscope (Zeiss, Germany).

SPA, the decrease in culturability of *Xtp* in these two microcosms occurred after only 21 days of incubation for *Xtp* in the copper-free original microcosm and 14 days of incubation *Xtp* in the 0.005 mM copper original microcosm (Fig. 6.6b). In the 0.01 mM cupric sulphate microcosm, cells showed only a transient, although significant, decrease in nonculturability on both NA and SPA immediately after the addition of cupric sulphate before gradually increasing to 10^6 CFU mL^{-1} after 7 days of incubation. The culturability on SPA decreased again to 10^5 CFU mL^{-1} after 14 days of incubation and remained at this level while on NA the decrease was observed again after 56 days. At 0.05 mM, cells became nonculturable on both media as soon as cupric sulphate was added (Fig. 6.7a and b). However, in replicate 2, culturability of *Xtp* was regained on NA after 56 days (10^5

CFU mL^{-1}) and on SPA after 70 days (10^4 CFU mL^{-1}) of incubation with cupric sulphate. In these cases, two types of colony were observed. Type 1 colonies, designated COL1, were convex and mucoid (Fig. 6.8a), typical of *Xtp* (Fig. 6.8c) while type 2 colonies, designated COL2, were smaller than *Xtp* and sticky (Fig. 6.8b) when picked up with a loop.

In this experiment, enumeration of total and viable cells in the microcosms was not possible as stained cells did not exhibit pure green or red colours. Similar results were obtained for stained cells of *Xtp* prepared from fresh culture and when a confocal microscope was used. Due to time constraints, the enumeration of total and viable cells was discontinued.


a
Log (CFU+1) of culturable cells mL-1
9
8
7
6
5
4
3
2
1
0
LSD (5%)
Initial culturable cell conc.
0
2
4
7
14
21
28
42
56
70
84
154
Incubation time in microcosms (days)

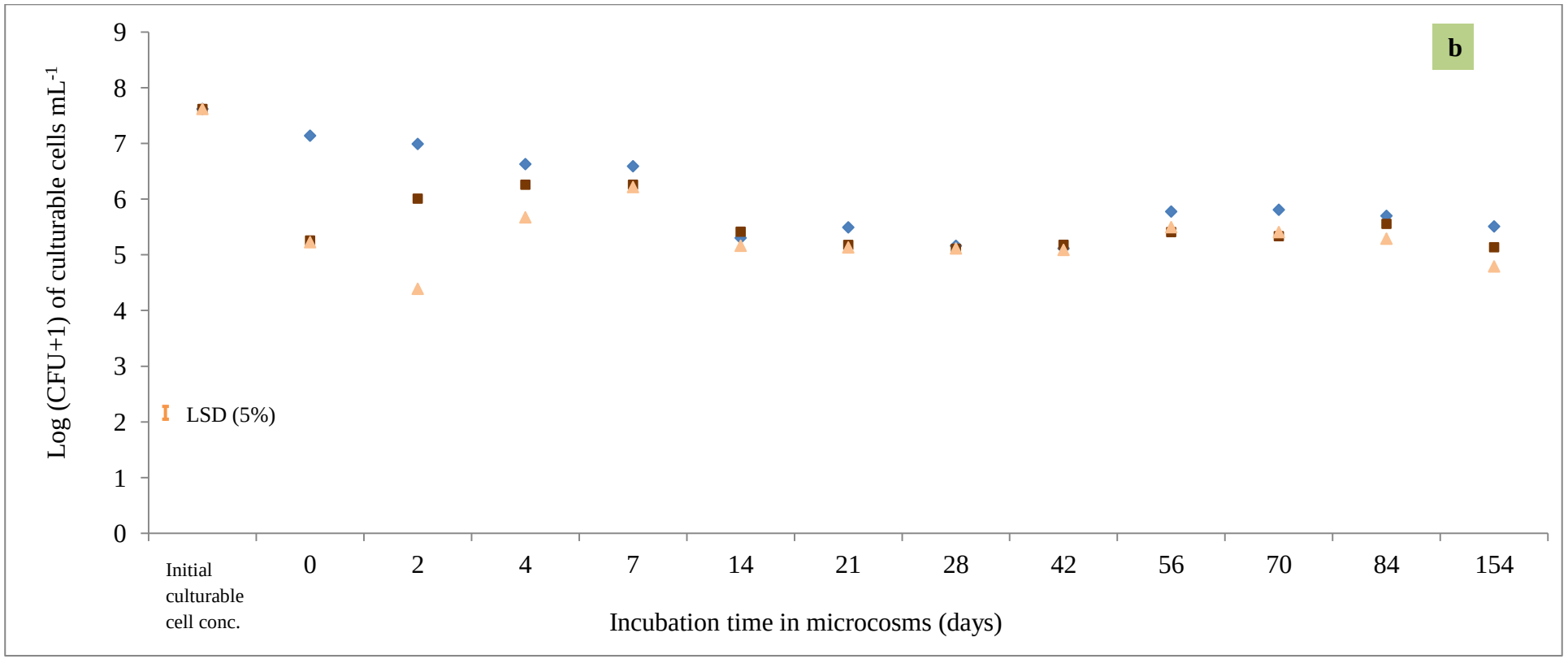


Figure 6.6 Culturability of *Xanthomonas translucens* pv. *pistaciae* in original microcosms containing cupric sulphate at 0 (negative control, blue diamond), 0.005 (brown square) and 0.01 (orange triangle) mM. Aliquots were removed from each microcosm from day 0 to day 154 to assay for colony forming units on (a) nutrient agar and (b) sucrose peptone agar. Mean of colony forming units following logarithmic transformation from two replicates after each incubation time in cupric sulphate is presented.

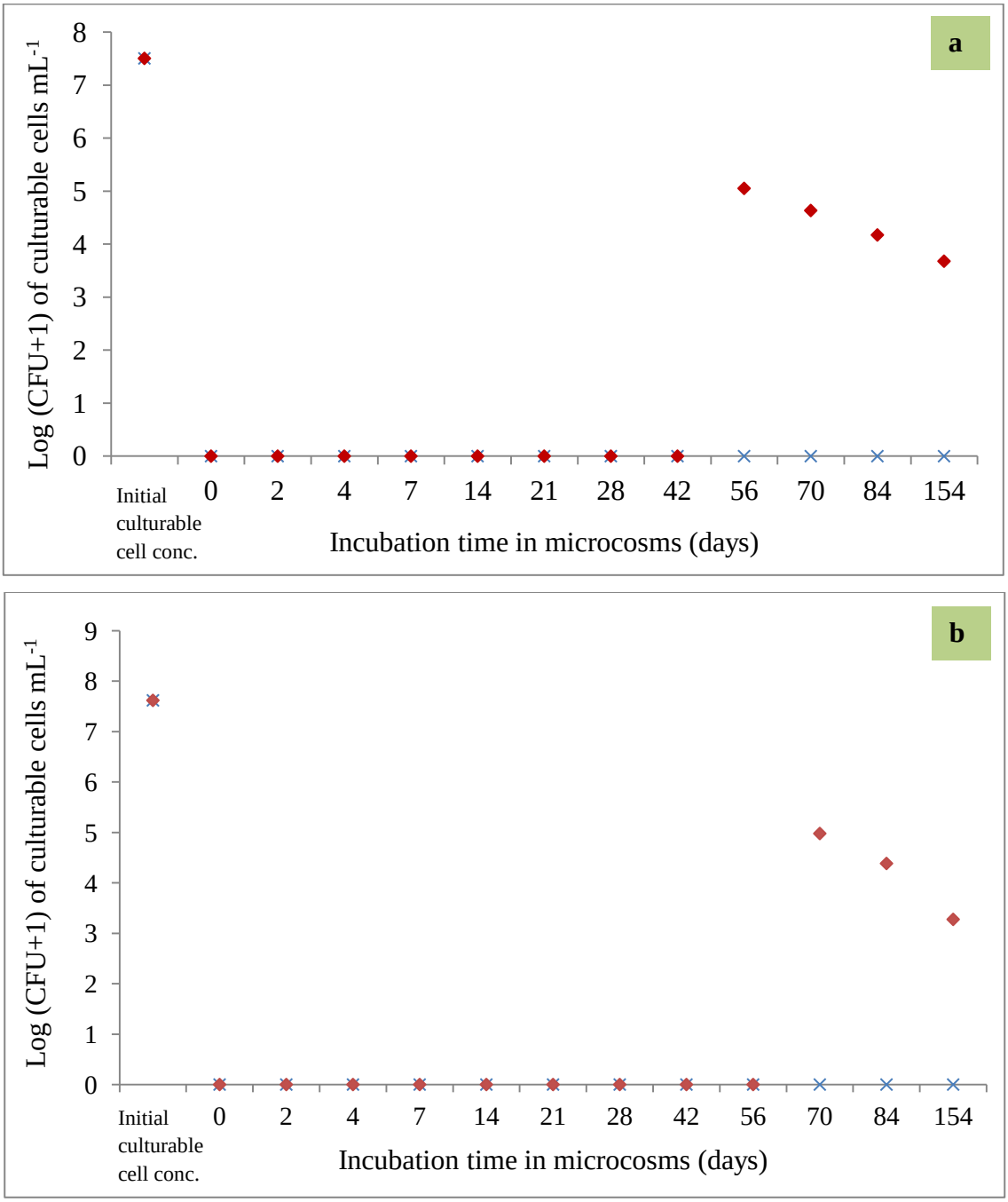


Figure 6.7 Culturability of *Xanthomonas translucens* pv. *pistaciae* in 0.05 mM cupric sulphate microcosms. Aliquots were removed from day 0 to day 154 to assay for colony forming units on (a) nutrient agar and (b) sucrose peptone agar. Colony forming units following logarithmic transformation from replicate 1 (blue cross) and replicate 2 (red diamond) after each incubation time in cupric sulphate is presented.

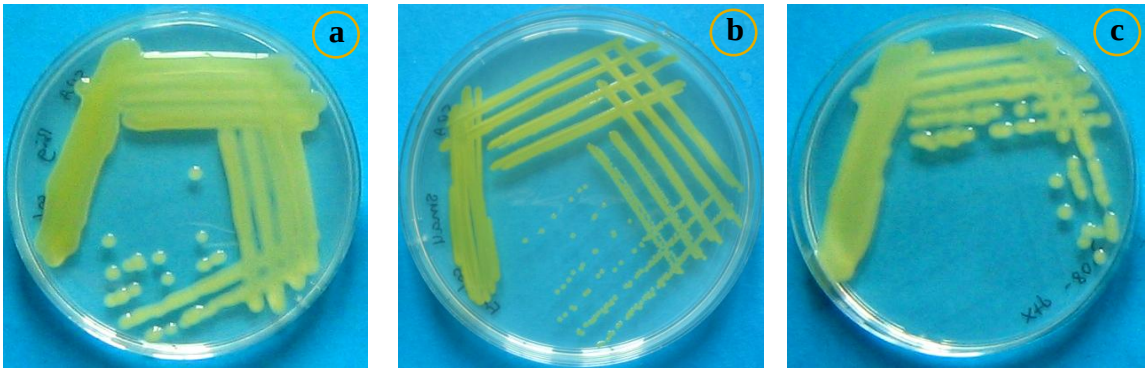
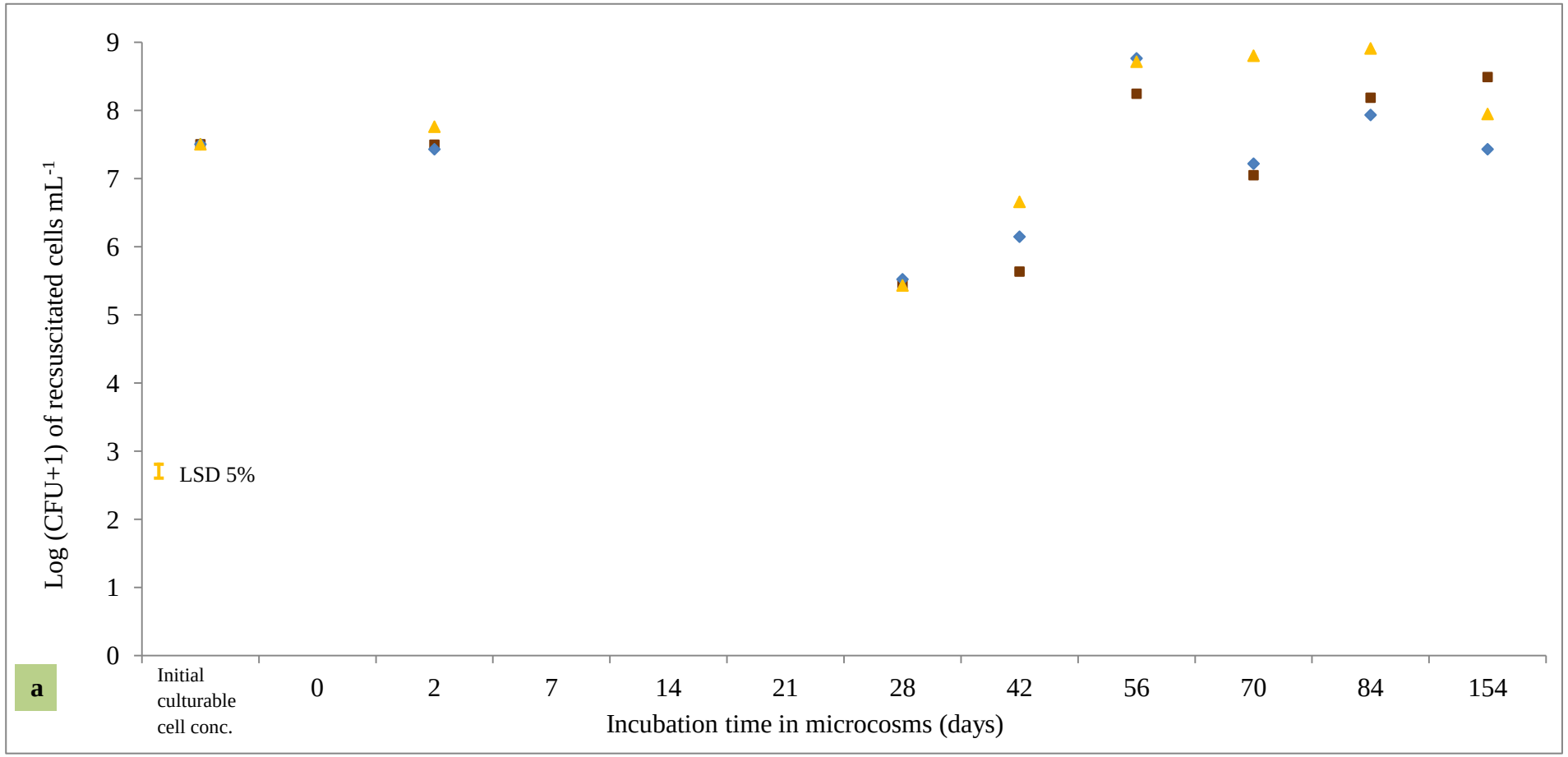


Figure 6.8 Colony types COL1 - convex and mucoid (a) and COL2 - small and sticky (b) yielded from microcosm, replicate 2, containing 0.05 mM cupric sulphate on sucrose peptone agar in comparison with colonies of *Xanthomonas translucens* pv. *pistaciae* prepared from fresh culture (c).

6.3.2.2 Resuscitation of culturability of cells in original microcosms

With the addition of 10-fold-diluted SPB, culturability of *Xtp* in the three original microcosms with 0, 0.005 and 0.01 mM cupric sulphate on day 2 returned to that prior to supplementation with cupric sulphate (10^7 CFU mL^{-1}) (Fig. 6.9). At the later dates, culturability in these microcosms after resuscitation was often very close to, equal to or greater than the population of culturable cells at time 0, except at weeks 4 and 6. On the contrary, SPB did not restore the culturability of cells in one replicate microcosm with 0.05 mM of cupric sulphate at any time or in replicate 2 on day 2 even when the aliquots of this microcosm were incubated in 10-fold-diluted SPB for 168 h (Fig. 6.10). However, resuscitation of culturability occurred in this replicate microcosm on days 28 and 42 (Fig. 6.10) even when no cells from the original microcosm developed into colonies on NA and SPA (Fig. 6.7). There was no significant difference ($P > 0.05$) in the recovery of culturability for any of the microcosms on SPA and NA. However, recovery of


Log (CFU+1) of recsuscitated cells mL-1
9
8
7
6
5
4
3
2
1
0
LSD 5%
a
Initial culturable cell conc.
0
2
7
14
21
28
42
56
70
84
154
Incubation time in microcosms (days)

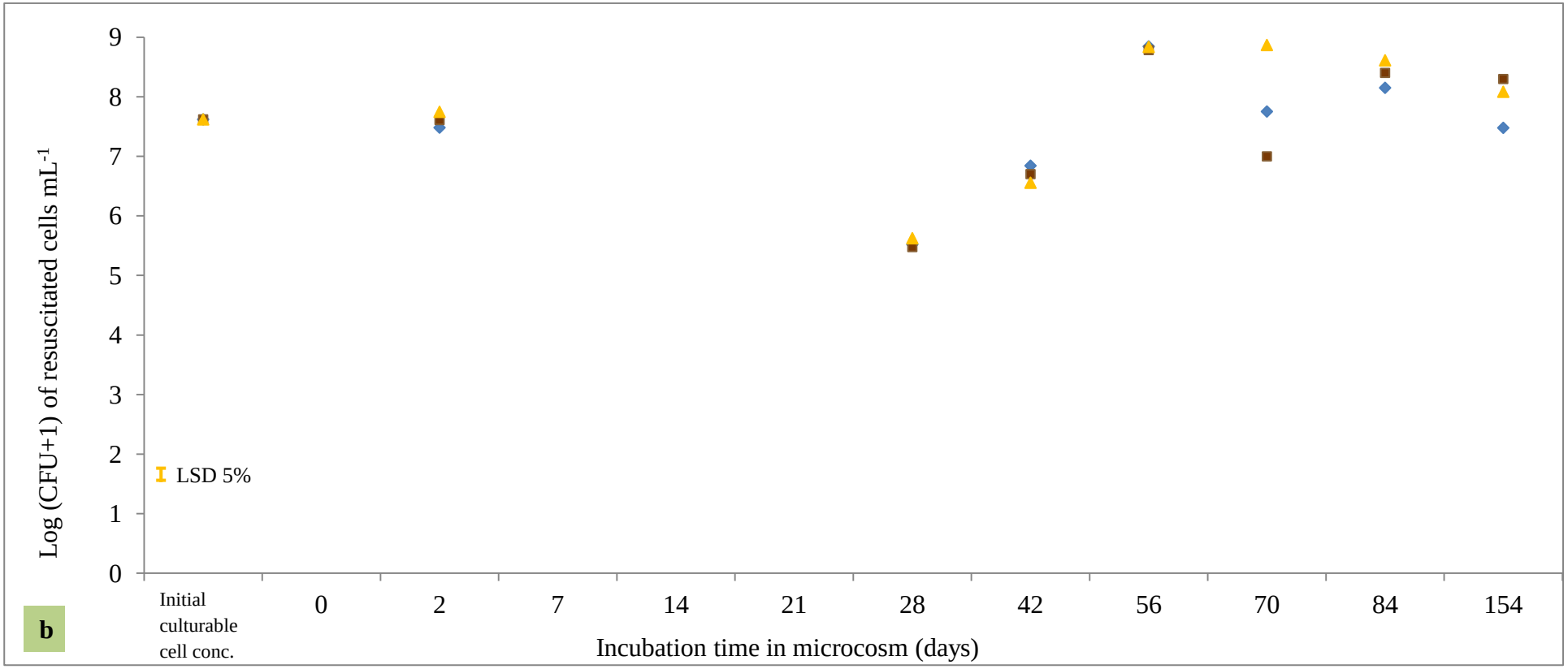


Figure 6.9 Resuscitation of nonculturable cells of *Xanthomonas translucens* pv. *pistaciae* in original microcosms containing no cupric sulphate (blue diamond), 0.005 (brown square) and 0.01 mM (orange triangle) cupric sulphate by addition of 10-fold-diluted sucrose peptone broth. Resuscitated cells were enumerated on (a) nutrient agar and (b) sucrose peptone agar. Mean of colony forming units following logarithmic transformation from two replicates after each incubation time in cupric sulphate is presented.

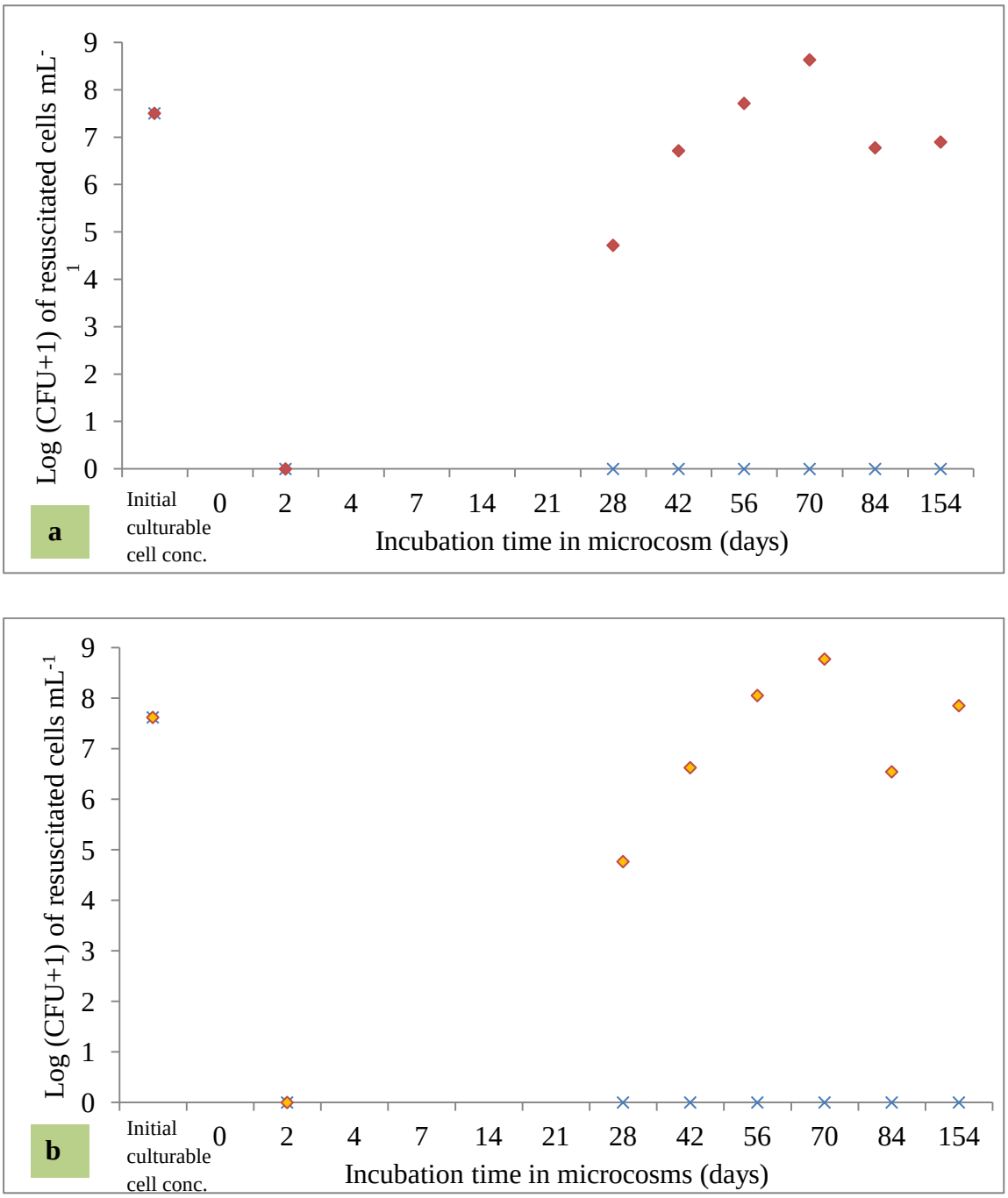


Figure 6.10 Resuscitation of nonculturable cells of *Xanthomonas translucens* pv. *pistaciae* in original microcosms containing 0.05 mM cupric sulphate by addition of 10-fold-diluted sucrose peptone broth. Resuscitated cells were enumerated on (a) nutrient agar and (b) sucrose peptone agar. Colony forming units following logarithmic transformation from replicate 1 (blue cross) and replicate 2 (red diamond) after each incubation time in cupric sulphate are presented.

culturability changed over time, depending on the concentration of cupric sulphate ($P < 0.001$).

In dilution studies, culturability of cells in resuscitated copper-free microcosms from undiluted up to dilution 10^{-6} was achieved at all times, except on day 154 when culturability was achieved only with resuscitated microcosms of dilutions up to 10^{-5}. Resuscitation of culturability from aliquots of 0.005 and 0.01 mM copper original microcosms varied. On some days, culturability was achieved for aliquots of undiluted up to dilution 10^{-4} while on other days culturability was observed even in resuscitated microcosms of dilutions 10^{-5} or 10^{-6}. Culturability in 0.05 mM copper original microcosms was not achieved at any dilution when no culturable cells were observed in the original microcosms on day 2, but were achieved up to dilution 10^{-5} on day 28 and 10^{-6} on day 42. From day 56, culturability of cells in resuscitated aliquots of this replicate microcosm was achieved for dilutions up to 10^{-4} or 10^{-6}.

6.3.2.3 Mutation in *Xtp*

Both colony types (COL1 and COL2) from replicate 2 of the 0.05 mM copper original microcosm in Experiment 2 (see Seection 6.3.2.1) produced the expected amplicon (331 bp) for *Xtp* in PCR using strain-specific primers. When examining the stained cells of each colony type under the microscope at 1000x, the shape and size of the cells were similar (Fig. 6.11), but some cells of COL2 tended to form short chains (Fig. 6.11b). In Experiment 3, COL1, COL2 and *Xtp* did not lose culturability on SPA or NA at time 0 regardless of the addition of cupric sulphate (Fig. 6.12). However, their culturability in microcosms containing cupric sulphate dropped to undetectable within 2 days. Culturability of the cells in the copper-free microcosms established from 9-time

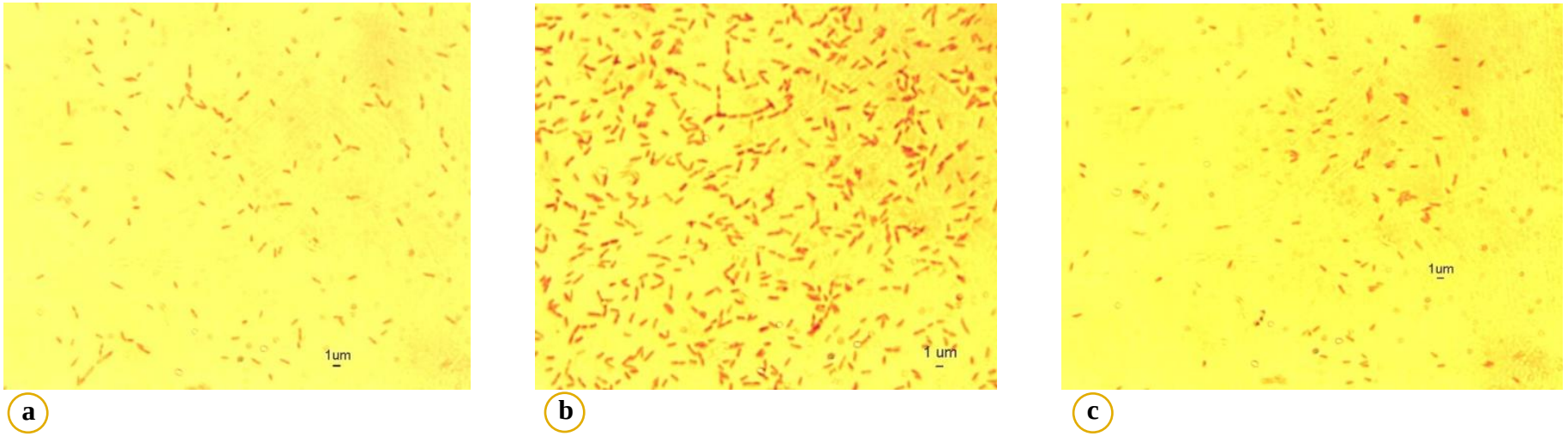


Figure 6.11 Gram stain and cell morphology of the two colony types obtained from microcosm, replicate 2, containing 0.05 mM cupric sulphate in comparison with cell morphology of *Xanthomonas translucens* pv. *pistaciae* prepared from fresh culture. a - cells of COL1, b - cells of COL2 and c - cells of *Xtp* prepared from fresh culture.

subcultured COL1 and COL2 on SPA decreased by about 2 and 3 logarithmic orders, respectively, while that from once subcultured COL1 and COL2 on SPA decreased by about 2 and 1 logarithmic orders, respectively, at day 56, compared with the concentrations of culturable cells at time 0. Similar decrease in culturability of the cells in the copper-free microcosms from once subcultured COL1 and COL2 was observed on NA, but decrease in culturability of the cells in the copper-free microcosms established from 9-time subcultured COL1 and COL2 was about 2 logarithmic orders.

In Experiment 4, additional enumeration of culturable cells at 3, 4, 5 and 6 h indicated various times of entry into nonculturability in the microcosms (Figs. 6.13 and 6.14). Overall, culturability of cells in each microcosm remained unchanged at time 0. Cells in copper-containing microcosms M2-COL2 and M2-COL1 lost culturability within 3 h, M1-COL2 after 3 h and M-*Xtp* after 4 h (Fig. 6.13). However, culturability of cells in one replicate of copper-containing microcosm M1-COL1 remained until 56 days after the addition of cupric sulphate (Fig. 6.14). The culturability in this copper-containing M1-COL1 was as high as that in the copper-free (control) M1-COL1. Nonetheless, no culturability in this copper-containing microcosm M1-COL1 and the copper-free M1-COL1 (control) was achieved on day 68.

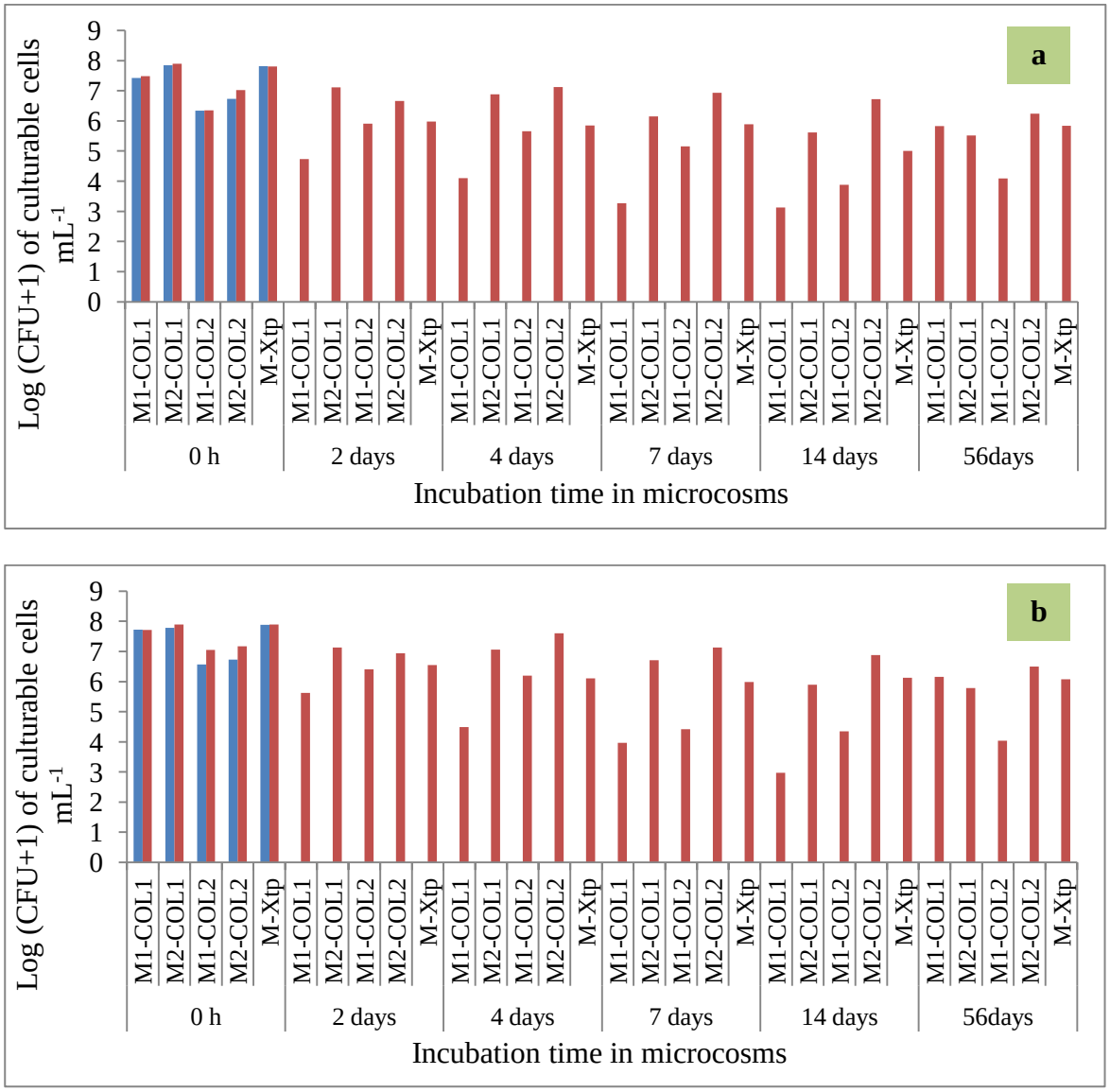
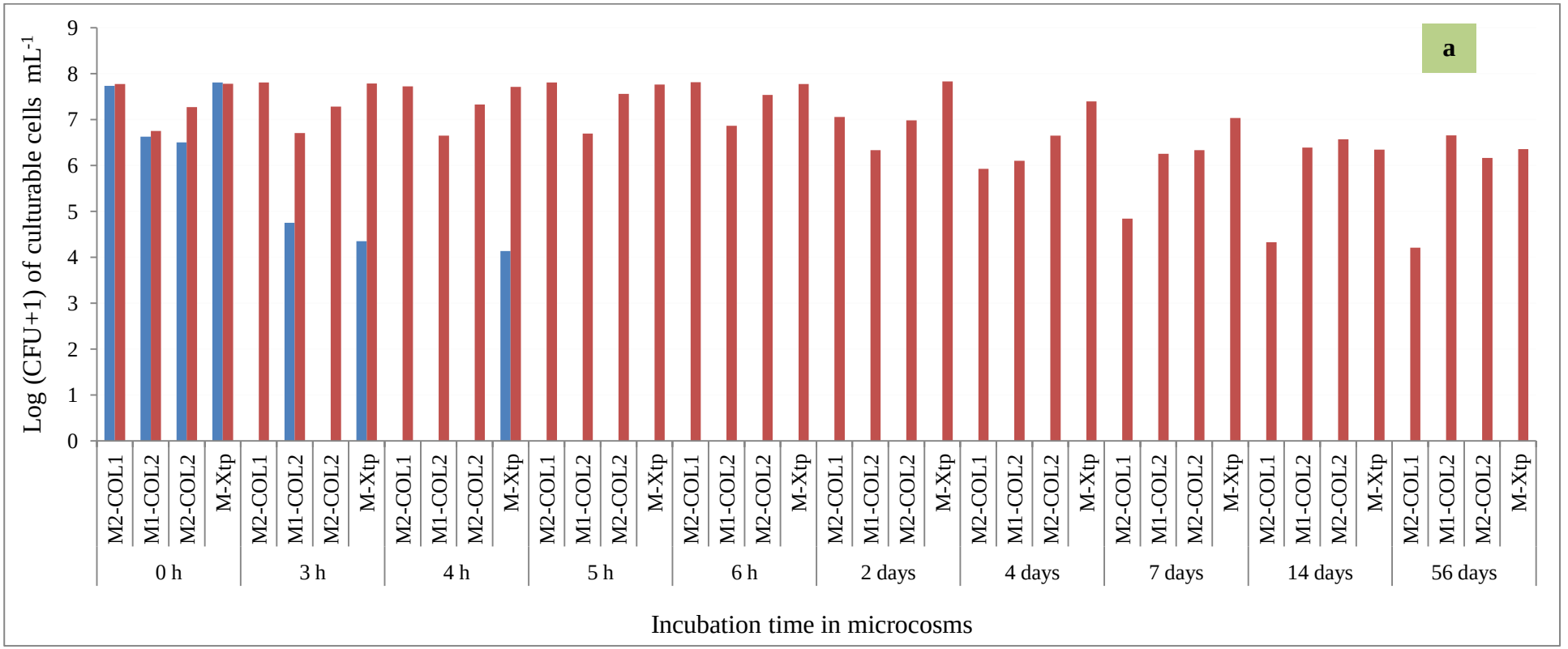


Figure 6.12 Culturability of cells in microcosms containing no (red) and 0.05 mM cupric sulphate (blue) from day 0 to 56 on (a) nutrient agar and (b) sucrose peptone agar. M1-COL1 and -COL2: microcosm established from a 9-time subcultured colony type 1 (COL1: convex and mucoid like *Xanthomonas translucens* pv. *pistaciae*) and 2 (COL2: small and sticky), respectively. M2- COL1 and -COL2: microcosm established from once subcultured colonies of type 1 and 2, respectively. Colonies did not develop from aliquots of any microcosm containing cupric sulphate at 0.05 mM from day 2 onwards.


a
Log (CFU+1) of culturable cells mL-1
9
8
7
6
5
4
3
2
1
0
M2-COL1
M1-COL2
M2-COL2
M-Xtp
0 h
3 h
4 h
5 h
6 h
2 days
4 days
7 days
14 days
56 days
Incubation time in microcosms

.

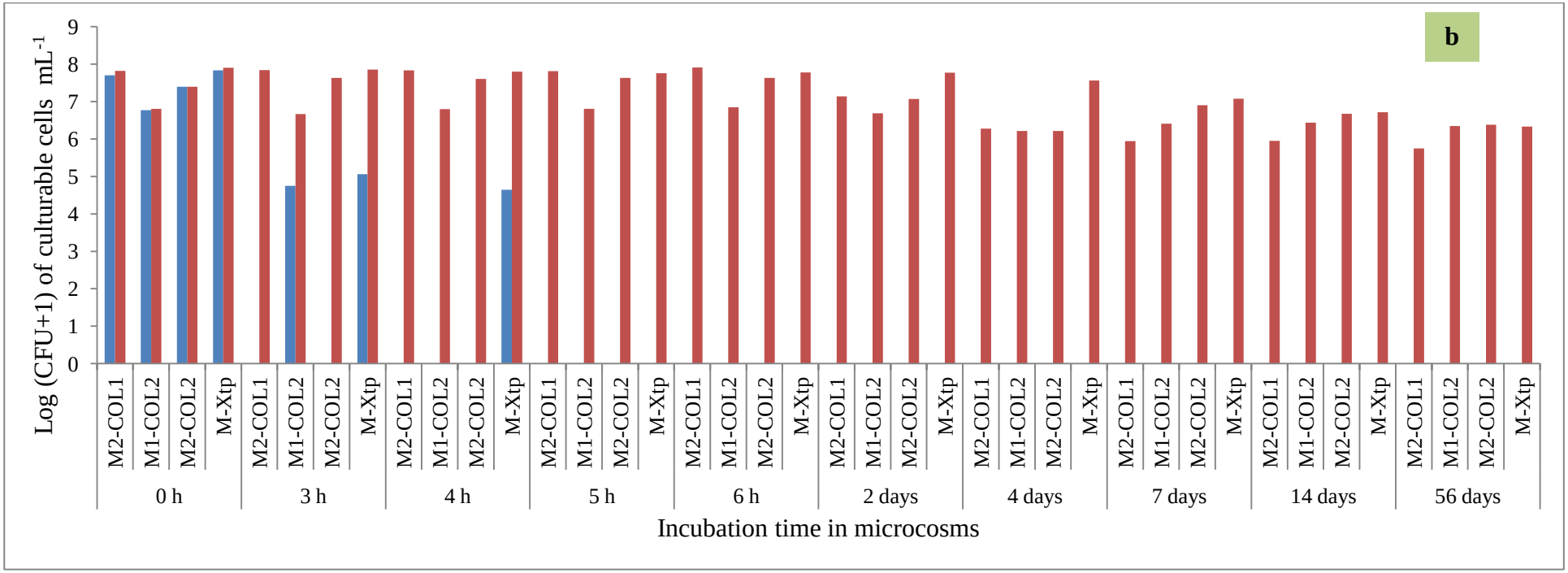


Figure 6.13 Culturability of cells in microcosms containing no (red) and 0.05 mM cupric sulphate (blue) from day 0 to 56 on (a) nutrient agar and (b) sucrose peptone agar. M2-COL1: microcosm established from a once subcultured colony type 1 (COL1: convex and mucoid like *Xanthomonas translucens* pv. *pistaciae*); M1- COL2: microcosm established from 9-time subcultured colonies of type 2 (COL2: small and sticky), and M2-COL1: microcosm established from a 1-time subcultured colony type 1 (COL1). Colonies did not develop from aliquots of M2-COL2 and M2-COL1 within 3 h, M1-COL2 after 3 h and M-*Xtp* after 4 h of addition of cupric sulphate.

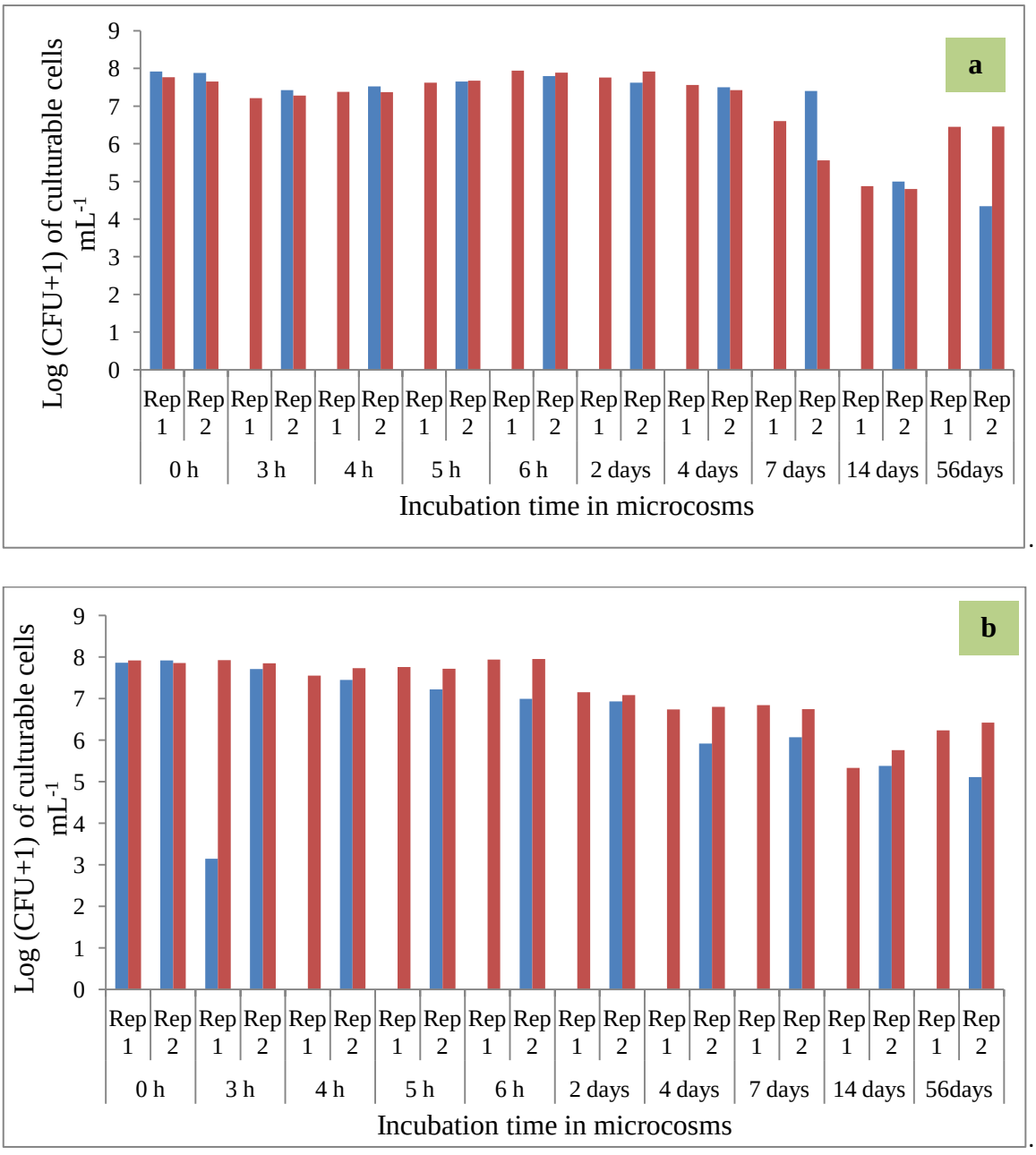


Figure 6.14 Culturability of cells in microcosms of 9-time subcultured (M1) colonies of type 1 (COL1: convex and mucoid like *Xanthomonas translucens* pv. *pistaciae*) containing no (red) and 0.05 mM cupric sulphate (blue) from day 0 to 56 on (a) nutrient agar and (b) sucrose peptone agar. Colonies did not develop from aliquots of M1-COL1 within 3 h and after 3 h of addition of cupric sulphate on nutrient agar and sucrose peptone agar, respectively.

6.4 Discussion

The present study investigated the potential of copper to induce the viable but nonculturable state in *Xanthomonas translucens* pv. *pistaciae* in liquid microcosms. The results indicated that cupric sulphate at 0.05 mM can induce an entire population of 10^7 CFU mL^{-1} of *Xtp* to become nonculturable within an hour of addition of cupric sulphate although viability was not confirmed by staining. In contrast, copper at 0.005 and 0.01 mM did not appear to induce nonculturability of *Xtp* cells. In addition, the gradual decrease in culturability of cells in copper-free microcosms suggested that copper was not the only factor to cause the cells to become nonculturable.

The rapid change of *Xtp* to a nonculturable state in the presence of copper at 0.05 mM in this study was in agreement with the finding by Ordax *et al.* (2006) for *Erwinia amylovora*. However, this change occurred sooner than that reported in *X. campestris* pv. *campestris*, which was within 2 days (Ghezzi & Steck, 1999). In contrast, while copper at 0.005 mM induced the cells of *X. campestris* pv. *campestris* to become VBNC within 2 days (Ghezzi & Steck, 1999), it did not induce all cells of *Xtp* to become nonculturable. Two different observations were made in this study. In Experiment 1, the earlier decrease (after 90 days) in culturability of cells in microcosms containing 0.005, 0.01 and 0.05 mM cupric sulphate than that (after 306 days) in copper-free microcosms suggested that copper at these concentrations might gradually induce a proportion of cells to become nonculturable. However, the ability of these copper concentrations to induce nonculturability of cells was not confirmed in Experiment 2. Although copper at 0.005 and 0.01 mM significantly decreased the culturability of cells in the original microcosms within an hour of the addition of cupric sulphate, a quick recovery of the culturability of cells close to the initial concentration may be explained as a transitional stage in which

cells gradually adapted to the new environment. Although a slight decrease in the culturability of cells in microcosms at these two concentrations was observed again from day 56, the culturability of cells in these original microcosms was always equal to that in copper-free microcosms, suggesting that copper at these concentrations might not be a factor to induce nonculturable cells. As the microcosms contained no nutrient, starvation might have induced a proportion of the cells to become nonculturable or even causing the death of some vulnerable *Xtp* cells. Regardless of whether the nonculturability in *Xtp* was induced by copper or starvation, the observation that some cells remained culturable for more than 2 years in microcosms containing no copper or $\leq$ 0.01 mM copper in Experiment 1 indicated the capability of *Xtp* to survive in unfavourable conditions.

Whether nonculturable cells are truly resuscitated or the recovery of culturability following the addition of nutrient is the result of the regrowth of a few undetected culturable cells has long been debated. In Experiment 1, due to the continued presence of culturable cells in the original microcosm with 0.01 mM copper, resuscitation assays were performed with the original microcosm diluted to 10^{-4}, the dilution that did not give rise to any colonies on culture media. In all cases, addition of 10-fold-diluted SPB into the diluted original microcosm restored the culturability. If this was a true resuscitation, it was expected that, in dilution studies, culturable cells would be recovered from all the dilutions when SPB broth was added to the original microcosm at each dilution and the final number of culturable cells would reach levels reflecting the dilution applied. However, in dilution studies the culturability was not restored for the microcosm at higher dilutions (from 10^{-5} to 10^{-7}), suggesting that the culturability of cells in the resuscitated 10^{-4}-diluted original microcosm might be the result of regrowth of a few undetected culturable cells. In Experiment 2, culturability of cells in the resuscitated microcosm (with SPB) was always detected at one to two dilutions higher than that in the original microcosms (without SPB), suggesting that resuscitation of culturability might have occurred. However, culturable

cells were not recovered from resuscitated microcosms at all dilutions, once again posing the question that the appearance of culturable cells might represent regrowth of a few undetected culturable cells. True resuscitation has been claimed for *Vibrio vulnificus* following a temperature upshift (Nilsson *et al.*, 1991; Whitesides & Oliver, 1997), for *Ralstonia solanacearum* by incubating in soil adjacent to roots of tomato plants (Grey & Steck, 2001) and for *E. amylovora* by adding copper-complexing compounds such as EDTA, citric acid, asparagines and 10-fold-diluted KB broth (Ordax *et al.*, 2006). However, Weichart *et al*. (1992) demonstrated that a few resuscitated cells or persistent culturable cells might evoke a response which mimicked resuscitation of the entire population following a temperature upshift to the range permitting growth, thus they argued that the resuscitation reported by Nilsson *et al*. (1991) might have represented regrowth. This argument might well be applicable to the resuscitation of *R. solanacearum* and *E. amylovora*. However, even if the restoration of culturability of *Xtp* was the result of regrowth of a few undetected culturable cells, the appearance of culturable cells of *Xtp* following the addition of nutrients, in this case SPB, would have to be taken into consideration for an effective control or eradication of the pathogen in the field.

In this study, resuscitation of the nonculturable cells in original microcosms with 0.05 mM was not achieved unless the original microcosms contained culturable cells, except on days 28 and 42 for one replicate microcosm in Experiment 2. A similar observation was reported by Morgan *et al.* (1991) for resuscitation of VBNC cells of *Aeromonas salmonicida* induced by river water at 10°C. While the ability to resuscitate VBNC cells might depend on bacterial species, duration of the VBNC state or resuscitation agents (nutrient, chelator or temperature), it has been reported that VBNC cells generally respond slowly to a reversal of the factor which initially induced the nonculturable state (Oliver, 1993). The time of incubation in, or exposure to, resuscitation agents required to allow the resuscitation of VBNC cells varies from 25 h (Roszak *et al.*, 1984) to a few days

(Oliver, 1993). However, with *Xtp*, even when aliquots of the 2-day microcosm with 0.05 mM copper were incubated in SPB for 168 h, resuscitation of nonculturable cells was not achieved. Ordax *et al.* (2006) also reported that resuscitation of VBNC cells of *E. amylovora* induced by 0.05 mM copper by 10-fold-diluted KB broth could be achieved only during the first 2 days after the cells had become nonculturable. They reasoned that rapid entry of the bacterium into the VBNC state might hinder the restoration of culturability. In their study, different copper-complexing compounds, including a chelator (EDTA) and nutrient (KB broth), were used to resuscitate the cells from the VBNC state. That the VBNC state induced by copper at 0.05 mM could not be resuscitated when the cells were in that state for more than 2 days indicates that the resuscitation from the VBNC state requires conditions other than simple addition of nutrient or a chelator. Whitesides and Oliver (1997), on the other hand, suggested that elevated nutrient content might be lethal or inhibit the resuscitation of the VBNC cells. This may help to explain why the culturability of the *Xtp* cells was not, or only poorly, restored in the present study when full strength KB broth and SPB were added to the original microcosms.

To determine the viability of nonculturable cells, viability assays using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit were conducted. However, in the second experiment, the stained cells failed to display the expected green for live and red for dead cells, even with living and dead cells of *Xtp* from a fresh culture. According to the manufacturer, the filter sets of the epifluorescence microscope used for the study were "long pass", which might have resulted in other colours, like orange or pink, instead of red and green. A long-pass filter is classified as blocking all "light below a certain wavelength and transmitting wavelengths longer than that cutoff" (Price & Jerome, 2011). However, the use of a confocal microscope, which allowed minimum and maximum wavelengths of the emission for the two stains to be set following the manufacturer's instructions, did not improve the situation. In addition, as noted by the manufacturer, the quality of black

polycarbonate membranes, especially the black dye in the membrane, used in this study might have interfered with viewing the colour of the stained cells, particularly as true green and red were observed in the initial validation test but not in the experiments with a new batch of membranes. This problem was not resolved in spite of discussion with the manufacturer.

Spontaneous recovery of culturable cells of *Xtp* in 0.05 mM copper microcosms after about 2 months in the two VBNC experiments raised the question of whether the cells were copper-resistant mutants or had just adapted to the presence of copper. Occurrence of copper-resistant strains has previously been reported for *Xanthomonas* (Ritchie & Dittspongpitch, 1991; Behlau *et al.*, 2011), but there has been no report on induction of copper-resistant mutants in any bacteria by copper used in VBNC assays. The observation of two different types of *Xtp* colonies but with microscopically similar cells agreed with the findings of Luria and Delbruck (1943) on mutant *Escherichia coli* resistant to bacteriophage. Two independent experiments were conducted based on the hypothesis that if the culturable cells observed in 0.05mM microcosms were copper-resistant mutants, they would retain their resistance to copper even if subcultured through many generations in the absence of copper, unless reverse mutation occurred (Luria & Delbruck, 1943). In addition, if the resistance was due to mutation, the proportion of resistant bacteria should have increased over time in a growing culture exposed to copper. In this study, loss of culturability in the microcosm containing cells of COL2 as quickly as 3 h after the addition of cupric sulphate at 0.05 mM excluded the possibility that these cells were resistant mutants. Instead, there might have been some robust cells in the population that had adjusted to the presence of copper, thus retaining culturability. In contrast, that cells of COL1, which were similar to colonies of *Xtp*, retained culturability in a microcosm (referred to as replicate 2 in section 6.3.2.3) at 0.05 mM copper suggested that copper might have produced a rare, resistant mutation in the population of *Xtp*. However, as the

culturability of cells in the other microcosm (replicate 1) was only achieved for up to 3 h after the addition of cupric sulphate, the mutations observed in this microcosm might be due to random, spontaneous mutation that occurred sometime during the growth of the culture prior to exposure to copper (Luria & Delbruck, 1943). That no culturability of cells from both the copper-free microcosm and the microcosm at 0.05 mM cupric sulphate (replicate 2) was achieved on day 68 hindered conclusions about whether spontaneous mutations had occurred in *Xtp*.

The primary conclusion of this study is that copper at 0.05 mM induced a nonculturable state in *Xtp*. However, due to the inability to demonstrate that the nonculturable cells of *Xtp* induced by copper were viable, no conclusion can be made as to the ability of *Xtp* to change to a VBNC state. At lower concentrations, copper might be able to induce a small part of the population of *Xtp* to become nonculturable. Further study should be conducted with a lower initial concentration of cells to elucidate the effect of copper to induce the nonculturable state in *Xtp* at lower concentrations of copper. Further studies are also required to confirm if spontaneous mutation may have occurred in the population of *Xtp* prior to the addition of copper. In addition, the report that the VBNC state of *X. axonopodis* pv. *citri* retained virulence (Del Campo *et al.*, 2009) supports the need for further study on virulence of nonculturable cells of *Xtp* induced by 0.05 mM copper if they are confirmed to be viable. Finally, the results of this study provided a basis for future research on the potential of *Xtp* to become viable but nonculturable.

Chapter 7 General discussion

The primary aim of an eradication programme is to eliminate a target pathogen. Therefore, an understanding of the factors influencing the survival of the target pathogen and how to promote factors that inhibit its growth is of paramount importance to the success of an eradication programme. This study focused on the effects of burning, high temperature and burial of infected plant materials on the survival of the pistachio dieback pathogen, *Xanthomonas translucens* pv. *pistaciae*, aiming to provide information on which to base management options for pistachio dieback and other systemic bacterial pathogens. The aim of this project was achieved through: (i) establishing the effectiveness of burning as an appropriate means of disposing of infected plant materials; (ii) identifying the thermal death point and thermal death time for *Xtp*; (iii) demonstrating that in a dry and warm environment, the pathogen was not eliminated by burial within 31 months but was rarely detected in infected materials on the soil surface; (iv) illustrating the potential of *Xtp* to enter a nonculturable state *in vitro* which might reflect the pathogen's evolution in response to conditions during burial.

Past eradication programmes, although reported to eradicate bacterial pathogens, did not provide details on the duration and temperature of burns nor potential of the pathogens to survive in non-burned infected materials (Sosnowski *et al.*, 2009). This study, apart from proving that complete burning was effective in eradicating *Xtp*, demonstrated that the pathogen could survive in infected plant materials that escaped burning or were exposed to a temperature for a duration less than that required to kill the pathogen (e.g. to 50 or 55°C for less than 120 min) (Chapter 4). According to Morita (1993), only one living cell is needed to ensure survival of a species if the cell encounters conducive environmental conditions, so propagules of pathogens that escape could become

a potential source for new infections and cause an eradication programme to fail (Janse & Wenneker, 2002). It might not always be feasible in a real eradication programme to determine the lethal temperature or duration of exposure to certain temperatures required to kill the target pathogen prior to burning infected materials. In such a situation, it is recommended that all infected materials be completely burned to reduce the likelihood of survival of bacterial cells, as viable *Xtp* was not detected in charcoal and ash in this study. As the temperature generated by fire when burning in the open is variable, as observed in this study and by Lanoiselet *et al*. (2005), disposal of infected and affected materials in a waste incinerator might be an option, especially for a small amount of material or if environmental pollution from open burning is of concern (Ebbels, 2003). Controllable temperature and even distribution of fire and heat inside an incinerator would help to eliminate the pathogen.

Factors contributing to incomplete eradication of a pathogen during a burn might include intensity and extent of fire and duration of exposure to the fire or fire heat. Being conducted in conditions of relatively high humidity, low temperature and light wind (Table 1, Chapter 4), the fires in 2009 did not spread well and were not intense enough to burn all the fuel materials and some samples in some locations furthest from the central pole, leaving them non-burned and allowing the pathogen to survive. Low intensity of fire was also observed by Raison *et al.* (1986) when they conducted a prescribed burning in a *Eucalyptus* forest in similar conditions of very light wind (< 1 km h^{-1}), low temperature (15°C), humidity (35%) and soil moisture ranged from 5 to 15% down to 10 cm below the surface. They reported that most of the plot was burnt slowly, which resulted in prolonged residence times of flames and complete incineration of the fuel. This supports the finding in the present study that slow burns in pits 2 and 3 were effective in eliminating the pathogen (Chapter 4). The isolation of viable *Xtp* in wood from locations where

temperature reached 200°C or more suggested that duration of the burn or exposure to heat play a vital role in the survival of the pathogen. It is worth highlighting that while the infected materials in mesh bags had been freshly cut, the fuel materials had been dried for some time before burning. Therefore, if fresh materials were to be disposed of in an eradication programme by burning, they would take longer and require more intense heat to be incinerated. In addition, the nature of materials being burned might influence the method and the duration of combustion. For example, dry grapevine canes caught fire quickly but did not keep burning, thus the flame-thrower was applied for 30 min. In contrast, dry pistachio wood comprising branches and twigs caught fire more slowly, but continued to burn once ignited. The remains in the mesh bags after the bonfire, which ranged from non-burned to ash, might reflect the reality in an eradication programme, especially when trunks or large branches are involved. As non-burned infected materials harboured viable cells of *Xtp*, the potential for the disease to be reintroduced remains. Therefore, in an eradication programme for *Xtp* or any other wood-inhabiting bacterial pathogen, the size and moisture content of infected materials should be taken into consideration to allow complete incineration.

While previous research on survival of bacterial pathogens in residues by burial mainly focused on pathogens of annual crops or in the case of citrus canker, leaves, this study provided information on survival of a systemic bacterium in infected wood of a perennial crop buried in or placed on the soil. In contrast to previous findings, *Xtp* was more persistent in wood buried in soil than that placed on the soil surface. This may reflect the nature of residue (pistachio wood), soil pH (alkaline) and moisture (dry), temperature (warm) and method of burial (mesh bag), as discussed in section 5.4. Additional factors which might hasten the decomposition process, for consideration in future survival studies and, where relevant, in eradication programmes involving burial are discussed below.

As *Xtp* has rarely been isolated from leaves (Facelli *et al.*, 2009) and the deciduous trees were leafless in winter, only infected branches were used in the present study. However, Collins *et al.* (1990) reported that, when buried, decomposition of mixtures of plant parts was about 25% faster than that of individual parts. As such, foliage might be included with wood in future experiments. In addition, that the infected branch segments or mulch in the present study had not decomposed 31 months after burial suggested that smaller size of disposal materials, which would increase the surface area exposed to decomposition (Brady & Weil, 2002), should be considered when burying resistant materials like pistachio wood. However, the escape of debris harbouring viable cells of *X. axonopodis* pv. *citri* during chipping the trees and emptying the trailers containing comminuted debris at the landfill site in an eradication programme for citrus canker in Florida (Graham *et al.*, 2004) suggested that the process must be implemented with great care.

Ideally, studies of burial as a means of disposal of infected materials should be done in a pit to simulate conditions of a real eradication programme. However, to aid the retrieval of samples, *Xtp*-infected wood was enclosed in mesh bags prior to burial in the present study, as were materials infected by various xanthomonads in other survival studies (Jones *et al.*, 1986; Schultz & Gabrielson, 1986; Graham *et al.*, 1987; Duffy, 2000). This approach has also been used to examine litter decomposition in terrestrial ecosystems (Wieder & Lang, 1982), although it was reported to underestimate the true total decomposition (Bocock & Gilbert, 1957), probably due to restricted soil-residue contact and decomposition activity by large arthropods and earthworms. Mesh size might also be relevant to consider in future survival studies involving burial. In this study and other survival studies, 1-mm-mesh bags were generally used. However, Edwards and Heath (1963) reported that the weight of oak leaves in 7-mm-mesh bags decreased three times faster than that in 0.5-mm-mesh bags, possibly because larger mesh-size (2 - 7 mm) might

have allowed access to all meso-fauna and macro-invertebrates (Tian *et al.*, 1992) while smaller mesh-size (0.5 - 2 mm) restricted access of such fauna (Swift *et al.*, 1979). Increasing the size of the mesh to 5 mm, which allowed access of most soil fauna (Tian *et al.*, 2007), could be considered for burial studies involving slow-to-decompose woody materials similar to pistachio wood. In addition, the mesh bag method might be optimised in future studies by mixing decomposing materials with soil before enclosing in mesh bags, or even better in mesh "cages", before placing in pot. By doing so, the materials could still be retrieved while having close contact with soil and soil fauna to enable more rapid decomposition of the materials.

Depth of burial of infected materials in survival studies has ranged from 5 to 30 cm (Schaad & White, 1974; Schultz & Gabrielson, 1986; Jones *et al.*, 1986; Graham *et al.*, 1987; Sikirou & Wydra, 2004), but an eradication programme requires a burial depth of at least 2 m to avoid movement by birds, animals or the elements (Ebbels, 2003). Although soil moisture may remain greater at this depth, activities of soil fauna and microbiota, key decomposers of plant residues, might be reduced compared to that in soil layers closer to the surface. In addition, materials have been either dumped directly into a landfill site (Graham *et al.*, 2004) or sealed in plastic bags prior to burial (Daly & Rodoni, 2007). As soil-residue contact, soil moisture and fauna play an important role in the decomposition process and from the observation of the resistance to decomposition of pistachio wood with little contact with soil in the present study, the addition of soil to the plant materials might be worthy of consideration in eradication programmes.

During burial, numerous fungi and bacteria were isolated from wood in contact with soil. Studies on the role of fungi in decomposition of pistachio wood and on survival of the dieback bacterium were not conducted due to time constraints. However, *in vitro* antagonism assays indicated the potential of some wood-associated bacteria to inhibit the

growth of *Xtp*, in some cases through antibiotic production. Initial *in vitro* investigation on the ability of two selected bacterial isolates, which gave large inhibition zones on a lawn of *Xtp* on agar, to colonise pistachio twigs and inhibit the growth of *Xtp* was conducted by a Masters student at the University of Adelaide. Results indicated that one isolate was able to colonise wood and suppress the growth of *Xtp* (N. Tran, pers. comm., 2011). A previous study also found one bacterium and peptide to inhibit the growth of *Xtp in vitro* (Salowi, 2010). Although they require to be validated in field conditions, these promising results provide additional knowledge for the Australian pistachio growers to further explore biological control and to integrate this approach into management strategies for pistachio dieback.

Survival of bacteria can be prolonged by production of extracellular polysaccharide (EPS) which protects the cells from adverse environmental factors, such as antibiotics and other antimicrobial agents, desiccation, heat and UV light (Leach *et al.*, 1957; Wiley *et al.*, 2007). In the present study, the protection of *Xtp* from heat in liquid culture with sucrose and in wood was attributed to the production of polysaccharides. Heating and desiccation of wood buried in or placed on the soil were likely to have occurred during the burial experiments. Although *Xtp* is known to produce EPS *in planta* (E. Facelli, pers. comm., 2008), that the pathogen was not isolated from wood placed on the soil surface raised questions of whether EPS would be produced at all times in infected organs, what factors would trigger or influence its production and what conditions would be suitable for it to confer protection. Environmental conditions such as temperature and relative humidity might influence the protective role of EPS. For example, Wilson *et al.* (1965) reported that survival of cells of *X. phaseoli* dried in their own exudates decreased when storage temperature and relative humidity increased and the survival of cells in slowly dried exudates became negligible after 14 days of storage at 20°C.

The production of EPS, as slime or capsule, is implicated in pathogenesis and is often correlated with virulence of plant bacterial pathogens, including *Xanthomonas* (Sutton & Williams, 1970; Ayers *et al.*, 1979; Denny & Baek, 1991; Denny, 1995; Poplawsky & Chun, 2002; Kemp *et al.*, 2004). EPS-deficient mutants were reported to be less virulent or unable to cause disease. For example, EPS-deficient mutants of *X. axonopodis* pv. *manihotis* induced symptoms only at the inoculation point without spreading (Kemp *et al.*, 2004) and those of *E. amylovora* were not able to multiply in host tissue and cause symptoms (Ayers *et al.*, 1979). Production of EPS may be affected by environmental conditions such as aeration, temperature and composition of the medium (Leach *et al.*, 1957; Lilly *et al.*, 1958; Souw & Demain, 1979), and is regulated by a number of genes, for example a cluster of genes *rpf*A-I (regulation of pathogenicity factors) (Barber *et al.*, 1997) or *pig*B (Poplawsky & Chun, 1997) in X. *campestris* pv. *campestris*. Strains with mutation at the *rpf*C, *rpf*G (Slater *et al.*, 2000), *rpf*B and *rpf*F (Poplawsky *et al.*, 1998) and *pig*B loci (Poplawsky & Chun, 1997) showed a decrease in production of EPS. However, production of EPS by the *pig*B mutant could be restored by growing it in proximity to wild-type strains, suggesting that the wild-type strains might have produced a diffusible factor (DF) to trigger the EPS production (Chun *et al.*, 1997). Similarly, production of EPS by *rpf* deletion mutants of a *X. campestris* pv. *campestris* strain was restored by adding purified or synthetic diffusible signal factor (DSF) (He *et al.*, 2006). DF and DFS are two distinct signals (Barber *et al.*, 1997; Poplawsky *et al.*, 1998) produced by various *Xanthomonas* species (Chun *et al.*, 1997; Chatterjee & Sonti, 2002; Thowthampitak *et al.*, 2008). DF and DSF are thought to be synthesised and secreted by bacteria in response to cell to cell communication, or quorum sensing, which permits them to assess their local population density (von Bodman *et al.*, 2003). The main pre-requisite for quorum sensing is growth in close proximity to other cells, such as in a biofilm or an enclosed environment (von Bodman *et al.*, 2003). Whether quorum sensing occurs between

cells of *Xtp* in culture or at any stage during pathogenesis or survival requires further investigation.

Apart from the knowledge that *Xtp* produces EPS in liquid culture and *in planta*, nothing is known of the role of the polysaccharide in virulence, pathogenesis or protection of *Xtp* in nature; further research may address this gap and strengthen understanding of the epidemiology of the pathogen.

Xtp appeared capable of entering into a nonculturable state in the presence of high concentration of copper and in nutrient-deficient microcosms. Although pistachio wood decomposed slowly during burial, it is probable that nutrients in the wood became depleted over time. Together with other factors such as fluctuations in temperature and desiccation during burial, it is possible that a proportion of *Xtp* cells residing in the infected pistachio wood buried in or placed on the soil surface entered into a VBNC state, which in turn could help explain the isolation of only very small numbers of colonies on culture media in the burial experiments. Further investigations are warranted to examine the possibility that temperature, desiccation and nutrient starvation might induce the VBNC state in *Xtp*.

Virulence of the nonculturable cells also merits further study. Del Campo *et al.* (2009) reported that a small proportion of cells of *X. axonopodis* pv. *citri* in the VBNC state produced lesions after infiltrating grapefruit plants. In addition, VBNC cells of *E. amylovora* could induce symptoms on pear fruits during the first 5 days after being in the VBNC state and resusciated cells could cause symptoms even 9 months after being in that state (Ordax *et al.*, 2006). It has been argued that the VBNC state might be an important means of survival of bacteria if the VBNC cells were able to remain virulent following resuscitation (Oliver, 2000b). Although nonculturable cells of *Xtp* were not resuscitated following addition of SPB or homogenised young pistachio leaves and shoots, the

existence of this state in *Xtp* should not be dismissed. If this state was to occur in field conditions, it would complicate the management of dieback, especially when the means of spread of the pathogen in nature is unknown. Further studies to determine if nonculturable cells of *Xtp* could be resuscitated and remain virulent are of importance in understanding the epidemiology of *Xtp.*

That *Xtp*, as well as other plant pathogens (Alexander *et al.*, 1999; Grey & Steck, 2001; van Overbeek *et al.*, 2004; Ordax *et al.*, 2006), was not culturable on routine bacteriological agar after being exposed to adverse environments suggested that diagnostic techniques other than the conventional plate count technique might need to be employed to avoid underestimation of the presence and population of the pathogens. Samples from natural environments are often heterogeneous, so direct methods to detect cells in a nonculturable state, for example direct viable count, measurement of respiration or vital staining, might be difficult. PCR methods, with primers specific for the target pathogen, are additional means of detection of the pathogen (Louws *et al.*, 1999). In the present study, *Xtp* was detected by PCR in wood from which the pathogen was isolated in the burning experiment in 2009 (see Chapter 4 and Appendix B). In contrast, viable *Xtp* was not detected in infected wood placed on the soil surface, whereas PCR assays indicated the presence of DNA of *Xtp* in some samples. However, PCR might also give "false positive" results as it also detects dead cells. Molecular methods such as PCR, reverse transcriptase PCR and nucleic acid sequence-based amplification have been assessed by Keer and Birch (2003) for their ability to determine viability of cells of environmental pathogens in the field of public health. Although these methods are often quicker, more sensitive and specific than classical methods, Keer and Birch (2003) noted that persistence of nucleic acids in cells post-death varied and the correlation between presence of DNA and RNA and viability was not clear. Therefore, they suggested using a combination of indirect methods; which should be considered in any studies of the VBNC state in *Xtp* and other

systemic bacterial plant pathogens. Recently, Golmohammadi *et al.* (2012) have developed RNA-based methods to detect and quantify viable *Xanthomonas citri* subsp. *citri*. With the advantage of detecting only viable cells, the methodology is worthy of investigation to examine viability of VBNC cells of *Xtp in vitro* as well as to quantify viable *Xtp* in debris buried in soil over time.

Although viable *Xtp* was only detected in one mulched wood sample in Experiment 1 and a few samples in infected twigs placed on the soil surface in Experiment 2 (see Section 5.3.2), this method is not recommended as a means of disposal of infected materials for a number of reasons. The first and most direct reason is the likelihood that the infected materials will harbour viable pathogen, thus they might act as source of inoculum. Second, in the present study, pots with infected wood on the surface were placed in an open area under direct sunlight. Intense heat, up to nearly 61°C, which proved lethal to *Xtp in vitro,* could eliminate the pathogen. However, survival of *Xtp* in infected materials placed on soil in shaded areas or less intense sunlight might have been different. Hsieh and Buddenhagen (1975) studied the effect of sunlight and shade on survival of *X. oryzae* in diseased rice leaves and reported that the pathogen survived longer in shade than under sunlight. The observation that bacteria, such as *Xtp,* in the present study, and *E. amylovora* (Ordax *et al.*, 2006), had the potential to enter a nonculturable state and that VBNC cells of both *X. axonopodis* pv. *citri* (Del Campo *et al.*, 2009) and *E. amylovora* (Ordax *et al.*, 2006) retained virulence and caused disease once again strongly suggests that placement of infected materials on the soil surface should be avoided. This might be relevant information for Australian pistachio growers to consider in their orchard management strategies, especially for the orchards where trees were shown to contain *Xtp*. Their current practice to mulch prunings and leave mulch on the soil surface could be risky, especially when the natural means of spread of the pathogen is not known. Disposal of prunings,

especially from trees infected with *Xtp,* by burning or burial might be worth practising to reduce the potential inoculum load.

At present, the natural dissemination and infection pathway of the dieback pathogen remains unknown. As the pathogen has not been isolated from surfaces of bark or lesions, and is infrequently recovered from leaves and bunches, the likely source of inoculum for new infection is diseased or infested wood. As rain, irrigation run-off, air and insects are not known to be means of dispersal (Sedgley *et al*, 2004, 2006), the risk of inoculum embedded in diseased or infected pistachio wood escaping and spreading to other planting areas in natural conditions is considered small. In addition, that *Xtp* was not isolated from roots and associated soil (Facelli *et al.*, 2009) suggests that dissemination of the pathogen below ground once woody debris has decomposed is improbable. In comparison, Gottwald *et al.* (2009) reported a low risk of spread of *Xanthomonas axonopodis* pv. *citri,* a pathogen that causes cankers on citrus foliage, stem and fruit, from discarded canker-affected fruit to surrounding trap plants in both natural conditions and extreme simulated wind and rain. However, inoculum of this pathogen was readily dispersed from leaf lesions in wind-driven rain over a long distance and a substantial period of time (Bock *et al.*, 2005).

Given that high temperature (50 - 65°C) reduced the population of or killed *Xtp* in infected wood and twigs, and pistachio wood is resistant to decomposition, it might be worth investigating a combination effect of heat and decomposition in a composting system as a means of disposal of dieback-affected materials. First of all, compost heaps contain numerous mesophilic microbes which assist in initial decomposition of the materials in the heaps (Sylvia *et al.*, 2005). Second, during a thermophilic phase in compost heaps, the temperature can reach between 50 and 80°C for days or even weeks (Brady & Weil, 2002; Zibilske, 2005), which are lethal to *Xtp,* other xanthomonads (Keck

et al., 1992; Sharma, 2006; Turechek & Peres, 2009), *E. amylovora* (Keck *et al.*, 1995) and *R. solanacearum* (Ryckeboer *et al.*, 2002). In addition, compost heaps are likely to contain microbial antagonists. For example, *Bacillus* spp. are commonly present in compost heaps during mesophilic and become more abundant during thermophilic phases (Zibilske, 2005) and *B. subtilis* has shown potential to inhibit growth of *Xanthomonas* (Lin *et al.*, 2001; Wulff *et al.*, 2002a). Finally, several plant bacteria have been eradicated during composting. For example, *E. amylovora* on cotoneaster shoots was eradicated after 7 days at 40°C (Bruns *et al.*, 1993) and *Pseudomonas savastanoi* pv. *phaseolicola* on bean leaves after 4 days at 35°C (Lopez-Real & Foster, 1985). There appears to be no information available in the literature on the fate of *Xanthomonas* pathogens in a compost heap. However, Coventry *et al.* (2004) conducted an experiment on the effect of composting conditions on survival of *X. campestris* and found that the pathogen was eradicated after incubating for 7 days at 50°C.

In conclusion, using the pistachio dieback bacterium as a model, this study demonstrated that complete incineration is an appropriate means of eradicating *Xtp* from infected wood and can be recommended for eradication of other systemic bacteria. This validates the principle laid out by Ebbels (2003) for disposal of infected or infested materials by burning. That temperatures between 50 and 60°C significantly reduced viable *Xtp* in pistachio twigs suggests that heat treatment might be tested as a means of thermotherapy for valuable budwood sources infected with *Xtp*. Disposal of bacterial pathogens of woody materials by burial might take a long time as decomposition of such material is slow and influenced by environmental conditions. In addition, *Xtp* might switch to a nonculturable state in unfavourable conditions, which might lengthen its survival and hinder its detection by conventional diagnostic methods. Overall, this study has improved knowledge of *Xtp*, which will help the Australian pistachio industry towards a better

management of dieback. It has also provided information to support and extend existing eradication response strategies for newly introduced or emerging bacterial plant pathogens.

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(Chapters 1 - 3 and 5 - 7)

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Appendices

Appendix A. Culture media

Dilute V-8 agar (Erwin *et al.*, 1987)

V-8 juice* (Campbell Australia Pty Ltd)	20 mL
$CaCO_3$	0.2 g
Agar (Bacto™ Agar, Difco)	17 g
Distilled water	to 1 L
Streptomycin sulphate (Sigma S0890)	30 mg L^{-1} in SDW

* V-8 juice was cleared by filtration through single then double layers of filter paper (Whatman, no. 41) before adding into the medium. Streptomycin was added aseptically after the medium had been autoclaved and cooled to 50°C in a water bath.

Luria-Bertani medium (Sambrook & Russell, 2001)

Bacto tryptone (Difco)	10 g
Yeast extract	5 g
NaCl	10 g
Sterile distilled water	to 1 L

Nutrient agar

Nutrient broth (Oxoid)	13 g
Technical Agar No 3 (Oxoid)	14 g
Distilled water	to 1 L

Nutrient agar with antibiotics (NA+A)

The following antibiotics were added to molten nutrient agar (as above) once the medium had cooled to 50ºC in a water bath.

Cephalexin (10 mg L^{-1})	1 mL
Ampicillin (1 mg L^{-1})	0.5 mL
Gentamycin (1.4 mg L^{-1})	0.14 mL

Preparation of antibiotic stock solutions:

Cephalexin (10 mg L^{-1}): Dissolve 250 mg of cephalexin hydrate (Sigma-Aldrich) in 25 mL of 75% ethanol.

Ampicillin (1 mg L^{-1}): Dissolve 20 mg of ampicillin (Sigma-Aldrich) in 10 mL SDW.

Gentamycin (1.4 mg L^{-1}): Dissolve 50 mg of gentamycin (Sigma-Aldrich) in 10 mL SDW.

Potato dextrose agar (PDA)

Potato dextrose agar (Difco)	39 g
Distilled water	to 1 L

Sucrose peptone agar (Hayward, 1960)

Sucrose	20 g
Bacteriological peptone (Oxoid)	5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.25 g
Technical Agar No 3 (Oxoid)	14 g
Distilled water	to 1 L

All culture media were autoclaved at 121ºC for 20 minutes and antibiotics were added, where required, to molten agar, once cooled to approximately 50ºC. Agar media were dispensed into 9 cm diameter Petri dishes and allowed to cool and solidify. When working with *Xanthomonas translucens* pv. *pistaciae*, the pH of all media was adjusted to 7.4 with KOH and a pH meter (Oakton®, Extech Equipment Pty Ltd, Australia or Activon Model 210, Australia) prior to autoclaving.

Appendix B. PCR results of burning experiment in 2009

Detection of *Xanthomonas translucens* pv. *pistaciae* by polymerase chain reaction from (a) infected wood in mesh bags and (b) infected wood in Petri dishes after the bonfire. Locations of positive samples are indicated with (⊕) and negative samples with (▭).