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Influence of *Vitis* xylem fluid and xylem fluid plus cecropin on growth of *Xylella fastidiosa*

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Summary

Colony growth of *Xylella fastidiosa* (UCLA PD and STL PD strains) was quantified after incubation for 48 h in xylem fluid of *Vitis rotundifolia* Michx. cv. Noble and *Vitis vinifera* L. cv. Chardonnay. Xylem fluid was collected from grapevines in the field (dormant and growing season) and from container-grown plants in a screen house (growing season). Colony forming units·ml^{-1} (cfu·ml^{-1}) were counted 15 d after plating on periwinkle wilt (PW$^+$) medium. Colony growth was promoted or inhibited compared to PW$^+$ medium, and was dependent on *X. fastidiosa* strain, plant species and source of xylem fluid. The efficacy of cecropin A and B was tested against this bacterium. Colony growth of *X. fastidiosa* was greatly inhibited after a 1-h-exposure to cecropin A or B. The minimum inhibitory concentration (MIC) of cecropin A or B for 100 % inhibition of *X. fastidiosa* was ≤ 1 µM. The activity of cecropin B in xylem fluid of *V. rotundifolia* cv. Noble was progressively reduced over time from 0.2 to 24 h. When 2 and 10 µM concentrations of cecropin A and cecropin B were mixed with xylem fluid for 24 h, a substantial amount of bacterial growth occurred after subsequent plating; shorter time intervals did not degrade the cecropins and did not prevent colony growth. Cecropin B (1 µM) added to xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay for 24, 48, 72 and 96 h did not prevent subsequent colony growth. Colony number tended to be higher for *V. rotundifolia* cv. Noble than *V. vinifera* cv. Chardonnay. Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) of cecropin B in xylem fluid showed that cecropin B degraded completely *(V. vinifera* cv. Chardonnay) or almost completely *(V. rotundifolia* cv. Noble) after 96 h.

K e y w o r d s : antimicrobial peptides, cecropins, lytic peptides, Pierce's disease, *Vitis rotundifolia*, *Vitis vinifera*.

Introduction

Pierce's disease (PD) of grapevine is caused by the xylem-limited bacterium *Xylella fastidiosa* which causes diseases of numerous plant species, *e.g.* plum leaf scorch, phony peach disease, almond leaf scorch (PURCELL and HOPKINS 1996; HOPKINS and PURCELL 2002). *X. fastidiosa* is vectored exclusively by xylem feeding leafhoppers. Xylem plugging and water stress are believed to result in marginal leaf necrosis and plant mortality. *X. fastidiosa* exists in a benign form in most plants that are endemic to its native range. *X. fastidiosa* has a negative impact on numerous ornamentals and tree species by causing scorch symptoms and/or plant mortality. For many plant species there is no resistant germplasm.

The PD strains of *X. fastidiosa* spread and multiply faster in xylem vessels of PD-susceptible than PD-resistant species of grapevines (HOPKINS 1984; FRY and MILHOLLAND 1990). Most PD strains do not move systemically in host species that do not show symptoms (HILL and PURCELL 1995; PURCELL and SAUNDERS 1999). The bacteria multiply within xylem vessels, and the result may be vascular system plugging and xylem dysfunction. Xylem vessels may be filled with pectins, tyloses, and gums that are produced by the host plant in response to invasion by the bacterium (FRY and MILHOLLAND 1990; PURCELL and HOPKINS 1996; HOPKINS and PURCELL 2002). The formation of tyloses and pectins is believed to be an adaptive plant response to infection and was reported to be more common in PD-resistant *V. rotundifolia* compared to PD-susceptible *V. vinifera* (MOLLENHAUER and HOPKINS 1976; HUANG *et al.* 1986; FRY and MILHOLLAND 1990). This bacterium is fastidious, as the name implies, and grows best on specialized media (CHANG and DONALDSON 1993; DAVIS *et a*l. 1980, 1981). Growth of *X. fastidiosa* may also be influenced by the chemistry of xylem fluid. The initial (or early) events associated with the growth and multiplication of *X. fastidiosa in planta* have not been resolved in *Vitis* or any other plant species. Similarly, the growth of *X. fastidiosa* in xylem fluid has not been related to the resistance/tolerance of *Vitis* species to PD.

One approach to reduce the titers of *X. fastidiosa* is by the use of lytic peptides. Many lytic peptides have been isolated from different kinds of organisms including bacteria, fungi, plants, arthropods, and vertebrates. Most of the lytic peptides appear to be major components of an antimicrobial defense system (BOMAN 1991). Cecropins belong to a family of homologous antibacterial peptides 35-37 amino acids long derived from the haemolymph of the giant silk moth, *Hyalophora cecropia.* Cecropins have much less inhibitory activity against eukaryotic cells than prokaryotic cells (DESTEFANO-BELTRAN *et al.* 1991; MILLS and HAMMERSCHLAG 1993; MILLS *et al.* 1994; MOURGES *et al*. 1998).

The amphipathic structure of lytic peptides enables the formation of ion channels in lipid bilayers. These channels induce a time and voltage dependent dissipation of membrane potential, proton leakage and cell death (CHRISTENSEN *et al.* 1988; CASTEELS and TEMPST 1994). Low concentrations

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of cecropin have also been shown to promote transcriptional activity and excessive production of stress proteins which results in cell death (OH *et al*. 1998). Synthetic cecropins have been produced with enhanced antibacterial activity against Gram negative and Gram positive bacteria (MERRIFIELD *et al.* 1982; DESTEFANO-BELTRAN *et al.* 1991, 1993). *V. vinifera* cv. Thompson Seedless grapevines have been genetically engineered with the Shiva-1 gene (SCORZA *et al.* 1996), and recently the technology has been patented to create PD-resistant grapevines (SCORZA and GRAY 2001).

The objectives of the present study were to 1) determine the influence of xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay on colony growth of *X. fastidiosa* 2) compare the bactericidal activity of cecropins A and B against *X. fastidiosa* 3) quantify the persistence of *X. fastidiosa* (as measured by colony forming units) after the *in vitro* incubation of cecropin in xylem fluid of susceptible and resistant *Vitis* species and 4) characterize the stability (as measured by Tricine SDS-Page electrophoresis) of cecropin B in xylem fluid.

Material and Methods

The collection and the chemical analysis of xylem fluid: Xylem fluid was collected from field-grown vines during the dormant season (27-29 March), during the summer (16 July) from field-grown vines and from container-grown vines located in a screen house during the summer. We collected xylem fluid from bleeding vines during the dormant season according to ANDERSEN and BRODBECK (1989, 1991). Vines did not bleed during the summer; therefore, xylem fluid was collected from field-grown and container-grown vines in a screen house using a pressure chamber apparatus (ANDERSEN *et al*. 1995). Extraxylellary tissue was stripped from the stem segment protruding from the pressure chamber. Xylem fluid from 4-6 terminal shoots was collected from 1200 to 1400 h for both species using a 0.25 MPa overpressure for 90 s (ANDERSEN *et a*l. 1993, 1995). All samples of xylem fluid were filtered through a 0.2 µm filter.

The concentrations of amino acids and organic acids in field- and container-grown vines were determined using high performance liquid chromatography (HPLC) (ANDERSEN *et al.* 1993, 1995). The samples from 3-5 plants were combined in order to obtain sufficient quantities of xylem fluid for analysis. Samples were centrifuged at 1200 x g and filtered through a 10,000 MW filter. Derivatization was performed with 2:2:1 ethanol:triethanolamine (TEA):H_2O. A 7:1:1:1 ethanol:TEA:H_2O:phenylisothiocyanate was added and reactions proceeded for 20 min. The eluent was 5 mM sodium phosphate buffer with 6 % acetonitrile. Quantification was via an HPLC gradient system equipped with an ultra violet (UV) detector on a Picotag column (Waters Division Millipore Corp. Milliford, MA, USA). Organic acid analyses were performed on a Waters HPLC equipped with a UV detector (Model 166, Beckman Corp. San Roman, CA, USA) and an a polymeric cation exchange column (Ion-300, Interaction Corp., San Jose, CA, USA). There was one replicate per treatment.

Growth of *X. fastidiosa* in xylem fluid: The *X. fastidiosa* strains used were UCLA-PD and STL-PD, and were provided by Dr. A. H. Purcell, University of California, Berkeley, California. *X. fastidiosa* was grown at 28 °C in liquid Periwinkle wilt plus (PW^+) medium (DAVIS *et al.* 1981). The treatments consisted of a 48 h incubation of xylem fluid plus *X. fastidiosa.* Inocula concentration was 10^5 colony forming units (cfu)·ml^{-1} ($A_{600\,nm}$ = 0.15) as measured with a Milton Roy Co. Spectronic 20 spectro-photometer (Spectronic Inc. Rochester, NY, USA). Suspensions of xylem fluid plus *X. fastidiosa* (10^3-10^4 cfu·ml^{-1}) were plated on PW^+ medium and the cfu·cm^{-2} were counted with a Bantex Colony Counter Model 920A after 15 d at 28 °C. Certain treatments resulted in extremely high colony numbers, and the maximum per plate (90 x 15 mm) was set at 2,000. Means $\pm$ 1 SE, n = 4 are presented.

Activity of cecropins after incubation in succinate citrate phosphate buffer: The minimum inhibitory concentration (MIC) for 100 % inhibition of cecropin A and cecropin B was determined. The *in vitro* activities of cecropin A and B (obtained from Sigma-Aldrich Chemical Co. Milwaukee, WI, USA) were determined by mixing these compounds in different concentrations for 1 h with suspensions of *X. fastidiosa* (UCLA-PD strain) in succinate citrate phosphate buffer (SCP). Inocula were 10^7 cfu·ml^{-1} ($A_{600\,nm}$ = 0.22) and were diluted to 10^4 cfu·ml^{-1} prior to mixing. The treated bacterial suspensions (100 µl) were plated with a loop on solid PW^+ medium. The cfu per plate were counted after 18 d at 28 °C. Cecropin A and B were tested at 0.0002, 0.001, 0.002, 0.01, 0.05, 0.1, and 1 µM. Values presented are the means $\pm$ 1 standard error (n = 5).

Activity of cecropin after incubation in xylem fluid: We examined whether there is loss of cecropin A and cecropin B activities after incubation in xylem fluid of *V. rotundifolia* cv. Noble. Xylem fluid was collected from bleeding field-grown vines in March while still dormant (ANDERSEN and BRODBECK 1991). Xylem fluid was frozen at -15 °C until experimentation in the summer. Samples were filtered through a 0.2 µm filter. The loss of activity of cecropin A and B was quantified by percentage inhibition of *X. fastidiosa* in xylem fluid compared to growth on PW^+ medium without cecropin. A stock solution of cecropin was prepared in SCP buffer. Solutions of cercopins A and B (2, 10 and 20 µM) and xylem fluid were mixed and were allowed to incubate for 0.2, 1, 2.5, 5 or 24 h. After the time intervals specified above, cell suspensions of *X. fastidiosa* were added ($A_{600\,nm}$ = 0.22, 10^7 cfu·ml^{-1}, 10^{-4} dilution = 10^3 cfu·ml^{-1}) and incubated in the cecropin A and B plus xylem fluid mixture for 1 h, and then plated on solid PW^+ medium. Cfu were counted 14 d later. The treatment was replicated once for each cecropin at each concentration at each time interval. Loss of activity was quantified by the reduction in percentage inhibition.

We also measured cfu of *X. fastidiosa* after incubation in xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay (collected from the container-grown plants in the screen house) with sublethal concentrations of cecropin B. Samples were filtered through a 0.2 µm filter. Suspensions of xylem fluid, cecropin B (1.0 µM) plus

X. fastidiosa UCLA-PD strain (A_{600nm} = 0.15, 10^5 cfu·ml^{-1}) were incubated continuously for 24, 48, 72 and 96 h and then diluted (10^3 to 10^4 cfu·ml^{-1}) and plated on solid PW$^+$ medium. Cfu per plate was determined after 15 d at 28 °C. Means ± 1 SE, n = 4 are reported.

Persistence of cecropin in xylem fluid as measured by tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS PAGE): The persistence of cecropin B in PW$^+$ medium and in xylem fluid of container-grown *V. vinifera* cv. Chardonnay and *V. rotundifolia* cv. Noble was determined by Tricine-SDS PAGE. Solutions of cecropin B were mixed 1:1 (v/v) with PW$^+$ medium or xylem fluid to create a cecropin B concentration of 50 µM. Solutions were incubated for 0, 1, 48 and 96 h at room temperature. The breakdown of cecropin was arrested by freezing at each time interval above, and samples were stored at -20 °C. Samples were mixed with a 2x Tricine sample buffer containing 2 ml of 0.5 M Tris.Cl (pH 6.8), 2.4 ml glycerol, 0.8 g sodium dodecyl sulfate and 2 mg Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chemical Co. Milwaukee, WI, USA). The samples were subjected to Tricine-SDS-PAGE (Schagger and von Jagow 1987) (50 V 10 mm for stacking/120 V for 70 mm for separation) using a 34 mm resolving gel. After electrophoresis, the peptide was covalently linked and prestained to the gel matrix with a solution of formaldehyde (5 %), ethanol (26 %) and Coomassie Brilliant Blue R-250 (0.1 %) for 1 h (Steck *et al.* 1980). Samples were destained overnight in a solution of 75 ml distilled water, 25 ml ethanol and 1 ml of 35 % formaldehyde.

Results and Discussion

Amino acids and organic acids are predominant organic compounds in xylem fluid of many woody species (Andersen and Brodbeck 1989, 1991; Andersen *et al.* 1993, 1995). The concentration of total amino acids was about 2-3-fold higher in xylem fluid of *V. rotundifolia* cv. Noble than *V. vinifera* cv. Chardonnay (Tab. 1). The concentration of glutamine/glutamic acid accounted for over 80 % of the amino acid profile. Glutamine predominated over glutamic acid for all treatments except *V. vinifera* cv. Chardonnay (dormant season) where deamination likely occurred. The remaining amino acids varied from 3-30-fold among treatments, but there was no clear relationship of individual amino acids to the growth of both strains of *X. fastidiosa* (data not shown). Tartaric and malic acids were the organic acids highest in concentration in the xylem fluid of most treatments, whereas citric acid was also in high concentration in *V. rotundifolia* (dormant season) (data not shown). The concentrations of total organic acids were between 6 and 26 % of total amino acids. The concentrations of total organic acids were lower in xylem fluid collected from vines during the dormant season.

Xylem fluid can be an excellent promoter of colony growth for *X. fastidiosa*. The ratio of colony counts in xylem fluid to colony counts formed after 48 h incubation in PW$^+$ medium was dependent on the interaction of strain, species and source of xylem fluid and varied between 0.07 and 21 (Tab. 2). The mean number of *X. fastidiosa* colonies varied between 16 and 2,000+ cfu per plate, depending upon treatment. The two treatments with a standard error of 0 (UCLA-PD strain, *V. rotundifolia* cv. Noble, field-grown, dormant; STL-PD strain, *V. vinifera* cv. Chardonnay container-grown, summer) indicate that all replicates had a colony number of at least 2,000. For the UCLA-PD strain, the highest and the lowest values were recorded when mixed with xylem fluid of field-grown dormant season and container-grown *V. rotundifolia* cv. Noble, respectively. By contrast, for the STL-PD strain, *V. vinifera* cv. Chardonnay xylem fluid collected from container-grown plants during the summer resulted in the highest colony number, and field-grown dormant plants of the same species produced the least colony growth. Surprisingly, there was not a consistent trend for the ratio of colony counts to be lower for PD-resistant *V. rotundifolia* cv. Noble compared to PD-susceptible *V. vinifera* cv. Chardonnay.

The composition of the first chemically defined medium (XF-26) for *X. fastidiosa* (Chang and Donaldson 1993) is somewhat similar to the composition of xylem fluid

Table 1

Concentrations of glutamine/glutamic acids, total amino acids and total organic acids in xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay. Xylem fluid was collected during the dormant season (field-grown vines) and during the growing season (field grown and container-grown vines in the screen house). Values consist of one replication

Species/source of xylem fluid	Glutamine/ Glutamic acid	Total Amino acids Concentration (µM)	Total Organic acids
V. rotundifolia cv. Noble			
Field-grown dormant	3674	4227	257
Field-grown summer	3069	3587	581
Container-grown summer	3725	4313	568
V. vinifera cv. Chardonnay			
Field-grown dormant	1015	1228	102
Field-grown summer	1653	1956	436
Container-grown summer	1808	2143	551

Table 2

The ratio of colony number after incubation in xylem fluid compared to colony number after incubation in PW+ medium for 48 h. *X. fastidiosa* UCLA-PD and STL-PD strains were incubated in xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay. Xylem fluid was collected during the dormant season (field-grown vines) and during growing season (field-grown vines or container-grown vines in a screen house). Means ±1 SE, n=4 are reported

Strain/species/source of xylem fluid	Colony number in xylem fluid/colony number in PW+ medium
UCLA-PD	
V. rotundifolia cv. Noble	
Field-grown dormant	21.06 ± 0
Field-grown summer	0.074 ± 0.02
Container-grown summer	15.79 ± 5.26
V. vinifera cv. Chardonnay	
Field-grown dormant	6.67 ± 2.41
Field-grown summer	5.43 ± 2.00
Container-grown summer	1.50 ± 0.10
STL-PD	
V. rotundifolia cv. Noble	
Field-grown dormant	1.88 ± 0.16
Field-grown summer	0.56 ± 0.25
Container-grown summer	0.89 ± 0.01
V. vinifera cv. Chardonnay	
Field-grown dormant	0.21 ± 0.09
Field-grown summer	3.63 ± 0.50
Container-grown summer	4.13 ± 0

(ANDERSEN and BRODBECK 1989, 1991; ANDERSEN *et al*. 1993, 1995). The *in vitro* nutritional requirements of *X. fastidiosa* are strain specific. The amino acid (and nitrogen compound) in highest concentration in xylem fluid of many species is glutamine, and it is also a major component of *Vitis* fluid, PW+ and PD media (DAVIS *et al.* 1980, 1981). The chemistry of xylem is not fixed and can vary with temperature (ANDERSEN and BRODBECK1989), time of year (BRODBECK *et al.* 1990; ANDERSEN and BRODBECK 1991), light conditions (ANDERSEN *et al*. 1993), water stress and soil nutrient status (ANDERSEN *et al*. 1995). Thus, we envision that *X. fastidiosa* is exposed to fluctuations in xylem chemistry *in planta*. Variations in the promotion/inhibition of xylem fluid of different chemistry (dependent on host species and source) is consistent with results of specific culture media that document strain specific variations in *X. fastidiosa* nutritional requirements. It was not possible to correlate the concentration of any compound that we detected to the promotion or inhibition of *X. fastidios*a. Xylem fluid contains a low diversity and concentration of secondary compounds, although proteins occur in low concentrations (BILES and ABELES 1991). The role of xylem fluid chemistry on *X. fastidiosa* multiplication and spread *in planta* should be an area of future investigation.

The effects noted were constitutive and not induced by living plants since we only tested the effects of extracted xylem fluid *in vitro*. One should not discount the formation of gums and tyloses by host plants as a resistance mechanism to PD (FRY and MILHOLLAND 1990). Gum formation in xylem vessels is associated with the accumulation of fibrillar material (partially esterfied pectic polysaccharides) in layers of parenchyma cells and then secreted across bordered pit membranes to xylem vessels (RIOUX *et al.* 1998). In addition, differences in anatomical characteristics of *Vitis* spp. have been suggested to be a component of PD resistance (MOLLENHAUER and HOPKINS 1976; HUANG *et al.* 1986; FRY and MILHOLLAND 1990).

The manipulation of xylem fluid chemistry may not be a viable technique to completely control *X. fastidiosa* and Pierce's disease. Alternatively, one tactic to control PD may be the development of transgenic plants (SCORZA *et al*. 1996; SCORZA and GRAY 2001). Cecropin A and cecropin B were very effective lytic peptides for inhibiting the growth of *X. fastidiosa* (Tab. 3). The minimum inhibitory concentrations (MIC) of cecropin A and cecropin B against *X. fastidiosa* were determined after 1 h of incubation in different concentrations of cecropin (0.0002, 0.001. 0.002, 0.01, 0.05, 0.10, 1.0) in SCP buffer, followed by 18 d of culture on PW+ media. For cecropin A and cecropin B, a MIC of 1 µM was recorded. The concentration of cecropin A that resulted in a 50 % growth inhibition (LC50) of *X. fastidiosa* was between 0.001 and 0.002; for cecropin B an LC50 of 0.001 µM was achieved; cecropin B at 0.1 µM resulted in a 99.9 % inhibition of growth. Magainin II, indolicidin and tetracycline were far less active against *X. fastidiosa* than were cecropin A and B (data not shown).

Table 3

Percentage inhibition of *X. fastidiosa* UCLA-PD strain incubated with cecropin A and cecropin B. Lytic peptides were incubated with *X. fastidiosa* in succinate citrate phosphate buffer for 1 h then plated on PW+ medium. The cfu were read 18 d later. Means ±1 SE, n=4 are reported

Compound	Concentration (µM)	Inhibition, %
Cecropin A	0.0002	12.8 ± 2.5
	0.001	43.9 ± 3.2
	0.002	61.8 ± 1.7
	0.01	82.0 ± 2.7
	0.05	88.1 ± 3.6
	0.10	96.6 ± 0.9
	1.0	100 ± 0
Cecropin B	0.0002	21.5 ± 1.5
	0.001	50.8 ± 1.3
	0.002	71.6 ± 5.7
	0.01	86.1 ± 2.0
	0.05	96.6 ± 0.6
	0.10	99.9 ± 0.1
	1.0	100 ± 0

LI and GRAY (2003) evaluated the activity of cecropin A, cecropin B, magainin I, magainin II and Shiva-1 in liquid PD-2 medium. They also found that cecropins were the most

potent lytic peptides with a zero increase in absorbance recorded at ≥ 0.5 µM. The MIC of the remaining compounds tested were ≥ 10 µM. However, it is not possible to accurately define MIC by increases in absorbance as bacteria may remain at initial levels or in low numbers at very low values of increased absorbance. For example, we found that 99.9 % growth inhibition of *X. fastidiosa* occurred at 0.1 µM. This would not be possible to quantify by absorbance. Nevertheless, our data showing a MIC of 1 µM in SCP buffer is not greatly different than that reported by Li and Gray (2003) in liquid PD-2 medium.

A relevant issue is to define the persistence of lytic peptides in xylem fluid (Jaynes *et al.* 1993; Mills and Hammerschlag 1993; Mills *et al.* 1994; Owens and Heutte 1997; Mourgues *et al*. 1998). The activity of cecropin A and B against *X. fastidiosa* was progressively reduced as a function of time in xylem fluid of *V. rotundifolia* cv. Noble after 15 d of growth on PW+ medium (Tab. 4). For cecropin A at 2 µM, percentage inhibition was 97 % after 1 h of incubation, but was reduced to 0 % after 24 h. Concentrations of cecropin A of 2, 10 and 20 µM after 5 h of incubation in xylem fluid failed to prevent the growth of *X. fastidiosa*. Cecropin B was a more effective bactericide than cecropin A when incubated in xylem fluid. The activity of cecropin B was retained at all concentrations when incubated for 5 h or less. There was a partial loss of activity at 24 h for all cecropin treatments. Thus, the activity of cecropin B persisted for a period between 5 and 24 h in xylem fluid, and there was 95 % growth inhibition at the highest concentration of cecropin B tested (20 µM) after 24 h.

Table 4

Percentage inhibition of *X. fastidiosa* after xylem fluid of *V. rotundifolia* cv. Noble was mixed with cecropin A and B (2, 10 and 20 µM) for 0.2, 1, 2.5, 5 and 24 h. *X. fastidiosa* was incubated in each of the treatments for 1 h, and the cfu were counted after 14 d of growth on PW+ medium. Values consist of one replication

Compound	Concentration (µM)	Inhibition, %				
		Time (h)				
		0.2	1	2.5	5	24
Cecropin A	2	97	79	63	47	0
	10	100	100	100	83	23
	20	100	100	100	98	78
Cecropin B	2	100	100	100	100	0
	10	100	100	100	100	39
	20	100	100	100	100	95

Cecropin B at 1 µM did not completely inhibit the growth of *X. fastidiosa* in xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay (Fig. 1). It should be pointed out that the incubation of *X. fastidiosa* was 10^5 cfu·ml^{-1} (or 10-100-fold higher) than in preceding cecropin experiments. The effectiveness of lytic peptides is stoichiometric, in that, it is dependent on the relative concentrations of lytic peptide and bacteria (Mills and Hammerschlag 1993). At higher concentrations of *X. fastidiosa* (10^6 cfu·ml^{-1}), the threshold MIC in SCP buffer was ≥ 10 µM and ≤ 10 µM, for cecropin A

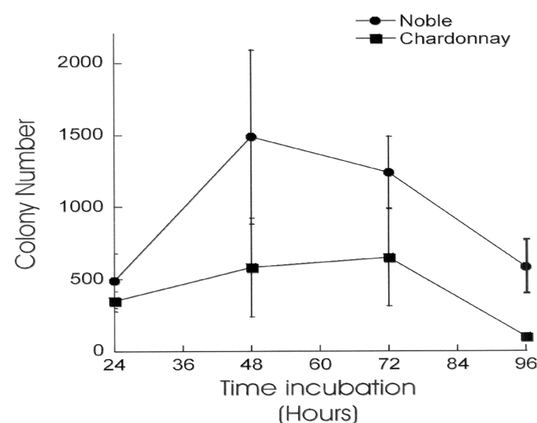

Fig. 1: The number of cfu per plate of *X. fastidiosa* incubated in xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay plus cecropin B (1 µM) for 24 to 96 h. After incubation for each time period, the solution was plated on PW+ and cfu were determined 15 d later. Means ± 1 SE, n=4 are reported.

and B, respectively (data not shown). Colony number of *X. fastidiosa* incubated in xylem fluid at concentrations of cecropin B below MIC values tended to be higher for *V. rotundifolia* cv. Noble than *V. vinifera* cv. Chardonnay, and the greatest numerical difference was achieved at 48-72 h. We presume that changes in the chemistry of xylem fluid over time induced a decline in the cfu at 96 compared to 72 h, but the reason for this is unknown.

Tricine-SDS PAGE analysis was used to monitor the degradation of cecropin B in PW+ medium and in xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay (Fig. 2). Results from Tricine-SDS PAGE corroborate that there was a breakdown of cecropin B in xylem fluid. There was a decline in concentration of cecropin B in all three treatments over time from 1 to 96 h; however, the band intensity was reduced more when cecropin B was incubated in xylem fluid compared to incubation in PW+ medium. For cecropin B, there were strong bands after 1 h of incubation in xylem fluid of both species. The band intensity was reduced, although still present at 48 h. The band corresponding to cecropin B was no longer visible after 96 h for *V. vinifera* cv. Chardonnay, although a very faint band remained after incubation in *V. rotundifolia* cv. Noble. Tricine-SDS PAGE results with cecropin B (50 µM) would likely reflect an increased persistence compared to lower concentrations of cecropin B. Recently, a more sensitive enzyme-linked immunoabsorbent assay has been developed for the detec-

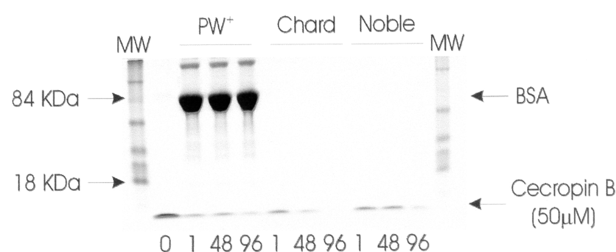

Fig. 2: Tricine SDS PAGE of cecropin B in PW+ medium or in xylem fluid of *V. vinifera* cv. Chardonnay (Chard) and *V. rotundifolia* cv. Noble. The persistence of cecropin B (50 µM) was determined after 1, 48 and 96 h in xylem fluid.

tion of Shiva-1 (0.1 µM) in transgenic *V. vinifera* cv. Thompson Seedless leaf tissue (LI *et al.* 2001).

The loss of activity of cecropin B in intercellular fluid over time has been attributed to proteolytic breakdown (MILLS *et al.* 1994; OWENS and HEUTTE 1997). However, some reduction in cecropin B concentration also occurred in the absence of proteolytic inhibition (in PW^+ medium), albeit at reduced levels compared to xylem fluid. Proteolytic inhibition may be mediated by peroxidases and other enzymes present in xylem fluid (BILES and ABELES 1991).

In conclusion, incubation of *X. fastidiosa* in xylem fluid of *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay was dependent on PD strain, *Vitis* spp. and source of xylem fluid. Concentrations of amino acids were 2-3-fold higher in xylem fluid of *V. rotundifolia* cv. Noble than *V. vinifera* cv. Chardonnay, although the concentrations of amino acids or organic acids were not correlated with growth promotion or inhibition. Since no xylem fluid treatment completely inhibited colony growth the antibacterial effects of cecropin were determined. Cecropin B was the most potent lytic peptide tested against *X. fastidiosa*. MIC of cecropin B was 1.0 µM and there was 99.9 % inhibition recorded at 0.1 µM. The antimicrobial activity of cecropin A and B was progressively reduced over time in xylem fluid. Tricine-SDS PAGE showed that the concentration of cecropin B was greatly reduced after 96 h in xylem fluid; the breakdown in cecropin B was greater in xylem fluid of *V. vinifera* cv. Chardonnay than *V. rotundifolia* cv. Noble.

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