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Zoosporicidal Activity of Polyflavonoid Tannin Identified in *Lannea coromandelica* Stem Bark against Phytopathogenic Oomycete *Aphanomyces cochlioides*

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In a survey of nonhost plant secondary metabolites regulating motility and viability of zoospores of the *Aphanomyces cochlioides*, we found that stem bark extracts of *Lannea coromandelica* remarkably inhibited motility of zoospores followed by lysis. Bioassay-guided fractionation and chemical characterization of *Lannea* extracts by MALDI-TOF-MS revealed that the active constituents were angular type polyflavonoid tannins. Commercial polyflavonoid tannins, Quebracho and Mimosa, also showed identical zoosporicidal activity. Against zoospores, the motility-inhibiting and lytic activities were more pronounced in *Lannea* extracts (MIC 0.1 μg/mL) than in Quebracho (MIC 0.5 μg/mL) and Mimosa (MIC 0.5 μg/mL). Scanning electron microscopic observation visualized that both *Lannea* and commercial tannins caused lysis of cell membrane followed by fragmentation of cellular materials. Naturally occurring polyflavonoid tannin merits further study as potential zoospore regulating agent or as lead compound. To the best of our knowledge, this is the first report of zoosporicidal activity of natural polyflavonoid tannins against an oomycete phytopathogen.

KEYWORDS: ***Lannea coromandelica*; polyflavonoid tannins; motility-inhibiting activity; *Aphanomyces cochlioides;* zoospore lysis**

INTRODUCTION

Oomycetes are phylogenetic relatives of brown algae that cause many destructive diseases of plants, as well as several animal and human diseases (*1*, *2*). Our knowledge of their biology is limited, but their physiology differs from that of fungi, and many fungicides are ineffective against oomycetes (*3*, *4*). New approaches are needed to find novel targets and to develop the "oomicides" for a sustainable and biorational management of those notorious phytopathogens (*4*). Among the oomycetes, *Aphanomyces* species cause some of the destructive plant and fish diseases in the world (*5*−*9*). Species of the phytopathogenic *Aphanomyces* exhibit high degree of specialization and can infect a limited number of plant species (*10*, *11*). For example, most of the plants are resistant to the strains of *A. cochlioides* that infect sugar beet, spinach, and a few other members of Chenopodiaceae and Amaranthaceae (*11*). This phenomenon of nonhost resistance, the ability of a pathogen to cause a disease in particular species but not in others, has always intrigued plant pathologists but remains poorly understood especially in oomycetes (*2*, *12*).

Accumulated evidence suggests that the motile zoospores of *A. cochlioides* locate their host by utilizing chemical signals released from the roots of host plant and then undergo a series of morphological changes as infection develops (*13*−*16*). In contrast to susceptible plants, we hypothesized that nonhost plants may have some chemical weapons for their resistance (*17*). We undertook a survey of physiologically active constituents in nearly 200 nonhost traditional medicinal plants guided by bioassay using zoospores of *A. cochlioides*. The motility and viability of the zoospores were markedly affected by some of the crude extracts. Among the activities of plant extracts, sudden inhibition of motility followed by characteristic lysis of zoospores by the stem bark extracts of *Lannea coromandelica* (Anacardiaceae) was noticeable. This interesting observation prompted us to characterize the motility-inhibiting and lytic factor of *Lannea* extracts by detailed bioassay-directed fractionation.

L. coromandelica L. is a deciduous tropical tree widely distributed in Bangladesh, India and in some other tropical countries. Plants belonging to this genus are used in folk medicine for treatment of elephantiasis, impotence, ulcers, vaginal troubles, halitosis, heart disease, dysentry, gout, and rheumatism (*18*, *19*). Reports on phytochemical investigations of *Lannea* genus are scanty, although some natural products including flavonoids, hydroquinones, and ferulic acid esters have been isolated from this genus (*18*−*21*).

Bioassay-guided fractionation and chemical characterization of *Lannea* extracts indicated that some polyflavonoid tannins are responsible for zoosporicidal activity. This paper describes

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the characterization of chemical properties of the zoosporicidal factor in *L. coromadelica* extracts and the bioassay results of *Lannea* extracts compared with two popular commercial polyflavonoid tannins, Quebracho and Momosa. In addition, the characteristic morphological changes of zoospores in the presence of polyflavonoid tannins are visualized by scanning electron microscopy. The potential role of condensed tannins in plant-pathogen compatibility is also discussed in relation to the biorational control of oomycete phytopathogens.

MATERIALS AND METHODS

Chemicals. Two commercial polyflavonoid tannins, namely, Quebracho (wood tannin of *Schinopsis balansae*) and Mimosa (bark tannin of *Acacia mearnsii*) for industrial use were received as a gifts from Dr. Kazuaki Takenouchi and Mr. Masahiro Sato, Graduate School of Agriculture, Hokkaido University, Japan. The commercial tannins were extracted with 80% acetone and successively fractionated as with *Lannea* tannin described below.

Plant Materials, Extraction, and Fractionation. The stem bark of *L. coromandelica* was collected from Mymensingh district of Bangladesh (*21*). The dried pulverized sample (1.9 kg) was successively extracted with 80% aqueous acetone and 60% aqueous MeOH to yield 280 and 271 g concentrated extracts after removing solvents in vacuo, respectively. The acetone extracts showed bioactivity and were successively fractionated according to solubility with *n*-hexane, diethyl ether, EtOAc, and MeOH. The motility inhibitory and lytic activity was observed in MeOH fraction (256 g) and was subjected to successive bioassay-directed fractionation using SiO_2 gel, Sephadex LH-20, and RP-18 reversed-phase column chromatography. However, none of the chromatographic techniques were found suitable for separating active constituents, and hence, the MeOH solubles were directly used for bioassay and MALDI-TOF-MS analysis.

Vanillin-sulfuric acid reagent spray on a silica gel thin-layer plate gave quick red coloration. The UV absorbances (at 280 nm) of MeOH solubles of *Lannea* extracts, Quebracho and Mimosa tannins, were ca. A_1^1 23.16, 17.73, and 9.02 (in MeOH) (absorbance of a 1% solution in a cell with 1-cm light path), respectively. Methylation and acetylation of the MeOH fraction of acetone extracts of *L. coromandelica* were carried out as described before (*17*), and the products were purified by SiO_2 gel column chromatography. Both mixture and purified acetylation and methylation products were bioassayed and were inactive up to 100 μg/mL concentration.

^{1}H and ^{13}C NMR spectra were measured in Me_2CO-d_6 at 270 MHz using TMS as the internal standard. Other instrumental analyses were conducted using a JEOL JSM AX-500 (FAB) and JEOL JSM-SX102A (FD) for mass spectrometry and a HITACHI U-3210 spectrometer for UV spectrometry (in MeOH). Spots were viewed on thin-layer plates (Merck RP-18 F_{254s} developed in CH_3CN-H_2O = 2:3) under 254-nm UV light and spraying with vanillin-sulfuric acid reagent followed by heat.

MALDI-TOF-MS. The tannin samples were dissolved in acetone (4 mg/mL). About 1 μL of sample (tannin extract) was placed on the MALDI target followed by equal volumes of NaCl and matrix (2,5-dihydroxybenzoic acid) solutions that were added on the same MALDI target (*22*). After evaporation of the solvent, the MALDI target was introduced into the spectrometer. The spectra were recorded on a Voyager DE-STR/15000 instrument. The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm. The measurements were carried out using the following conditions: polarity-positive, flight path-linear, mass-high (20 kV acceleration voltage), scans averaged 256. The delayed extraction technique was used applying delay times of 256 ns.

Production of Zoospores and Bioassay. *A. cochlioides* (AC-5) was a gift from Prof. R. Yokosawa and was isolated from the soil of sugar beet field. Culture of *A. cochlioides* and production of zoospores and bioassay was carried out as reported previously (*17*, *23*). Quantitative bioassay for *Lannea* extracts and two commercial tannins were carried out as follows. Tannin extracts were first dissolved in small quantities of DMSO and then diluted with distilled water. Appropriate amounts

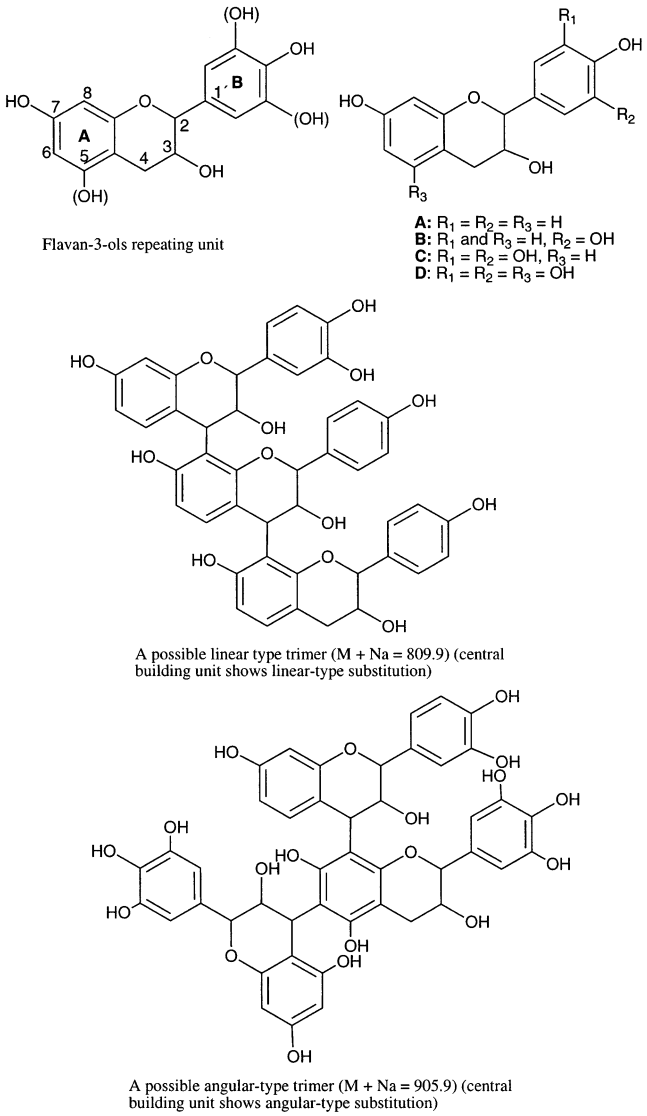

Figure 1. Possible structural units (A–D) of *Lannea coromandelica* (stem bark) polyflavonoid tannins and structures of typical linear- and angular-type trimers.

of sample suspension were directly added to the zoospore suspension taken in one dish of Nunc Multidish (NUNC) to make final volume as 200 μL and quickly mix well by a glass rod. The final concentration of DMSO in the zoospore suspension was maintained less than 1% in all treatments. 1% aqueous DMSO was used as a control. The halted zoospores rapidly settled at the bottom of a Petri dish and then started to burst. Time-course changes of the number of settled and burst spores were counted microscopically ($\times$20 magnification). Each treatment was replicated thrice and the mean value (%) of each treatment was compared to that of the control. The percent of halted and burst zoospores was calculated by the following formula:

$$\%\ \text{of halted zoospores} = \frac{H_t - H_c}{H_v} \times 100$$

$$\%\ \text{of burst zoospores} = \frac{H_b}{H_v} \times 100$$

where H_t is the average number of halted spores per microscopic field in tannin-treated dish, H_c is the average number of halted spores per microscopic field in control dish, H_b is the average number of burst spores per microscopic field in tannin-treated dish, and H_v is the average number of halted spores per microscopic field in dish containing equal volume of vortexed (30 s) zoospore suspension. Vortexing of 30 s caused 100% halting in 1 min.

Table 1. MALDI-TOF-MS Peaks for *Lannea coromandelica* Stem Bark Extract with Their Possible Repeat Units[a]

		unit type			
$M + Na^+$ experimental	$M + Na^+$ calculated	A	B	C	D
		Monomer			
314.2	313.3			1	
		Dimer			
552.2	553.6	1	1		
568.2	569.6		2		
586.2	585.6		1	1	
602.3	601.6			2	
618.2	617.6			1	1
634.0	633.6				2
		Trimers			
810.1	809.9	2	1		
890.3	889.9		1	1	1
		or,		3	
904.1	905.9		1		2 angular tannin
		or,		2	1 angular tannin
920.0	921.9			1	2 angular tannin
		Tetramers			
1178.3	1178.2			4	
		or,	1	2	1
		or,	2		2
1194.3	1194.2			3	1 angular tannin
		or,	1	1	2
1211.5	1210.2			2	2 angular tannin
		or,	1		3 diangular tannin
		Pentamers			
1450.0	1450.5				
1466.8	1466.5				
1482.2	1482.5				
1499.1	1498.5				
		Hexamers			
1753.8	1754.8				
1770.5	1770.8				
1786.8	1786.8				
		Heptamers			
2042.9	2043.1				
2059.0	2059.1				
2074.3	2075.1				
		Octamers			
2298.2	2299.7				
2347.2	2347.7				
2365.0	2363.7				
2381.2	2379.7				
2397.0	2395.7				
		Nonamer			
2506.3	2507.7				

[a] The predominant repeat units in this tannin is 288 Da, indicating that this tannin is predominantly prorobinetinidin.

Spores were counted from at least five microscopic fields in each dish and were averaged.

Scanning Electron Microscopy. Appropriate amount of tannin extract was directly added to a zoospore suspension taken on a SEMpore membrane (pore size 0.6 μm, JEOL). After a set time (5, 30, and 60 min for *Lannea* extracts, 10 and 30 min for commercial tannins) of treatments, the specimen was fixed with 2% buffered glutaraldehyde (TAAB, Berkshire, U.K.) at room temperature (about 23 °C). After dehydration in a graded acetone series (50%, 70%, 90%, 95%, and 99.5%), the spores were critical-point dried using liquid CO_2 and were coated with 10-nm-thick platinum−palladium using a sputter coater. The coated spores were observed under a JSM-6301F, JEOL scanning electron microscope with accelerating voltage of 5 kV (*15*).

RESULTS AND DISCUSSION

Isolation of the Factor Responsible for Motility Inhibition and Lysis of Zoospores. The MeOH fraction of acetone extract of *Lannea* stem bark showed bioactivity and hence was subjected to bioassay-guided fractionation by different column chromatography including SiO_2 gel, Sephadex LH-20, and RP-18 CC (data not shown). However, none of the chromatographic techniques were suitable for separating the active factor because of its high polarity and complex behavior in chromatography. The active fraction (MeOH solubles from the 80% acetone extracts) was tan-colored amorphous powder soluble in 80% aqueous acetone and gave a sharp UV absorption maxima at 280 nm (in MeOH). The FD-MS and FAB-MS were ineffective to get information of the molecular weight of bioactive constituents in the column fractions. ^{1}H NMR of the bioactive SiO_2 gel column fractions or MeOH solubles gave an identical very broad peak at the aromatic region. The ^{13}C NMR also gave some broad peaks at δ 25−85 ppm and 96−160 ppm indicating the presence of polyflavonoid tannins in the bioactive fractions (data not shown) (*24*). On the basis of the physicochemical properties including ^{1}H and ^{13}C NMR data of the active fractions, we assumed that the active principle in *Lannea* extracts might be a mixture of complex polyflavonoid tannins, which was highly stable in hydrolysis. To get definite evidence, we tested the activity of two popular commercial tannins (Quebracho and Mimosa) in our bioassay. Interestingly, 80% acetone extracts of both commercial tannins showed identical halting

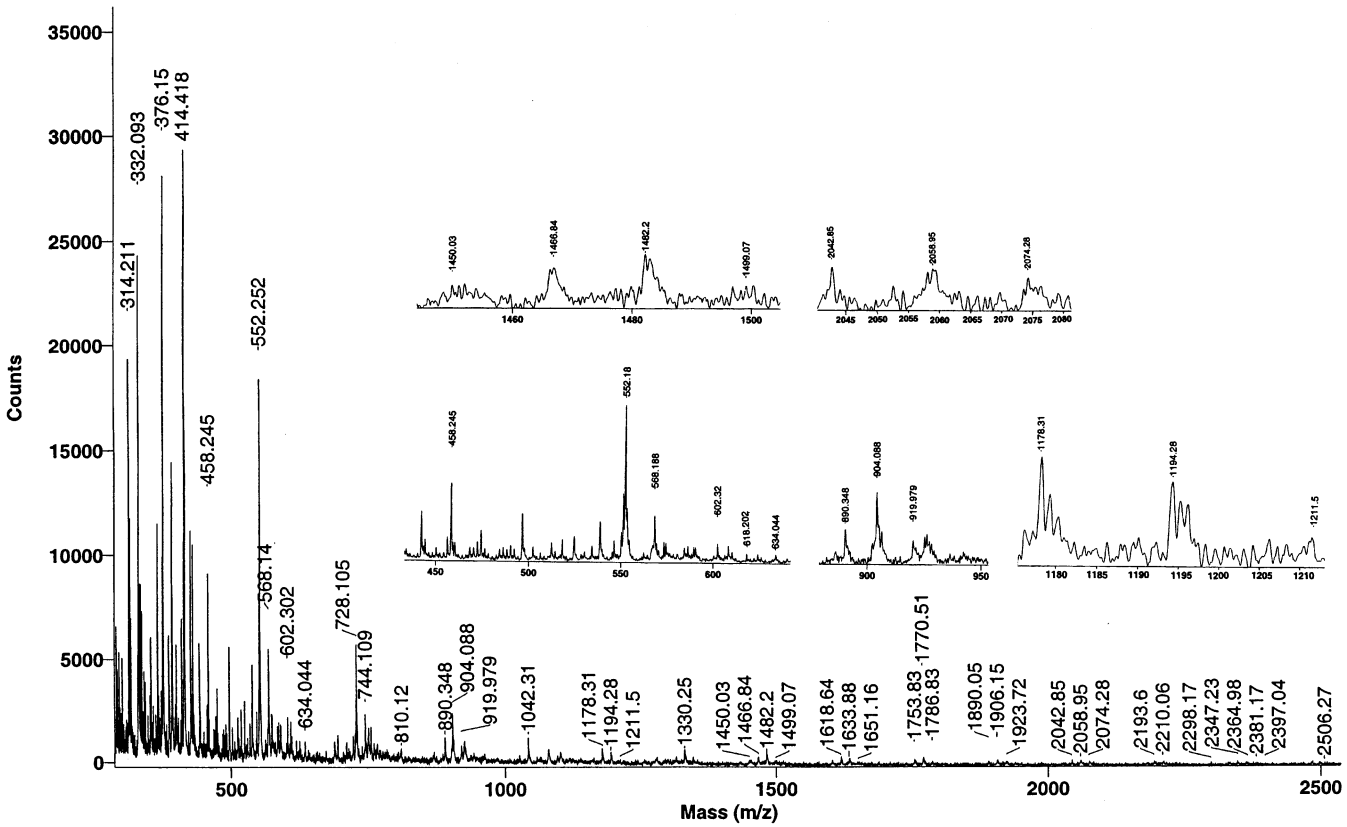

Figure 2. MALDI-TOF mass spectrum of *Lannea coromandelica* extract. Insets showing expanded form of some important parts of the MALDI-TOF mass spectrum. For each oligomer, substructures with mass increments of 16 Da appear, indicating different combinations of various substructures (Please see **Table 1**).

Table 2. Zoosporicidal Activity of *Lannea coromandelica* Extract and Two Commercial Plant Polyflavonoid Tannins against *Aphanomyces cochlioides*

		zoosporicidal activity (% ± SE)[a]							
		15 min		30 min		45 min		60 min	
name of tannins/extract	dose (μg/mL)	halted	burst	halted	burst	halted	burst	halted	burst
Lannea extract	0.1	10 ± 3	0 ± 0	19 ± 8	2 ± 0	22 ± 4	5 ± 3	25 ± 7	7 ± 4
	0.5	36 ± 4	5 ± 3	48 ± 7	14 ± 5	55 ± 3	18 ± 6	68 ± 8	28 ± 5
	5.0	80 ± 8	16 ± 4	88 ± 7	21 ± 8	95 ± 5	39 ± 4	100 ± 0	57 ± 8
	50.0	100 ± 0	42 ± 6	100 ± 0	68 ± 4	100 ± 0	71 ± 10	100 ± 0	89 ± 7
Quebracho	0.1	2 ± 0	0 ± 0	4 ± 0	0 ± 0	6 ± 2	0 ± 0	10 ± 4	0 ± 0
	0.5	12 ± 6	0 ± 0	26 ± 5	5 ± 2	31 ± 4	7 ± 2	40 ± 7	10 ± 2
	5.0	47 ± 4	9 ± 5	63 ± 3	18 ± 7	75 ± 8	22 ± 5	79 ± 7	35 ± 6
	50.0	96 ± 3	26 ± 10	100 ± 0	46 ± 8	100 ± 0	51 ± 8	100 ± 0	60 ± 5
Mimosa	0.1	4 ± 0	0 ± 0	5 ± 0	0 ± 0	4 ± 0	0 ± 0	9 ± 2	0 ± 0
	0.5	15 ± 6	0 ± 0	19 ± 2	2 ± 1	36 ± 7	10 ± 5	45 ± 5	15 ± 2
	5.0	61 ± 9	12 ± 4	70 ± 8	19 ± 4	78 ± 2	30 ± 4	81 ± 5	39 ± 9
	50.0	77 ± 4	23 ± 7	82 ± 11	47 ± 4	98 ± 2	66 ± 6	100 ± 0	71 ± 4
control		3 ± 1	0 ± 0	5 ± 2	0 ± 0	6 ± 1	0 ± 0	9 ± 3	0 ± 0

[a] Data presented here are the average value ± SE of at least three replications in each dose of polyflavonoid tannin. Both Mimosa and Quebracho caused granulation or deformation of almost all rounded cysts especially at higher concentration (>5.0 ppm). Some of the pre-encysted spores were not burst completely but became enlarged and they did not germinate or regenerate up to 24 h. SE = standard error.

and lysis activities and supported our assumption that the active principle in *L. coromandelica* extract is also a polyflavonoid tannin.

Characterization of *Lannea coromandelica* Polyflavonoid Tannin by MALDI-TOF-MS. Recently, MALDI-TOF mass spectrometry has successfully been used in determination of aspects of the structure and characteristics of the polyflavonoid tannins, which are too difficult to determine by other techniques (*22*). We applied this new method to determine the structural features of *Lannea* tannin along with a known commercial Quebracho tannin (*22*). The Quebracho tannin gave clear spectrum showing the degree of polymerization of the building units and oligomer series with masses of the repeat units of 272.3 and 288.3 Da (*22*). The predominant repeat units in this tannin are 272 Da, indicating that this tannin is predominantly consisting of profisetinidin (data not shown). The flavonoid repeating units present in the polyflavonoid tannins could be A, B, C, and D having masses of 258.3, 274.3, 290.3, and 306.3 Da, respectively (**Figure 1**). Combinations of these masses can be used to calculate the masses of the profisetinidin/prorobinetinidin type of polyflavonoid tannin oligomer peaks in the spectra according to the expression, $M + Na^+ = 23.0$ (Na) + 2.0 (endgroups, 2×H) + k(256.3A) + l(272.3B) + m(288.3C) + n(304.3D) (k, l, m, n are integral numbers) (**Table 1**) (*22*).

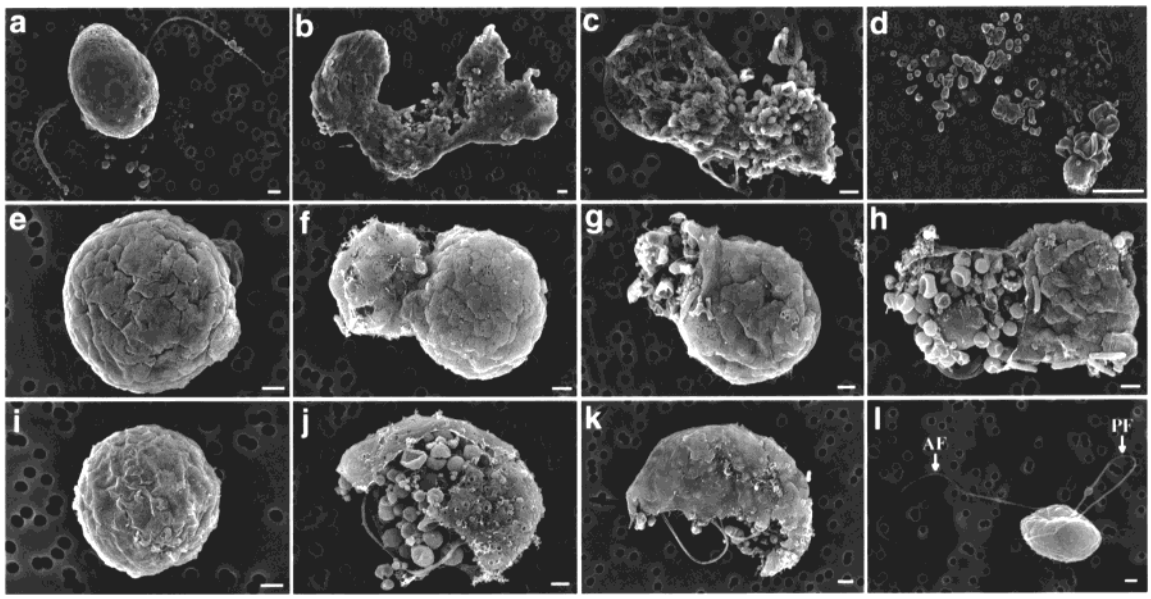

Figure 3. Scanning electron micrographs showing zoosporicidal activity of *L. coromandelica* extracts (5 μg/mL) (**a**–**d**) and two commercial polyflavonoid tannins (10 μg/mL) (**e**–**h**, Quebracho; **i**–**k**, Mimosa) toward zoospores of the *A. cochlioides*. (**a**) fine structures of the flagella of a zoospore are precipitated (5 min), (**b**) a zoospore burst after fragmentation of cellular materials (30 min), (**c**) a lysed zoospore (30 min), (**d**) characteristic fragments of a lysed zoospore (60 min), (**e**–**h**) characteristic zoosporicidal effects of Quebracho tannin (**e**, 10 min; **f**–**h**, 30 min), (**i**–**k**) typical zoosporicidal effects of Mimosa tannins (**i**, 10 min; **j**, **k**, 30 min), (**l**) a bi-flagellated (AF, anterior flagellum; PF, posterior flagellum) zoospore (control). Scale bars, **a**–**l** all 1 μm except d, 10 μm.

As can be seen in the spectra, there are more peak series, which are due to different endgroups. They have the same repeat units, for example, 586−314 and 1450−1178 Da in **Figure 2**.

MALDI-TOF analysis of *Lannea* extracts gave clear spectra exhibiting the degree of polymerization of the building units and oligomer series with masses of the repeat units of 256.3, 272.3, 288.3, and 304.3 Da (**Figure 2** and **Table 1**). For each oligomer, subsets of spectra with mass increments of 16 Da appear, suggesting different combinations of various substructures (*22*). The MALDI-TOF analysis also indicates the presence in the tannin of oligomers to the maximum of nonamer (2506.3 Da). **Table 1** indicates that many valid combinations of different repeating units are possible.

The MALDI-TOF analysis shows the existence of possible fragments of angular tannins by the presence of intense peaks at 904.1, 1178.3, 1194.3, and 1211.5 Da in *Lannea* tannin extract (**Figure 2**). All these fragments were found in Mimosa tannin and suggested to be indicative of angular tannin (*22*). Thus, the *Lannea* tannin is an angular-type one which is partly similar to Mimosa but rather different from Quebracho (*22*). The predominant repeat units in *Lannea* tannin are 288 Da, indicating it to be predominant prorobinetinidin-type polyflavonoid tannin. Although the presence of a high proportion of phlobatannin in *L. coromandelica* stem bark has been reported earlier (*18*), so far, report on the structural characterization of *Lannea* tannin has not been published. Therefore, it is the first report on the structural features of the polyflavonoid tannin present in *L. coromandelica*.

Motility Inhibition and Lytic Activities of Polyflavonoid Tannins against Zoospores. Light microscopic observation revealed that the *Lannea* extract and commercial ploy flavonoid tannins show zoosporicidal activity against *A. cochlioides* almost in a similar manner. In all cases, initially zoospores were halted and the cellular materials rapidly fragmented and formed globular structures. These globular structures achieved Brownian movement and finally dispersed into the surrounding water medium by bursting the cell membranes within 60 min. The time-course motility inhibition and lytic activities of *Lannea* and commercial polyflavonoid tannins are presented in **Table 2**. Apparently, both *Lannea* extracts and commercial tannins caused motility inhibition followed by lysis of zoospores in a dose-dependent manner at a range of 0.1−50 μg/mL concentration (**Table 2**). Among the three extracts, *Lannea* showed the higher halting and lytic activity (MIC 0.1 μg/mL) than the two commercial tannins extracts (both MIC 0.5 μg/mL). Both halting and lytic activity was increased with time and the highest activity was achieved within 60 min of treatment. When previously encysted spores (cystospores) were exposed to 5 μg/mL of *Lannea* or commercial tannin extracts, they were deformed and did not germinate or regenerate zoospores even after 12 h, seemingly being killed (data not shown). Methylation or acetylation of *Lannea* extracts yielded completely nonactive products (inactive at 100 μg/mL) indicating that hydroxyl groups in the polyflavonoid tannin may be essential for motility inhibition and lysis activity.

Previously, one natural product avenacin (a saponin) from the oat roots also exhibited motility inhibition followed by lysis of some oomycetes zoospores, but the active mechanism of that compound is yet to be known (*25*). Recently, we found that nicotinamide from a nonhost *Amaranthus gangeticus* showed potent motility-inhibiting activity against *A. cochlioides* zoospores (*23*). Interestingly, nicotinamide induced halted zoospores were encysted and then regenerated zoospores instead of germination or lysis. Motility-inhibiting activity of zoospores was also observed by the interactions of two chemically different factors isolated from another nonhost *Portulaca oleracea*, where all halted zoospores were first encysted and then germinated (*26*).

Morphological Changes of Zoospores Interact with Polyflavonoid Tannins. To get more insights of the zoosporicidal activity of *Lannea* and commercial tannin extracts, we studied the morphological changes of zoospores by scanning electron microscopy (SEM) (**Figure 3**). Time-course SEM observation revealed that both *Lannea* extract and commercial polyflavonoid tannins caused lysis of zoospores in a similar manner (**Figure**

3a−**k**). Apparently, tannin extracts first attacked the tripartite tubular hairs (TTHs) (characteristic hairy structures responsible for swimming) of the anterior flagellum as well as the fine structures of the posterior flagellum (responsible for swimming) (*15*). The tannins reacted with TTHs and precipitated them within 5 min (**Figure 3a**). Thus, zoospores became paralyzed and rapidly halted. The surface of the affected zoospores became relatively smooth and rounded (**Figure 3e, i**) and rapidly burst (**Figure 3b, f**−**h, j**). The inner cellular materials of the spores fragmented and formed unique globular structures and soon dispersed into surrounding medium within 30 min (**Figure 3c, d, h, j**). The morphological changes (fragmentation of cellular materials and formation of unique structures) of zoospores by polyflavonoid tannins observed in this experiment are similar to the characteristic features of apoptosis (*27*). Recently, gallotannin was found to induce apoptosis in a human colon cancer line (T-84) at 10 μg/mL (*28*). The preformed cystospores were also affected by the extract where they completely deformed and cracked down but no clear fragmentation of cellular materials was observed (photomicrgraph not shown). The fate of zoospores interacting with commercial tannins was identical to *Lannea* extracts indicating that the same active mechanism may be involved in both cases.

Tannins are secondary metabolites distributed widely in the plant kingdom, which have been closely associated with plant defense mechanisms toward phytopathogens, insects, and mammalian herbivores. Recently, direct anthelmintic effects of condensed tannins toward different gastrointestinal nematodes of sheep have been demonstrated (*29*). Kiuchi et al. (1988) also found that tannins, both condensed and hydrolyzable, caused bursting of the second-stage larvae of dog roundworm (*Toxocara canis*) (*30*). The characteristic zoosporocidal activity of *Lannea* and other two commercial polyflavonoid tannins shown in this paper has not been reported for any other oomycete zoospores.

In conclusion, polyflavonoid tannins from *L. coromandelica* and commercial sources have motility-inhibitory and unique lytic effects in vitro against the zoospores of a phytopathogenic oomycete *A. cochlioides*. Plant polyflavonoid tannins could be useful for managing the infestation of crops by the notorious oomycete phytopathogens. Further studies on the zoosporicidal mode of action of polyflavonoid tannins and their effects on other phytopathogenic oomycetes are needed for considering their practical use as a naturally occurring oomicidal agent.

ABBREVIATIONS USED

MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MIC, minimum inhibitory concentration; SEM, scanning electron microscopy; CC, column chromatography.

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