JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Dietary Toxicity of Decomposed Arborescent Leaf Litter against Larval Mosquito: Involvement of a Lignin–Polypeptidic Complex

MATHIEU TILQUIN, JEAN-CLAUDE MEYRAN,* AND GÉRARD MARIGO

Laboratoire "Ecosystèmes et Changements Environnementaux", EA 3112, Centre de Biologie Alpine, Université Joseph Fourier, BP 53, F-38041 Grenoble Cedex 9, France

To characterize the toxic compounds involved in the dietary toxicity of decomposed arborescent leaf litter against larval mosquito, a toxic fraction was extracted from crude leaf litter by using hot water. Preliminary characterization of this fraction, called the insoluble fraction (IF) because it progressively precipitates after extraction, has suggested the involvement of lignin-like compounds in the toxicity. Further analyzes are currently being performed by using additional phytotoxicity-based methods. The involvement of lignin-like compounds in the toxicity was indicated by both the comparative effects of different enzymatic oxidative treatments and reversed-phase high-performance liquid chromatography analysis of the phenolic aldehydes and acids obtained after alkaline nitrobenzene oxidation. However, these lignin-like compounds may not be involved alone in the toxicity, as no specific feature of those components was associated with the toxicity. Among the possible compounds associated with lignin-like compounds in the toxicity, peptidic compounds were suggested by comparative determination of the C/N ratio and then revealed by denaturation experiments, use of specific binding protein molecules, and thin-layer chromatography analysis. A possible role of these peptidic compounds associated with lignin-like compounds in the dietary toxicity of the leaf litter against the larval mosquito midgut is discussed.

KEYWORDS: Leaf litter; larvicidal activity; lignin-like compounds; peptides; denaturation; HPLC; thin-layer chromatography; bioassays; *Aedes aegypti*

INTRODUCTION

Whereas the phytoxicity of green leaves against terrestrial herbivorous Arthropoda has been extensively investigated (*1*), toxicological interactions between leaf detritus and arthropodan fauna are poorly documented. This is true in freshwater ecosystems, where decomposed leaf litter is a main food source for many detritivorous larval insects (*2*).

These dietary interactions are particularly important in subalpine mosquito breeding sites, where the leaf litter originating from environmental vegetation is involved in ecological discrimination among mosquito species through differential toxicity against detritivorous larvae (*3*). Such larvicidal effects were evidenced against the midgut epithelium of sensitive species after experimental dietary use of decomposed arborescent leaf litter (*4*, *5*). These deleterious effects are comparable to those obtained after intoxication with the bacterio-insecticide *Bacillus thuringiensis* var. *israelensis* (*6*). This strong dietary toxicity was attested by the very small amount of leaf litter ingested per larva to reach lethality (*7*). The use of dietary decomposed arborescent leaf litter was thus proposed as a new larvicidal strategy against anthropophilic vector-competent mosquitos that are resistant to conventional insecticides (*8*). Operational use of such a natural toxic food source may be facilitated by its insolubility in water and its attractiveness for the larvae (*9*).

Leaf litter larvicidal compounds were first recognized as insoluble polyphenols included within the cell-wall fraction of a crude decomposed matrix (*7*). They were then hypothesized as lignin-like compounds (*10*). Partial hot water extraction of the toxic compounds from crude material allowed us to significantly concentrate the toxicity into an insoluble toxic fraction (IF). Preliminary characterization confirmed the water insolubility of the toxicity as well as the involvement of lignin-like compounds (*11*).

Additional phytotoxicity-based investigations are needed in order to further characterize the toxic fraction and its involvement in the dietary toxicity against the mosquito larval midgut epithelium. Therefore, this fraction was further examined by using new phytochemical tools monitored by toxicological checking.

MATERIALS AND METHODS

Plant and Insect Materials. Poplar leaf litter samples were selected within the whole old leaf litter layer (*13*) collected from five standardized plots (50 × 50 cm) after 10 months of decay in two

* To whom correspondence should be addressed. Telephone: (33) 4 76 51 44 59. Fax: (33) 4 76 51 44 63. E-mail: jcmeyran@ujf-grenoble.fr.

10.1021/jf0205155 CCC: $22.00
Published on Web 09/17/2002

different woodland sites, submitted to periodic flooding, in the Rhône-Alpes region (France). These sites were known to produce toxic or nontoxic leaf litter (*12*). Samples were air-dried, reduced in 0.5 mm mesh homogeneous powder, and then preserved deep-frozen until use. Preliminary toxicological checking, using the standard method of David et al. on larval *Aedes aegypti* (*4*, 5, *10*), revealed a strong larvicidal effect in the samples originating from one site (thus called toxic leaf litter) and no toxicity in the samples originating from the other site (thus called nontoxic leaf litter) (data not shown).

Toxic and nontoxic leaf litter materials were then comparatively used under two different forms. Standard lignin fraction (SLF) samples were obtained by sequential extraction of 70 mg of crude leaf litter with water, absolute ethanol, acetone, benzene, and Triton X-100 according to the procedure of Alibert et al. (*14*), as modified by David et al. (*10*). SLF was used for lignin monomeric analysis. Standard IF samples were obtained from 70 mg of crude leaf litter according to the procedure of Tilquin et al. (*11*). Briefly, crude leaf litter samples were stirred in 15 mL of distilled water maintained at 60 °C for 1 h. The IF, which precipitated within the aqueous extract during 24 h of cooling, was then recovered by centrifugation (5 min at 10 000 rpm). Each standard IF sample thus obtained was then used for an additional biochemical and toxicological characterization.

Toxicity controls of extracted and treated IF samples were comparatively performed on *A. aegypti* L. fourth instar larvae (Bora-Bora strain) raised in our laboratory (*15*) following the procedure of David et al. (*10*), as modified by Tilquin et *al. (11*). Larval *A. aegypti* was chosen as the standard because of its continuous availability during the whole year and its standardized tolerance to toxic leaf litter, in contrast to autochthonous field larval populations (*10*). After experimental treatments, the IF samples were collected by centrifugation (5 min at 10 000 rpm) and then rinsed three times with 1 mL of distilled water. Bioassays were then conducted at 25 °C on homogeneous samples including 20 same-sized larvae, in glass vials containing the IF sample resuspended in 10 mL of distilled water, buffered to pH 7.5. Larval mortality was measured after 4 h of exposure, and the results were corrected eventually by Abbott's formula (*16*). In these conditions, larval mortality was observed neither in tap water (i.e., blank control) nor in the presence of nontoxic IF (i.e., nontoxic control). When the bioassays were carried out in the presence of toxic IF (i.e., toxic control), the mortality ranged from 80 to 100%.

Involvement of Lignin-like Compounds in the Toxicity. The involvement of lignin-like compounds in the toxicity was investigated in two different ways.

Toxic IF samples were submitted to oxidative enzymatic treatments checked by bioassays according to the following protocol: each standard sample was treated with 1 mL of the enzymatic solution for 4 h at 35 °C. The reaction was stopped by centrifugation (5 min at 10 000 rpm), and the IF was water-rinsed three times and then resuspended in 10 mL of distilled water before bioassay. Enzymatic treatments were performed in 50 mM Mes/Tris medium buffered to pH 6, using phenolase (Sigma, 50 units/mL), tyrosinase (Sigma, 125 units/mL), peroxidase (Sigma, 50 units/mL), or the laccase-*N*-hydroxybenzotriazole (HBT) delignifying system (*11*). The peroxidase treatment was performed in the presence of 25 mM H_2O_2. Concurrently, standard nontoxic IF samples were submitted to the same enzymatic treatments as the control experiments.

The monomeric composition of the SLF originating from toxic and nontoxic leaf litter samples was comparatively analyzed by reversed-phase high-performance liquid chromatography (HPLC) after alkaline nitrobenzene oxidation (*17*). Nitrobenzene oxidation yields the major lignin monomeric compounds (i.e., phenolic aldehydes and acids), which are then separated from the whole nitrobenzene extract with diethyl ether, either at pH 8.5 (extraction of phenolic aldehydes) or at pH 2.5 (extraction of phenolic acids). HPLC, which enables qualitative and quantitative characterization of these compounds (*18*), was performed by using a reversed-phase system including a Waters Spherisorb 5 μm ODS-2 column (250 × 4.6 mm i.d., Alltech, Deerfield, IL) equipped with a 5 mm precolumn, a Dionex 4500i gradient pump, a Shimadzu SIL-9A autoinjector, a Shimadzu SPD 6A UV detector, and Shimadzu Workstation Class CR-10 integration software. Aldehydes were identified and quantified using commercial standard solutions.

Table 1. Effects of Oxidation Enzyme Treatments on the Toxicity of the Insoluble Fraction

toxic IF treatment	toxicity (% mortality ± SE[a])
phenolase	87 ± 5
tyrosinase	100
peroxidase	90 ± 3
laccase-HBT	0
control (untreated IF)	92 ± 6

[a] SE, standard errors calculated from triplicate and inferior to 2% are not shown. No significant mortality (>5%) was observed in the controls conducted with nontoxic IF submitted to the same enzymatic treatments.

Involvement of Peptidic Compounds in the Toxicity. The possible involvement of peptidic compounds in the toxicity was investigated by comparison of the C/N ratio between IF and crude leaf litter. This ratio was measured on 5 mg samples run in a CHONS Microanalyser NA 1 500 (Carlo Erba Strumentazione).

The effects on the IF toxicity of protein-denaturing agents and general or specific binding protein molecules were tested. The following protocol was used: a standard toxic IF sample was treated with 1 mL of reagent for 4 h at 35 °C. After centrifugation (10 000 rpm for 5 min), the pellet was water-rinsed three times and resuspended in 10 mL of distilled water before toxicity control. Chemical denaturation was performed with absolute ethanol, 2% sodium dodecyl sulfate (SDS, Sigma), an anionic detergent widely used for protein denaturation, and an aqueous solution (2% w/v) of tannic acid (Prolabo), a NH_2 groups complexant. Protein binding experiments were performed with 5 mM ninhydrin (Prolabo) in distilled water, 1.5 mM dansyl chloride (DC, Sigma) in 0.2% DMSO, 1 mM *N*-ethylmaleimide (NEM, Sigma) in 0.2% DMSO, 1 mM *p*-chloromercuribenzenesulfonic acid (*p*-CMBS, Aldrich) in distilled water, 5 mM diethyl pyrocarbonate (DEPC, Sigma) in distilled water, 0.5 mM diisothiocyanatostilbene disulfonic acid (DIDS, Sigma) in 0.2% DMSO, and 1 mM phenylglyoxal (PG, Sigma) in distilled water.

The presence of peptidic compounds within the toxic IF was characterized by thin-layer chromatography. A standard toxic IF sample, previously extracted from crude leaf litter as indicated above, was submitted again to a hot water treatment (1 h at 50 °C in 30 μL of distilled water) in order to solubilize the toxic components. After centrifugation (10 000 rpm for 5 min), the hot aqueous extract was laid on a reversed-phase silica chromatographic support (silice RP 18 F 254, sds, France). The plate was eluted with SDS (2% in water) and stained by 0.1% ninhydrin (Merck) in absolute ethanol.

RESULTS

Involvement of Lignin-like Compounds in the Toxicity. Among the different oxidation enzymatic treatments performed on the IF (**Table 1**), only the laccase−HBT delignifying system, which is a strong lignin-degrading agent, appeared to be able to affect significantly the toxicity of this fraction. Phenolase, tyrosinase, and peroxidase, which oxidize only the OH phenolic groups, did not alter the IF toxicity. Control experiments, carried out on standard nontoxic IF samples submitted to the same enzymatic treatments, did not reveal any significant toxicity (i.e., >5% mortality) (not shown).

Lignin-like constituents were also characterized in the SLF after alkaline nitrobenzene oxidation, which yielded both phenolic aldehydes (**Table 2**) and acids (data not shown). However, comparison of the monomeric composition between toxic and nontoxic SLF revealed no significant difference in their aldehyde content (**Table 2**). In both cases, vanillin appeared as the major constituent, and *p*-hydroxybenzaldehyde and syringaldehyde were found at very similar values. In the same

Table 2. Benzoic Aldehydes Composition of Toxic and Nontoxic Standard Lignin Fractions after Alkaline Nitrobenzene Oxidation

	benzoic aldehydes[a] (mg/g of dry SLF ± SE[b])		
	p-HB	V	S
toxic SLF[c]	1.32 ± 0.33	5.57 ± 0.35	1.70 ± 0.35
nontoxic SLF	1.30 ± 0.14	5.65 ± 3.18	1.45 ± 0.92

[a] *p*-HB, *p*-hydroxybenzaldehyde; V, vanillin; S, syringaldehyde. [b] SE, standard errors calculated from triplicate. [c] SLF, standard lignin fraction.

Table 3. C/N Ratio Measured on Samples of Crude Leaf Litter and Insoluble Fraction

	C/N ratio ± SE[a]	
	toxic samples	nontoxic samples
crude leaf litter	18.6 ± 0.2	16.8 ± 0.3
IF[b]	4.3 ± 0.3	3.9 ± 0.1

[a] SE, standard errors calculated from triplicate. [b] IF, insoluble fraction.

way, the HPLC profiles of phenolic acids within the toxic and nontoxic SLF appeared to be nearly identical (not shown). The peaks observed in both toxic and nontoxic SLF profiles occurred with similar retention times, indicating the presence of the same compounds in both extracts. Quantitatively (i.e., in terms of absorbance, which reflects the concentration of each compound), the differences observed between the two profiles remained slight and were not reproducible. As no difference was observed between profiles of toxic and nontoxic samples, the involvement of lignin-like compounds alone in the toxicity appeared to be improbable.

Involvement of Peptidic Compounds in the Toxicity. The C/N ratio appeared far higher in both toxic and nontoxic crude leaf litter than in the corresponding IFs (**Table 3**), with N percentages shifting from around 3% in the crude leaf material to around 11% in the IF of both toxic and nontoxic samples (not shown). This preferential enrichment of nitrogen compounds from the crude material to the IF together with the toxicity increase from one fraction to the other (see above) suggested the involvement of some peptidic forms in the toxicity. Such an occurrence of peptidic compounds within the toxic IF was evidenced by general protein-denaturing agents (e.g., absolute ethanol, tannic acid, and SDS), which appeared to alter strongly the toxicity (**Table 4**). Moreover, when the toxic IF was treated by general binding protein molecules such as DC and ninhydrin, which are able to fix amino and carboxyl groups of proteins, around 65% toxicity loss was observed.

However, the differential results obtained with more specific binding protein molecules (i.e., those able to bind particular amino acids) indicated the involvement of specific proteins in the toxicity (**Table 4**). Interestingly, treatment with DIDS, a specific amino acid binding molecule targeting lysine, led to a complete toxicity loss, whereas no obvious alteration occurred with NEM and *p*-CMBS, which are SH groups binding molecules, nor with DEPC and PG, which target histidine and arginine, respectively. No larval mortality was observed when control experiments were carried out with nontoxic IF submitted to the same treatments (not shown).

Five peptidic compounds extracted from the toxic IF were evidenced by thin-layer chromatography (**Figure 1a**). Those fragments were separated only after migration in SDS; no migration occurred with the usual solvents such as water and water/ethanol (20/80 v/v) or any migration buffer adjusted to pH 7. The peptidic nature of the compounds was attested to by

Table 4. Effects of Denaturing Agents and Binding Protein Molecules on the Toxicity of the Insoluble Fraction

	treatment[a]	toxicity (% mortality ± SE[b])
denaturing agents	absolute ethanol	22 ± 6
	tannic acid	27 ± 4
	SDS	0
binding protein molecules	ninhydrin	30 ± 10
	DC	35 ± 8
	NEM	97 ± 3
	p-CMBS	88
	DEPC	90 ± 5
	DIDS	0
	PG	92
control (untreated IF)		95 ± 3

[a] SDS, sodium dodecyl sulfate; DC, dansyl chloride; NEM, *N*-ethyl maleimide; *p*-CMBS, *p*-chloromercuribenzene sulfonic acid; DEPC, diethylpyrocarbonate; DIDS, diisothiocyanatostilbene disulfonic acid; PG, phenyl glyoxal. [b] SE, standard errors calculated from triplicate are not shown when less than 2%. No significant mortality (>5%) was observed in the controls conducted with nontoxic IF submitted to the same denaturing treatments.

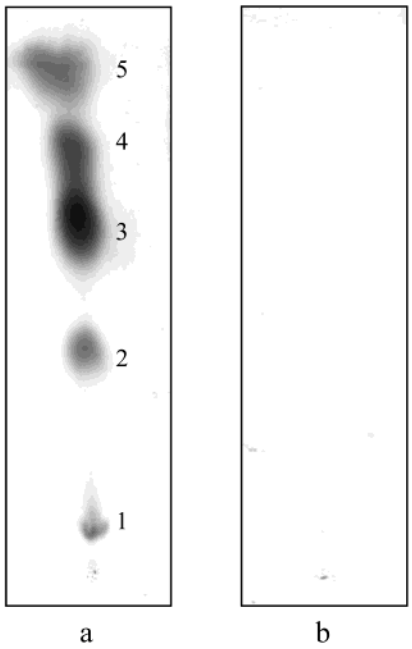

Figure 1. Chromatographic profiles of the peptidic forms within the toxic insoluble fraction (a) before and (b) after the action of the laccase-HBT delignifying system. Spots 1–5 correspond to the peptidic forms revealed after miration in SDS and ninhydrine staining.

their intense appearance after ninhydrin staining. Concurrently, the total disappearance of these spots after digestion of the IF with the laccase-HBT delignifying system (**Figure 1b**) suggested that these peptidic compounds may be bound to lignin-like compounds and, thus, involved in the IF toxicity.

DISCUSSION

The toxic IF extracted by the hot water procedure from decomposed arborescent leaf litter was recognized previously as a lignin-like fraction (*11*). Further investigation of the IF currently suggests that this toxicity may be due to the association between lignin-like compounds and polypeptidic structures.

Involvement of Lignins-like Compounds in the Toxicity. Our preliminary characterizations, based upon purification of toxic crude leaf litter, suggested the involvement of lignin-like compounds, first indirectly among the components of a purified

cell wall fraction (*7*), and then more directly within a hot-water-extracted IF (*11*). This IF, far more toxic than crude leaf litter, appeared to include lignin-like components with monomers of *p*-hydroxybenzaldehyde, as shown indirectly after alkaline nitrobenzene oxidation (*11*). The involvement of lignin forms in the IF toxicity is demonstrated further by our comparative oxidative enzymatic treatments checked by bioassays. Whereas phenolase, tyrosinase, and peroxidase, which only oxidize the OH phenolic groups into the corresponding quinones, did not alter the IF toxicity, the laccase-HBT delignifying system, which fragments the lignins themselves (*19*), is strongly efficient against the IF toxicity. This indicates that those lignin forms would be involved through their own structural features rather than in terms of phenolic activity. This is in contrast to numerous cases of known dietary phytotoxicity which are monitored only by phenolic activity (*20*).

Such an involvement of lignin-like compounds in the toxicity of decomposed leaf litter, associated with the necessity of a 10-month-long decaying process to generate the toxicity in nature (*7*, *10*), has suggested the hypothesis of complex biotransformation of nontoxic forms from green leaves into toxic ones in leaf detritus (*11*). As no significant difference appears in the lignin monomeric compositions of toxic and nontoxic leaf litter, it has been assumed that no difference would occur in the corresponding purified IF. Thus, the toxicity was supposed not to be exclusively related to the structure of the lignin-like compounds.

Involvement of Peptidic Compounds Associated with Lignin-like Compounds in the Toxicity. Although the same nitrogen enrichment was observed after hot water extraction of the IF from either toxic or nontoxic crude material, the involvement of peptidic compounds in the toxicity appears to be effective. This is clearly evidenced by the efficiency of general protein denaturing agents and binding protein molecules. However, some particular peptidic forms should be involved in the toxicity, as suggested by the discriminating action of specific binding protein molecules. Whereas the strong inhibiting effect of DIDS suggests the involvement of lysine residues in the toxicity, the ineffectiveness of some other tested effectors must be interpreted with caution. Many factors, particularly the compact three-dimensional structure of the proteins, can interfere with and prevent these molecules from accessing their target site (*21*). The presence of polypeptidic compounds in the toxic IF is also attested to by their direct chromatographic characterization using ninhydrin. As ninhydrin is strongly efficient against the IF toxicity when used as a protein effector, its use is of interest for revelation of the toxic polypeptides. Moreover, the association between those polypeptides and lignin-like compounds in the toxic IF is clearly evidenced by the action of the laccase on our chromatographic profiles.

The occurrence of peptidic compounds associated with lignin-like compounds in the IF is not surprising since such associations are common in the vegetable cell wall fraction (*22*). In particular, some glycoproteins such as peroxidases and oxidases are known to be cross-linked to lignin while catalyzing its deposition (*23*). Moreover, such peptidic compounds may play a key role in the toxicity of the dietary leaf litter which is exerted against the midgut epithelium (*4*, *7*). These peptidic compounds may constitute the binding sites of the toxic lignin−polypeptide complexes against membrane receptors of the midgut epithelium, following a general pattern observed, for example, in the dietary toxicity of bacterio-insecticides such as *Bacillus thuringiensis* (*24*) and *Bacillus sphaericus* (*25*). Moreover, the association between lignin-like and peptidic compounds appears to be conditional in the effectiveness of the toxicity. This is suggested by the effect of the laccase-HBT delignifying system which, while fragmenting lignins through drastic radical-based oxidative reactions (*26*), may prevent the lignin−peptide binding, thereby canceling the toxicity. Lignin forms may thus appear as carriers, not only in the expression of the toxicological process itself in the larval midgut, but also during the genesis of the toxicity within the foliar matrix. During the 10-month-long decaying process in the water of the breeding sites (*7*, *10*), numerous complex molecular recombinations and disappearances may occur among the different compounds of the cell wall, including lignin and polypeptidic compounds. The persistence of such polypeptidic compounds in the fully decomposed leaf litter may be due to their strong binding to lignins, which are the most stable cell wall components, because of their resistance to the main biotic and abiotic processes involved in leaf litter processing (*27*).

Further investigations, however, are necessary to better characterize the polypeptidic fraction, which could have a key involvement in the toxicity against the mosquito larval midgut epithelium.

ACKNOWLEDGMENT

We thank A. B. Failloux (Institut Pasteur, Paris) for supplying *A. aegypti* strain, M. Asther (Faculté des Sciences, Luminy) for supplying highly purified laccase, and J. Patouraux and G. Girard for technical assistance.

LITERATURE CITED

(1) Bernays, E. A.; Chapman, R. F. Plant secondary compounds and grasshoppers: Beyond plant defenses. *J. Chem. Ecol.* **2000**, *26*, 1773−1794.

(2) Yanoviak, S. P. Effects of leaf litter species on macroinvertebrate community properties and mosquito yield in Neotropical tree hole microcosms. *Oecologia* **1999**, *120*, 147−155.

(3) Rey, D.; Martins, D.; David, J. P.; Pautou, M. P.; Long, A.; Marigo, G.; Meyran, J. C. Role of vegetable tannins in habitat selection among mosquito communities from the Alpine hydrosystems. *C. R. Acad. Sci. Paris, Sci. Vie* **2000**, *323*, 1−8.

(4) David, J. P.; Rey, D.; Pautou, M. P.; Meyran, J. C. Differential toxicity of environmental vegetation on some aquatic dipteran larvae of mosquito breeding sites. *J. Invertebr. Pathol.* **2000**, *75*, 9−18.

(5) David, J. P.; Rey, D.; Cuany, A.; Amichot, M.; Meyran, J. C. Comparative ability to detoxify alder leaf litter among different mosquito ecotypes. *Arch. Insect Biochem. Physiol.* **2000**, *44*, 143−150.

(6) Rey, D.; Long, A.; Pautou, M. P.; Meyran, J. C. Comparative histopathology of *Bacillus thuringiensis* var. *israelensis* on some dipteran larvae and Crustacea of aquatic hydrosystems. *Entomol. Exp. Appl.* **1998**, *8*, 255−263.

(7) David, J. P.; Rey, D.; Marigo, G.; Meyran, J. C. Larvicidal effect of a cell-wall fraction isolated from alder decaying leaves. *J. Chem. Ecol.* **2000**, *26*, 901−913.

(8) Rey, D.; David, J. P.; Besnard, G.; Jullien, J. L.; Meyran, J. C. Comparative sensitivity of larval mosquitoes to vegetable phenolics versus conventional insecticides. *Entomol. Exp. Appl.* **2001**, *98*, 361−367.

(9) David, J. P.; Ferran, A.; Gambier, J.; Meyran, J. C. Leaf litter feeding pattern of *Aedes* larvae in subalpine mosquito breeding sites surrounded by vegetation. *J. Chem. Ecol.* **2002**, *28*, 983−995.

(10) David, J. P.; Rey, D.; Meyran, J. C.; Marigo, G. Involvement of lignin-like compounds in the toxicity of dietary alder leaf litter against mosquito larvae. *J. Chem. Ecol.* **2001**, *27*, 161−174.

(11) Tilquin, M.; Meyran, J. C.; Marigo, G. Hot extraction and characterization of a lignin fraction involved in the larvicidal effects of vegetable leaf litter against mosquito. *J. Chem. Ecol.* **2002**, *28*, 1497−1510.
(12) Rey, D. Un exemple de coévolution plante-arthropode: étude de la phytotoxicité des composés phénoliques de la litière d'*Alnus glutinosa* (Bétulacées) et perspective d'application en démoustication. Thèse, Université Grenoble, 2000.
(13) Green, R. N.; Trowbridge, R. L.; Klinka, K. Towards a taxonomic classification of humus forms. *Forest Sci. Monogr.* **1993**, *29*, 1−49.
(14) Alibert, G.; Marigo, G.; Boudet, A. Recherches sur la biosynthèse des composés aromatiques chez les végétaux supérieurs. Identification des acides phénoliques de *Quercus pedunculata* Ehrh. *Physiol. Vég.* **1969**, *7*, 57−68.
(15) Rey, D.; Pautou, M. P.; Meyran, J. C. Histopathological effects of tannins on the midgut epithelium of aquatic Diptera larvae. *J. Invertebr. Pathol.* **1999**, *73*, 173−181.
(16) Abbott, W. S. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **1925**, *18*, 265−267.
(17) Hartley, R. D. Improved methods for the estimation by gas−liquid chromatography of lignin degradation products from plants. *J. Chromatogr.* **1970**, *54*, 335−344.
(18) Villeneuve, F.; Abravanel, G.; Moutounet, M.; Alibert, G. General scheme of analysis of phenolic compounds in plant extracts by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **1982**, *234*, 131−140.
(19) Sealey, J.; Ragauskas, A. J. Investigation of laccase/N-hydroxybenzotriazole delignification of kraft pulp. *J. Wood Chem. Technol.* **1998**, *18*, 403−416.
(20) Barbehenn, R. V.; Martin, M. M. Tannin sensitivity in larvae of *Malacosoma disstria* (Lepidoptera): Role of the peritrophic envelope and midgut oxidation. *J. Chem. Ecol.* **1994**, *20*, 1985−2001.
(21) Pelmont, J. Marquage de site, inhibition, covalence. In *Enzymes: Catalyzeurs du Monde Vivant*; Bornarel, J., Ed.; Presses Universitaires: Grenoble, 1995; pp 589−617.
(22) Wallace, G.; Fry, C. Phenolic components of the plant cell wall. *Int. Rev. Cytol.* **1994**, *151*, 229−267.
(23) McDougall, G. J. Cell-wall proteins from Sitka spruce xylem are selectively insolubilised during formation of dehydrogenation polymers of coniferol alcohol. *Phytochemistry* **2001**, *57*, 157−163.
(24) Schnepf, E.; Crickmore, N.; Van Rie, J. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 775−806.
(25) Charles, J. F.; Silva-Filha, M. H.; Nielsen-Leroux, C. Mode of action of *Bacillus sphaericus* on mosquito larvae; incidence of resistance. In *Entomophagic Bacteria: From Laboratory to Field Application*; Charles, J. F., Delécluse, A., Nielsen-Leroux, C., Eds.; Kluwer Academic Publishers: Dordrecht, 2000; pp 237−252.
(26) Poppius-Levlin, K.; Wang, W.; Tamminen, T.; Hortling, L.; Viikari, L.; Niku-Paavola, M. L. Effects of laccase/HBT treatment on pulp and lignin structures. *J. Pulp Pap. Sci.* **1999**, *25*, 90−94.
(27) Criquet, S.; Farnet, A. M.; Tagger, S.; Le Petit, J. Annual variations of phenoloxidase activities in an evergreen oak litter: influence of certain biotic and abiotic factors. *Soil Biol. Biochem.* **2000**, *32*, 1505−1513.

Received for review May 6, 2002. Revised manuscript received July 16, 2002. Accepted July 19, 2002. This investigation was supported by a grant from Entente Interdépartementale pour la Démoustication (EID) Rhône-Alpes.

JF0205155