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Amatoxins in wood-rotting *Galerina marginata*

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Abstract: Amatoxins, bicyclic octapeptide derivatives responsible for severe hepatic failure, are present in several Basidiomycota species belonging to four genera, i.e. *Amanita, Conocybe, Galerina* and *Lepiota*. DNA studies for *G. autumnalis, G. marginata, G. oregonensis, G. unicolor* and *G. venenata* (section *Naucoriopsis*) determined that these species are the same, supporting the concept of *Galerina marginata* complex. These mostly lignicolous species are designated as white-rot fungi having a broad host range and capable of degrading both hardwoods and softwoods. Twenty-seven *G. marginata* basidiomes taken from different sites and hosts (three sets) as well as 17 *A. phalloides* specimens (three sets) were collected in French locations. The 44 basidiomes were examined for amatoxins and phallotoxins using high-performance liquid chromatography. Toxinological data for the wood-rotting *G. marginata* and the ectomycorrhizal *A. phalloides* species were compared and statistically analyzed. The acidic and neutral phallotoxins were not detected in any *G. marginata* specimen, whereas the acidic (β-Ama) and neutral (α-Ama and γ-Ama) amanitins were found in all basidiomes from either Angiosperms or Gymnosperms hosts. The *G. marginata* amatoxin content varied from 78.17 to 243.61 $\mu g.mg^{-1}$ of fresh weight and was elevated significantly in one set out of three. The amanitin amounts from certain *Galerina* specimens were higher than those from some *A. phalloides* basidiomes. Relationship between the amanitin distribution and the chemical composition of substrate was underlined and statistically validated for the white-rot *G. marginata*. Changes in nutritional components from decayed host due to enzymatic systems and genetic factors as well as environmental conditions seem to play a determinant role in the amanitin profile. Variability noticed in the amanitin distribution for the white-rot *G. marginata* basidiomes was not observed for the ectomycorrhizal *A. phalloides* specimens.

Key words: amanitins, Basidiomycota, *Galerina*, white-rot fungi

INTRODUCTION

Amatoxins belong to a family of bicyclic octapeptide derivatives composed of an amino acid ring bridged by a sulphur atom and characterized by differences in their side groups (Wieland and Faulstich 1991, Zanotti et al 1992). The main amatoxins, α-, β-, and γ-amanitins inhibit eukaryotic DNA-dependent RNA polymerase II and are hepatotoxic (Faulstich and Wieland 1996, Faulstich and Zilker 1994, Wieland and Faulstich 1983). These toxins first are extracted from the poisonous mushroom *Amanita phalloides* Fr. and then from other *Amanita* species. Smaller amounts also are found in species of other genera, such as *Conocybe, Galerina* and *Lepiota* (Ammirati et al 1985, Block et al 1955, Brady et al 1975, Bresinsky and Besl 1990, Gérault and Girre 1977, Lincoff 1998, Wieland 1986). Phallotoxins are bicyclic heptapeptide derivatives present in *A. phalloides* basidiomes and related species (Wieland 1983, Wieland and Faulstich 1983). Minor quantities of phallotoxins recently were detected in *Conocybe lactea* (J.E. Lange) Métrod (Hallen et al 2003).

The toxicity of certain *Galerina* species is well known. Early in the 20th century, Peck (1912) reported a human poisoning case due to *G. autumnalis*

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(Peck) A.H. Sm. & Singer. Later, Grossman and Malbin (1954) reported a poisoning produced by *G. venenata* A.H. Sm. (Smith 1953). Over two decades, 10 cases caused by amatoxin-containing Galerinas were mentioned: (i) three European cases, two from Finland (Elonen and Härkönen 1978) and one from France (Bauchet 1983) due to *G. marginata* (Batsch) Kühner and *G. unicolor* (Vahl) Singer, respectively; (ii) seven North American exposures, two fatalities from Washington due to *G. venenata* (McKenny and Stuntz 1987) and five cases reacting positively to treatment, four caused by *G. autumnalis* from Michigan (two reports) and Kansas (two reports), respectively (Trestrail 1991, 1994), and one by *Galerina* sp. from Ohio (Trestrail 1992). A clinical analysis of 12 Chinese patients poisoned with *G. autumnalis* also was published (Yin and Yang 1993). Over three decades, 53 Japanese patients poisoned by *G. fasciculata* Hongo, including five fatal cases, were reported (Ishihara and Yamaura 1992). A 6-year-old boy also developed severe hepatic failure after eating a mushroom morphologically identified as *G. fasciculata* (Kaneko et al 2001).

Using thin-layer chromatography, α-amanitin (α-Ama) and β-amanitin (β-Ama) were detected in the fruiting bodies of *G. autumnalis, G. marginata* and *G. venenata* (Tyler and Smith 1963, Tyler et al 1963). Both amanitins were quantified in *G. autumnalis* (1.5 $mg.g^{-1}$ dry weight; Johnson et al 1976) and *G. marginata* (1.1 $mg.g^{-1}$ dry weight; Andary et al 1979). α-Ama and γ-amanitin (γ-Ama) were produced by fermentation from American *G. marginata* (Benedict and Brady 1967). Then, experiments confirmed the occurrence of α-Ama and β-Ama in German basidiomes of *G. autumnalis* and *G. marginata* and revealed the presence of the three amanitins (α-Ama, β-Ama and γ-Ama) in the fruiting-bodies of *G. beinrothii* Bresinsky, *G. sulciceps* (Berk.) Singer and *G. unicolor.* Furthermore, *G. marginata* mycelium could produce the three amanitins whereas *G. beinrothii* and *G. unicolor* mycelia yielded β-Ama and α-Ama, respectively (Besl et al 1984). Recently, α-Ama, β-Ama, and γ-Ama were found in cultured mycelia of *G. fasciculata* and *G. helvoliceps* (Berk. & M.A. Curtis) Singer (Muraoka et al 1999, Muraoka and Shinozawa 2000).

Nine amatoxin-containing *Galerina* species—*G. autumnalis, G. badipes* (Fr.) Kühner, *G. beinrothii, G. fasciculata, G. helvoliceps, G. marginata, G. sulciceps, G. unicolor* and *G. venenata*—are cited in the literature (Ammirati et al 1985, Benjamin 1995, Bessette et al 1997, Bresinsky and Besl 1990, Courtecuisse and Duhem 2000, Enjalbert et al 2002, Kaneko et al 2001, Lincoff 1998, Muraoka et al 1999). All the toxic *Galerina* species belong to section *Naucoriopsis* Kühner included in the genus *Galerina* Earle (Smith and Singer 1964) classified into the family either of *Strophariaceae* (Agaricales; Kühner 1980) or *Cortinariaceae* (Agaricales; Singer 1986, Kirk et al 2001) or *Crepidotaceae* (Cortinariales; Bon 1992, Courtecuisse and Duhem 2000). American species are distributed in three stirpes: (i) stirps *Autumnalis* with *G. autumnalis*; (ii) stirps *Marginata* including *G. marginata, G. helvoliceps* and *G. venenata*; (iii) stirps *Cedretorum* consisted of *G. badipes* and *G. sulciceps* (Singer 1986, Smith and Singer 1964). European toxic species also are classified in these stirpes: (i) stirps *Autumnalis* corresponding to *G. autumnalis* and *G. beinrothii*; (ii) stirps *Marginata* composed of *G. marginata, G. sulciceps* and *G. unicolor* (Bon 1992).

Analyses of rDNA sequences carried out on the 35 European and North American *Galerina* collections belonging to five amatoxin-containing taxa revealed no significant distinctiveness between the species referred to two stirpes, *Marginata* and *Autumnalis*. According to Gulden et al (2001), these analyses support the concept that: (i) the boreal Northern Hemisphere constitutes one mycogeographical region; (ii) the North American and European species—*G. autumnalis, G. marginata, G. unicolor* and *G. venenata*—should be treated as one species named *G. marginata,* leaving *G. badipes* as the other distinct species.

The *Galerina marginata* complex is widespread in the Northern Hemisphere—Europe and North America (Gulden and Vesterholt 1999) and in Asia (Imazeki et al 1987). Macroscopic and microscopic characters of *G. marginata* are described, and its lignicolous habit also is reported (Bon 1990, Gulden et al 2001, Singer 1986).

A review on the distribution of the 1669 wood-rotting fungi from North America reports that: (i) 93% of these species are white-rotters; (ii) the studied brown-rot fungi do not belong to families of *Cortinariaceae* and *Strophariaceae* (Gilbertson 1980). According to the current classifications, *G. marginata* is a species belonging to one of these two families; it is likely that it could be designated as a white-rot Basidiomycota.

To our knowledge, there is no recent study on the toxin content and profile for the wood-rotting *G. marginata.* For this reason, *G. marginata* basidiomes were collected from deciduous and coniferous hosts in French forests and investigated for amatoxins and phallotoxins using high-performance liquid chromatography. Furthermore, toxinological data of the wood-rotting *G. marginata* and the ectomycorrhizal *A. phalloides* were compared and the results were validated by statistical analysis.

MATERIALS AND METHODS

Collection conditions of G. marginata *and* A. phalloides.—Twenty-seven *G. marginata* basidiomes at various stages of development were collected on decayed wood from three locations in two French regions—Alsace-Lorraine and Franche-Comté in Oct 1996 and 1998, respectively. Sixteen specimens were gathered on fallen trunks of beech (*Fagus sylvatica* L.) found in two sites from the Lorraine forest of Languimberg and Sarrebourg (Gal L1: n = 10; Gal L2: n = 6). This forest of beech and hornbeam is on either neutral or calcareous soil (at 450 m). Eleven mushrooms (Gal FC) were collected on moss-covered rotted spruce (*Picea abies* [L.] Karsten) in a mixed pine/spruce/beech wood called the Bois des Tilles (Franche-Comté) at an altitude of 630–675 m on rauracien limestone with pH = 6.8. The three *Galerina* sets are listed in TABLES I and II. The mushrooms were identified on the basis of macro and micromorphological descriptions (Bon 1992, Courtecuisse and Duhem 2000, Gulden and Vesterholt 1999).

Furthermore, 17 *A. phalloides* basidiomes collected from three French locations during the same years were: (i) six specimens from the Alsatian Haguenau forest (Aph A); (ii) three samples gathered from the Languimberg and Sarrebourg forest (Lorraine) (Aph L3); (iii) eight basidiomes (Aph FC) from the Bois Rodolphe situated in Franche-Comté (TABLES I and II).

Analytical procedure.—Each basidiome was weighed, frozen in liquid nitrogen and ground. Amatoxins and phallotoxins were extracted by sonication and separated by reversed-phase liquid chromatography. Identification of both classes of toxins was based on retention times and UV spectral data; the absorbance of the eluate was monitored at 285 nm for the phallotoxins and 305 nm for the amatoxins (Wieland 1986, Wieland and Faulstich 1983). The toxin detection limit for both amatoxins and phallotoxins was 0.5 $ng.g^{-1}$ of fresh fungal material. The concentration of amatoxins (α-Ama, β-Ama and γ-Ama) and phallotoxins (phalloidin, phallisin, phalloin, phallacidin and phallisacin) from the 44 basidiomes (27 Galerinas and 17 Amanitas) was determined by means of analytical procedure as previously reported (Enjalbert et al 1992). The amatoxin content corresponding to the sum of the three amanitin amounts assayed in each basidiome (n = 44) taken from the different sites (n = 6) was expressed in μg of toxin per gram of fresh weight of tissue (F.W.). The amanitin concentrations were expressed as a percentage to determine the toxin profile in the basidiomes of both species *G. marginata* and *A. phalloides*. The ratio β-Ama/(α-Ama + γ-Ama) between the acidic and neutral amanitin amounts was calculated for each specimen. The mean data of the five variables—amatoxin content, β-Ama, α-Ama and γ-Ama concentrations as well as β-Ama/(α-Ama + γ-Ama) ratio—found for the 27 Galerinas (three sets) and the 17 Amanitas (three sets) were analyzed statistically. This data allowed the comparison of amatoxin content and amanitin profile between the wood-rotting and ectomycorhizal species.

Statistical analyses.—The effect of "Origin" factor on the amatoxin content and amanitin distribution in the 27 *G. marginata* basidiomes (Gal L1, Gal L2, Gal FC; site/host factor) and the 17 *A. phalloides* specimens (Aph A, Aph L3, Aph FC; site factor) respectively, was determined by an ANOVA analysis of variance (P^A significance level). Then, one-way ANOVA analysis with combination of two factors (i.e. Species-Origin) allowed comparison between the toxinological data from *G. marginata* and *A. phalloides* (n = 44). Furthermore, the Newman-Keuls multiple range tests were used to compare the toxin distribution between the six sets taken two by two (significance level). $P < 0.05$ indicated a significant effect of the factor. The statistical analysis was carried out using Statgraphics software (SIGMA-PLUS).

RESULTS

Toxin content and amanitin distribution for G. marginata.—The mean weight (g of fresh basidiomes ± sem) for each set of *G. marginata* was of 0.92 ± 0.11 g (from 0.5 to 1.7 g), 2.90 ± 0.66 g (from 1.2 to 5.5 g) and 1.01 ± 0.24 g (from 0.35 to 2.71 g) for Gal L1, Gal L2 and Gal FC, respectively (TABLE I). The acidic (phallacidin and phallisacin) and neutral (phalloidin, phallisin and phalloin) phallotoxins were not present at the toxin HPLC detection limit of 0.5 $ng.g^{-1}$ F.W. in any *G. marginata* basidiome collected on rotten wood from deciduous and coniferous trees during different years. The amatoxin content corresponding to the sum of three amanitins (mean amounts ± sem) for Gal L1, Gal L2 and Gal FC sets was of 243.61 ± 16.54 $\mu g.g^{-1}$, 78.17 ± 10.08 $\mu g.g^{-1}$ and 96.88 ± 12.82 $\mu g.g^{-1}$ F.W., respectively (TABLE I). It was equivalent to 0.024%, 0.008% and 0.009% for Gal L1, Gal L2 and Gal FC, respectively. The statistical analysis by one-way ANOVA showed a significant effect of "Origin" ($P^A < 10^{-4}$) for the three sets of *G. marginata*. The mean amanitin concentrations for the Gal L1 set presented significant difference ($P^{NK} < 10^{-4}$) when compared to nearly equivalent amounts for both sets Gal L2 and Gal FC (TABLE I, FIG. 1).

The mean concentrations of β-Ama, α-Ama and γ-Ama (expressed as percentage) as well as the mean values of β-Ama/(α-Ama + γ-Ama) ratio in the specimens belonging to the three sets, i.e. Gal L1, Gal L2 and Gal FC are listed in TABLE II. The acidic and neutral amanitin distribution in the *G. marginata* species obviously was affected by both site and host (TABLE II, FIG. 2a, b) because significant variation ($P^A < 10^{-4}$) relative to the Origin factor was found. The Newman-Keuls multiple range tests showed a significant difference ($P^{NK} < 10^{-3}$) between the mean concentrations taken two by two for either the three amanitins or the ratios displaying the predominance of acidic and neutral toxins. As regards the Gal L1 and Gal L2 sets, β-Ama and α-Ama were the major toxins but not similarly distributed in both sets. The former,

TABLE 1. Collection, biomass and amatoxin contents for *Galerina marginata* (Gal) and *Amanita phalloides* (Aph) sets

Species	Origin (site/host)	Set name	Sample size n	Weight (g F.W.[c])	Amatoxin content ($\mu g \cdot g^{-1}$ F.W.)
G. marginata	L1[a]/Beech	Gal L1	10	0.92 ± 0.11	243.61 ± 16.54
G. marginata	L2[a]/Beech	Gal L2	6	2.90 ± 0.66	78.17 ± 10.08
G. marginata	FC[a]/Spruce	Gal FC	11	1.01 ± 0.24	96.88 ± 12.82
					P^{A} value[d] $< 10^{-4}$
A. phalloides	A[b]/Mixed wood	Aph A	6	20.81 ± 5.91	367.09 ± 62.38
A. phalloides	L3[b]/Beech	Aph L3	3	32.60 ± 12.74	172.07 ± 56.32
A. phalloides	FC[b]/Mixed wood	Aph FC	8	18.42 ± 4.78	247.87 ± 14.67
					P^{A} value[d] = 0.04

[a] L1, L2 = Lorraine (Forest of Languimberg and Sarrebourg, France), FC = Franche-Comté (Bois de Tilles, France).
[b] A = Alsace (Forest of Haguenau, France): *Carpinus betulus* L., *Quercus robur* L., L3 = Lorraine (Forest of Languimberg and Sarrebourg, France), FC = Franche-Comté (Bois Rodolphe, France): *Fagus sylvatica* L., *Carpinus betulus*, *Quercus robur*.
[c] Fresh Weight (mean ± sem).
[d] P^{A} value: level of significance of ANOVA. P^{A} value $< 10^{-4}$ between Gal L1, Gal L2 and Gal FC sets; P^{A} value = 0.04 between Aph A, Aph L3 and Aph FC sets.

distinguishable by a high toxin content (243.61 ± 16.54 $\mu g.g^{-1}$), displayed: (i) an elevated mean concentration of β-Ama (58.69 ± 1.09%); (ii) a small mean concentration of γ-Ama (2.34 ± 0.15%); (iii) a $\beta/(\alpha + \gamma)$ ratio of 1.44 ± 0.07 (from 1.21 to 1.38). The latter was characterized by: (i) β-Ama and α-Ama mean concentrations almost identical; (ii) a high mean γ-Ama concentration (9.47 ± 0.33%); (iii) a reversed $\beta/(\alpha + \gamma)$ ratio 0.76 ± 0.05 (from 0.61 to 0.87) (TABLE II, FIG. 2a, b). An elevated mean percentage of neutral toxins (α-Ama = 63.43 ± 2.39%, γ-Ama = 13.44 ± 1.08%) and a small value of $\beta/(\alpha + \gamma)$ ratio equivalent to 0.31 ± 0.03 (from 0.20 to 0.52) differentiated the Gal FC set (TABLE II, FIG. 2a, b).

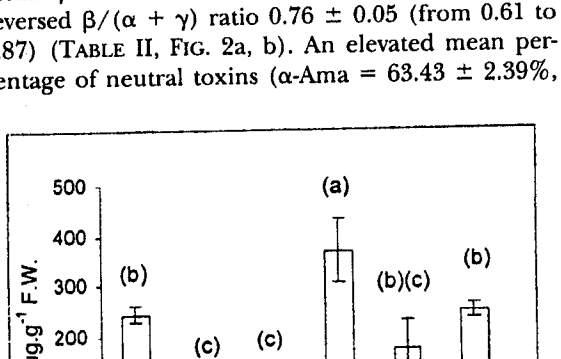

FIG. 1. Amatoxin contents in *Galerina marginata* and *Amanita phalloides* sets ($\mu g.g^{-1}$ Fresh Weight, brackets indicate sem).
Gal L1, Gal L2, Gal FC: *Galerina marginata* set names (Gal) from Lorraine (L1, L2) and Franche-Comté (FC).
Aph A, Aph L3, Aph FC: *Amanita phalloides* set names (Aph) from Alsace (A), Lorraine (L3) and Franche-Comté (FC).
(a) Aph A set; (b) Gal L1, Aph FC and Aph L3; (c) Gal L2, Gal FC and Aph L3: amatoxin contents divided into three groups ($P^{NK} < 10^{-2}$).

Toxin content and amanitin distribution for A. phalloides.—The mean weight (g of fresh weight ± sem) for each set of *A. phalloides* was of 20.81 ± 5.91 g F.W., 32.60 ± 12.74 g F.W. and18.42 ± 4.78 g F.W for Aph A, Aph L3 and Aph FC, respectively (TABLE I). Phallotoxins and amatoxins were detected by HPLC in all 17 specimens collected from the three sites; amatoxin content and amanitin distribution were listed solely (TABLES I and II, respectively).

The mean amatoxin content of the Aph A, Aph L3 and Aph FC sets was of 367.09 ± 62.38 $\mu g.g^{-1}$, 172.07 ± 56.32 $\mu g.g^{-1}$ and 247.87 ± 14.67 $\mu g.g^{-1}$ F.W., respectively (TABLE I, FIG. 1). One-way ANOVA showed significant difference between the three sets of *A. phalloides* (Aph A: n = 6; Aph L3: n = 3; Aph FC: n = 8; $P^{A} = 0.04$). Significant variation in the amatoxin contents was observed only between the Aph A and Aph L3 sets ($P^{NK} = 0.04$). Origin factor (site) did not affect the amanitin profile of this ectomycorrhizal species because the β-Ama, α-Ama and γ-Ama concentrations as well as $\beta/(\alpha + \gamma)$ ratios from the three sets were not statistically different ($P^{A} > 0.05$; TABLE II, FIGS. 2a, b).

Statistical comparison of the toxinological data of G. marginata *and* A. phalloides.—The analysis of variance combining Species and Origin as factors was performed on the six sets constituted of 44 specimens (27 Galerinas, three sets; 17 Amanitas, three sets). Significant difference ($P^{A} < 10^{-4}$) between the six amatoxin contents was observed. The Newman-

TABLE II. Amanitin distribution in *G. marginata* (Gal) and *A. phalloides* (Aph) sets

Set name	Sample size n	β-Amanitin (%)[c]	α-Amanitin (%)	γ-Amanitin (%)	β/(α + γ)
Gal L1[a]	10	58.69 ± 1.09	38.97 ± 1.03	2.34 ± 0.15	1.44 ± 0.07
Gal L2[a]	6	43.18 ± 1.49	47.35 ± 1.55	9.47 ± 0.33	0.76 ± 0.05
Gal FC[a]	11	23.13 ± 1.80	63.43 ± 2.39	13.44 ± 1.08	0.31 ± 0.03
		$P^{\wedge}$ value[d] $< 10^{-4}$	$P^{\wedge}$ value[d] $< 10^{-4}$	$P^{\wedge}$ value[d] $< 10^{-4}$	$P^{\wedge}$ value[d] $< 10^{-4}$
Aph A[b]	6	52.88 ± 4.08	34.77 ± 2.93	12.48 ± 1.82	1.20 ± 0.20
Aph L3[b]	3	52.13 ± 1.46	40.77 ± 2.26	7.10 ± 1.11	1.08 ± 0.06
Aph FC[b]	8	46.27 ± 2.98	42.43 ± 2.95	11.30 ± 0.77	0.93 ± 0.12
		$P^{\wedge}$ value[e] = 0.33	$P^{\wedge}$ value[e] = 0.19	$P^{\wedge}$ value[e] = 0.08	$P^{\wedge}$ value[e] = 0.44
G. marginata	27	40.76 ± 3.20	50.8 ± 2.39	8.44 ± 1.06	0.83 ± 0.10
A. phalloides	17	49.64 ± 2.08	39.43 ± 1.90	10.98 ± 0.86	1.05 ± 0.09
		$P^{\wedge}$ value[f] $< 10^{-4}$	$P^{\wedge}$ value[f] $< 10^{-4}$	$P^{\wedge}$ value[f] $< 10^{-4}$	$P^{\wedge}$ value[f] $< 10^{-4}$

[a] L1, L2 = Lorraine (Forest of Languimberg and Sarrebourg, France), FC = Franche-Comté (Bois de Tilles, France).

[b] A = Alsace (Forest of Haguenau, France), L3 = Lorraine (Forest of Languimberg and Sarrebourg, France), FC = Franche-Comté (Bois Rodolphe, France).

[c] Mean concentrations expressed as percentage ± sem.

[d] $P^{\wedge}$ value: level of significance of ANOVA. $P^{\wedge}$ value $< 10^{-4}$ between Gal L1, Gal L2 and Gal FC sets for β-Amanitin, α-Amanitin and γ-Amanitin percentages as well as for β/(α + γ) ratios.

[e] $P^{\wedge}$ value > 0.05: no statistical difference between Aph A, Aph L3 and Aph FC sets for β-Amanitin, α-Amanitin and γ-Amanitin percentages as well as for β/(α + γ) ratios.

[f] $P^{\wedge}$ value $< 10^{-4}$ between for the six sets of *G. marginata* and *A. phalloides* for the amanitin mean concentrations and β/(α + γ) ratios.

Keuls tests divided the experimented sets into three groups ($P^{NK} < 10^{-2}$, FIG. 1). The Aph A set representing the first group (a) contained the highest mean amanitin amount (367.09 ± 62.38 μg.g^{-1} F.W.) whereas the third (c) was formed by the Gal L2 and Gal FC sets exhibiting amanitin amounts approximately four times less (78.17 ± 10.08 μg.g^{-1} and 96.88 ± 12.82 μg.g^{-1} F.W., respectively). The second group (b) consisted of the Gal L1 and Aph FC sets showing nearly the same amatoxin content (243.61 ± 16.54 μg.g^{-1} and 247.87 ± 14.67 μg.g^{-1} F.W., respectively). Concerning the Aph L3 set, the basidiomes were classified either in the third (c) or second group (b) (172.07 ± 56.32 μg.g^{-1} F.W.).

The comparison between the amanitin profiles for the six sets of *G. marginata* and *A. phalloides* revealed significant difference ($P^{\wedge} < 10^{-4}$) in the mean amanitin concentrations and mean values of ratio (TABLE II). β-Ama concentration from Gal FC set statistically was lower than that from each of the three Amanitas series ($P^{NK} < 0.01$). β-Ama concentrations from Gal L1 and Gal L2 sets were significantly different from only those for Aph FC ($P^{NK} < 0.01$) and Aph A ($P^{NK} < 0.05$), respectively. Significant difference was recorded between the α-Ama concentrations: (i) higher concentration from Gal FC than those from all Amanitas analyzed ($P^{NK} < 0.01$); (ii) higher concentration from Gal L2 than that from Aph A ($P^{NK} < 0.05$). Significant difference also was revealed between the γ-Ama concentrations: (i) lower concentration from Gal L1 than those from all Amanitas ($P^{NK} < 0.01$); (ii) higher concentration from Gal FC than that from Aph L3 ($P^{NK} < 0.01$) (TABLE II, FIG. 2a). Last, the β/(α + γ) ratio calculated for Gal FC was lower than each one established for the Amanitas series ($P^{NK} < 0.01$) whereas the low ratio distinguished Gal L1 only from Aph FC ($P^{NK} < 0.05$) (TABLE II, FIG. 2b).

Furthermore, the comparison between the amanitin mean concentrations from the three sets of *G. marginata* showed a significant difference ($P^{\wedge} < 10^{-4}$) revealing that the Origin (site/host) clearly affected the ligninolytic *G. marginata* species. The same analysis carried out on the three sets of *A. phalloides* showed no statistical difference reporting that the amanitin distribution in the ectomycorrhizal species was not influenced by the Origin (site), as seen in FIG. 3.

DISCUSSION

Occurrence of G. marginata *white-rot fungi.*—*G. marginata* was reported as wood-rotting fungi occurring predominantly on conifers (Bon 1990, 1992; Courtecuisse and Duhem 2000; Lincoff 1998; Singer 1986). In general, white-rot fungi predominantly de-

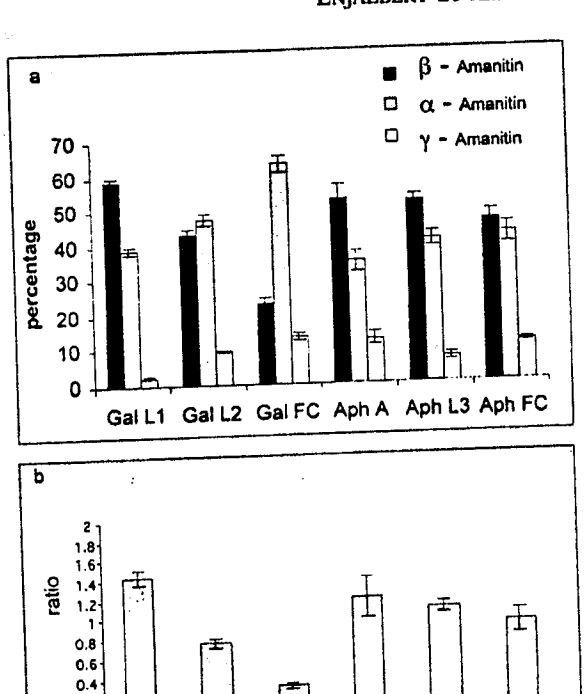

FIG. 2. a. Distribution of the three amanitins in *Galerina marginata* and *Amanita phalloides* sets (concentrations expressed as percentage, brackets indicate sem); b. β-amanitin/(α-amanitin + γ-amanitin) ratio (brackets indicate sem).
Gal L1, Gal L2, Gal FC: *Galerina marginata* set names (Gal) from Lorraine (L1, L2) and Franche-Comté (FC).
Aph A, Aph L3, Aph FC: *Amanita phalloides* set names (Aph) from Alsace (A), Lorraine (L3) and Franche-Comté (FC).

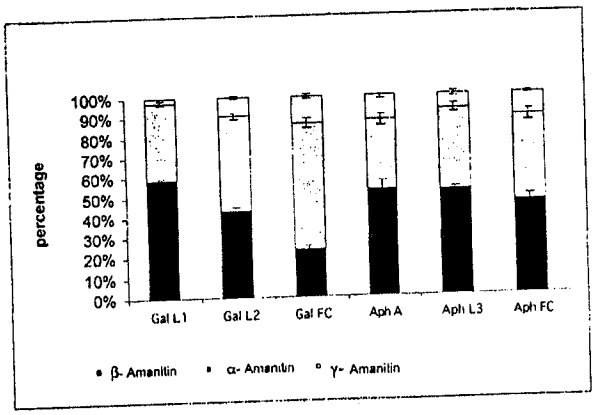

FIG. 3. Amanitin profile (expressed in percentage, brackets indicate sem) for *Galerina marginata* and *Amanita phalloides* sets from various collections.
Gal L1, Gal L2, Gal FC: *Galerina marginata* set names (Gal) from Lorraine (L1, L2) and Franche-Comté (FC).
Aph A, Aph L3, Aph FC: *Amanita phalloides* set names (Aph) from Alsace (A), Lorraine (L3) and Franche-Comté (FC).

grade hardwood. However some of them having a broad host range attack both softwood and hardwood (Blanchette 1991, Rypáček 1977). It is well known that certain ligninolytic fungi, either white-rot or brown-rot as *Serpula lacrymans* (Wulfen) J. Schröt., *Coniophora puteana* (Schumach.) P. Karst. and *Gloeophyllum trabeum* (Pers.) Murrill, grow on coniferous as well as on deciduous trees (Zaremski 1996).

Our investigated specimens were collected on hosts belonging to either hardwoods (*Fagus*) or softwoods (*Picea*); so *G. marginata* can be designated as both a hard- and softwood degrader. European and North American *Galerina* material known as amatoxin-containing species analyzed for DNA studies also was taken from hosts belonging to Gymnosperms and Angiosperms (Gulden et al 2001).

Different characteristics distinguish softwoods and hardwoods: (i) microstructure of wood (Montgomery 1982); (ii) structural elements building lignin (Blanchette 1991, 2000; Tuor et al 1995); (iii) sugars constituting hemicelluloses (Levy 1982, Montgomery 1982): it should be noted that the major carbohydrates—D-mannose (hardwoods) and D-xylose (softwoods)—provide the energy source for the decay process (Blanchette 1991, Tuor et al 1995). Moreover, the lowest pH values of natural substrates are reported for softwoods (Scheikl 1994). Last, it is well known that the chemical composition of coniferous wood differs also from that of deciduous wood in its content of volatile monoterpenes (Hintikka 1982). Given the variations in microstructure and chemical components between softwoods and hardwoods, differences in physiology and biochemistry could be expected for wild mushrooms growing on either one or the other substrate.

Amatoxin content of G. marginata *and* A. phalloides.—The three amanitins (α-Ama, β-Ama, γ-Ama) were identified in each analyzed *G. marginata* basidiome from hardwoods (Gal L1: n = 10; Gal L2: n = 6) and softwoods (Gal FC: n = 11). Differences in chemical composition of two substrates could explain the significant variation ($P^{NK} < 10^{-4}$) in the mean amatoxin contents between Gal L1 (243.61 ± 16.54 $\mu g.g^{-1}$ F.W.), collected on a decayed beech and Gal FC (96.88 ± 12.82 $\mu g.g^{-1}$ F.W.) taken from rotten spruce. On the other hand, variation in amatoxin contents between Gal L1 and Gal L2 could be due, at least in part, to the mean weight of fresh basidiomes forming the both sets, 0.92 ± 0.11 g and 2.90 ± 0.66 g, respectively. Approximately 56% of the total

nitrogen in wood is bound to lignin; lignin degradation with simultaneous nitrogen utilization would contribute to mycelial growth (Dill et al 1984). As reported by Muraoka et al (2000), changes in timing between biomass and intracellular α-Ama production are observed for fermentation of *G. fasciculata* strain GF-060. Furthermore, other parameters such as extrinsic factors (environmental conditions) and intrinsic factors (genetic properties) could contribute to the significant difference between amatoxin contents for Gal L1 and either Gal FC or Gal L2.

Amatoxin content was different between the three *A. phalloides* sets (Aph A, Aph L3, Aph FC; TABLE I) and as high as those reported in literature (Enjalbert et al 1993, Stijve and Seeger 1979, Tyler et al 1966). Variations in the amanitin mean amounts also were due to different factors. The mean weight of fresh basidiomes of 20.81 ± 5.91 g, 32.60 ± 12.74 g and 18.42 ± 4.78 g, respectively, probably should be involved as suggested above for *G. marginata*. Moreover, the environment (seasonal conditions of growth, moisture content) and diversity of genetic inheritance play a determinant role on amatoxin content for *A. phalloides* from different collections (Enjalbert et al 1993, Stijve and Seeger 1979, Tyler et al 1966).

It is worthy of note that certain *G. marginata* specimens were more toxic than some *A. phalloides* basidiomes, European species considered as the richest in amanitins (TABLE I, FIG. 1). The amatoxin content in Gal L1 (243.61 ± 16.54 $\mu g.g^{-1}$ F.W.) was as high as that in Aph FC (247.87 ± 14.67 $\mu g.g^{-1}$ F.W.) and more elevated than that in Aph L3 (172.07 ± 56.32 $\mu g.g^{-1}$ F.W.). Given the lethal dose of amatoxins estimated to be about 0.1 $mg.kg^{-1}$ human body weight, or even lower (Wieland 1986), the ingestion of 10 *G. marginata* basidiomes containing about 250 μg of amanitins per g of fresh tissue should poison a child weighing approximately 20 kg. *G. marginata* is a toadstool easily confused with such edible mushrooms as *Kuehneromyces mutabilis* (Schaeff.) Singer & A.H. Sm. and *Armillaria mellea* (Vahl) P. Kumm. In a 20-year retrospective recording clinical data from 2108 amatoxin exposures in North America and Europe, few cases due to ingestion of Galerinas were listed. Scarcity of these wood-rotting fungi often unobserved by collectors explains the infrequency of poisoning. Moreover, it has been shown that 21% of amatoxin poisonings are caused by unidentified species (Enjalbert et al 2002).

As regards comparison of the amatoxin contents between *G. marginata* and other species of *Galerina* genus, our findings are in agreement with the Czech survey of amanitin-containing mushrooms, recording that *G. sulciceps* is more toxic than *A. phalloides* (Klán 1993). Furthermore, according to Muraoka et al (2000), the amanitin mean amount in the strains of both Japanese *G. helvoliceps* (n = 3; 207.7 ± 130.11 $\mu g.g^{-1}$ F.W., from 47.81 to 556.22 $\mu g.g^{-1}$) and *G. fasciculata* (n = 18; 245.96 ± 20.58 $\mu g.g^{-1}$ F.W., from 222.33 to 259.96 $\mu g.g^{-1}$) could be as high as that from *A. phalloides*. Numerous fatalities consequently are caused by *G. fasciculata* in Japan (Ishihara and Yamaura 1992).

Toxin distribution of G. marginata *and* A. phalloides.—Regarding the toxin profile, any acidic and neutral phallotoxins were not detected in the 27 French *G. marginata* specimens using HPLC analyses. Our results confirmed that these bicyclic heptapeptide derivatives related to amatoxins are not present in the Galerinas (Wieland 1983, Wieland 1986, Wieland and Faulstich 1983). On the other hand, the γ-Ama previously HPLC detected in only one specimen (Enjalbert et al 1992) was identified, associated with β-Ama and α-Ama, in each *G. marginata* basidiomes (n = 27) from either hardwoods or softwoods. Significant variations ($P^{M} < 10^{-4}$) between the β-Ama, α-Ama and γ-Ama concentrations as well as the values of the β/(α + γ) ratio observed for the three sets underlined the effect of Origin factor (site/host) on the amanitin distribution in *G. marginata*. Predominance of β-Ama (acid amanitin) distinguished Gal L1 growing on hardwoods whereas that of α-Ama + γ-Ama (neutral amanitins) individualized Gal FC collected from the most acidic substrate (softwoods; FIG. 2b). Relationship between the distribution of either acidic or neutral toxins and pH of the collection site is observed for *A. phalloides*. Basidiomes of the ectomycorrhizal species collected from acidic soil (siliceous soil and clay and chert; pH = 4.5–5) are distinguishable by the predominance of phalloidin, a neutral phallotoxin (Enjalbert et al 1996, 1999).

Difference between amanitin profiles for the two sets (Gal L1 and Gal L2) growing on *Fagus* hosts could not be due to the chemical composition of natural substrate but to the different components from decayed wood. Indeed, the decomposition process leads to various chemical patterns of substrate (Barrasa et al 1992). White-rot Basidiomycota have the capacity to degrade the three wood polymers (lignin, cellulose, hemicellulose) at different rates and extent. Within that group, fungi are either simultaneous degraders of lignin along with wood polysaccharides or selective lignin degraders or may show both types of decay (Blanchette 1991, Eriksson et al 1990, Solov'ev et al 1985). Many enzyme systems leading to oxidative and reductive conversions are involved in lignin biodegradation. Enzyme multiplicity can explain the heterogeneity of the substrate at suc-

cessive stages of wood decay (Tuor et al 1995, Watanabe and Kuwahara 2000). Advances on the molecular genetics of ligninolytic fungi have shown that different genes encoded enzymatic system responsible for lignin degradation (Blanchette 1991, Cullen 1997, Varela et al 2000). Furthermore, environmental conditions, such as temperature, humidity, microclimate, low nitrogen content, elevated carbon dioxide and pH values, must be crucial in governing the selectivity of fungal biodegradation of wood components. Such factors giving rise to series of ecological niches filled by a range of micro-organisms improve carbohydrate degradation (Blanchette 2000, Levy 1982). Fungal lignin degradation results in the formation of low molecular weight compounds, mostly aromatic carboxylic acids (oxalic, citric, formic and butyric acids) metabolized by bacteria (Tuor et al 1995). Changes in glucide and organic acid contents of substrate affect the production and regulation of secondary metabolites (Frank 1998) and therefore could play a role in amanitin profile of *G. marginata*. Experiments on the three strains of *G. fasciculata* and 18 strains of *G. helvoliceps* have shown that fermentation conditions are involved in the distribution of acidic and neutral amanitins. In liquid-cultured mycelia, α-Ama and small γ-Ama amounts are accumulated whereas β-Ama is only detectable; in solid-cultured mycelia, α-Ama and β-Ama are the main toxins and only trace of γ-Ama is detected (Muraoka and Shinozawa 1999, 2000). Significant difference in amanitin distribution between the three sets of *G. marginata* due to components of substrate is in agreement with the findings for the Japanese *Galerina* species considered generally as wood-rotting fungi. Unlike the DNA studies performed on the *G. marginata* complex showing the absence of correlation between the substrate and the distribution of genetic types (Gulden et al 2001), our findings pointed out the relationship between the substrate and distribution of amanitins being nitrogen-containing toxins. In the same way, changes in the chemical components of edible *Pleurotus* sp. mainly the protein content are correlated with the substrate composition (Sturion and Oetterer 1995).

Concerning the toxin distribution for the three *A. phalloides* sets (Aph A, Aph L3 and Aph FC), our HPLC results showed that phallotoxins and amatoxins were detected in all specimens (n = 17). The occurrence of both groups of toxins for this species is consistent with literature (Wieland 1983, Wieland 1986, Wieland and Faulstich 1983). Amounts of acidic and neutral phallotoxins assayed in many French *A. phalloides* specimens are indicated (Enjalbert et al 1989, 1993). Unlike American materials of *A. verna* Fr. (Benedict et al 1970, Tyler et al 1966), no amanitin-free *A. phalloides* specimen from either our collections or various French locations was found (Enjalbert et al 1992, 1996, 1999). The major amanitins were always β-Ama and α-Ama; predominance of acidic over the neutral toxins seems to be the rule as previously reported (Enjalbert et al 1993, Stijve and Seeger 1979, Tyler et al 1966, Wieland 1986). Studies concerning this ectomycorrhizal species revealed that associated woody plants do not appear to have an effect on the toxin distribution and that the geological and pedological characteristics of the collection sites have a greater influence on phallotoxin profile than amanitin distribution (Enjalbert et al 1996, 1999). No significant difference was found between the amanitin distributions for the *A. phalloides* sets taken from the three sites having nearly the same type of soil.

Overall, our findings underlined intraspecific variability in the amanitin profile for the wood-rotting *G. marginata* resulting from the host/fungus combination. Relationship between the chemical composition of the substrate and the expression of toxic secondary metabolites depends on many intrinsic and extrinsic factors. Structural and chemical differences in wooden substrate (hardwoods and softwoods) should constitute only a part of the parameters determining the nitrogen-containing toxin pattern. Decaying process leading to changes in nutritional substrate certainly linked on host/fungus gene activity also could be considered as crucial factors in the acidic and neutral amanitin production. An extensive research should be done to validate these hypotheses. First, analyses of additional *G. marginata* specimens from various hosts and sites over the years should be carried out to verify the variability in the amanitin distribution. Second, a thorough understanding of enzyme system from the white-rot fungi should lead to identify the nutritional components from the substrate participating in the regulation of toxic secondary metabolites for *G. marginata*.

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