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Toxin composition of *Amanita phalloides* tissues in relation to the collection site

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Abstract: The influence of different factors on amatoxin and phallotoxin composition of *Amanita phalloides* was studied. The concentration of eight toxins (three amatoxins and five phallotoxins) in six parts of the carpophore, namely, the pileus, lamellae, annulus, stipe, volva and bulb was evaluated in an effort to establish a relationship between the type of tissue and its toxin composition. The possible influence of the collection site on the distribution of amatoxins and phallotoxins was investigated by analyzing the tissues of 27 expanded carpophores and 4 buttons harvested from four geologically different soils located in the same region (Franche-Comté, France): Jurassic limestone, siliceous soil, clay with chert and gley-like argillaceous sand. Toxin concentrations were measured by using high-performance liquid chromatography and the data analyzed statistically using hierarchical clustering analysis, principal component analysis and discriminant analysis. Significant differences in the amounts of amatoxins and phallotoxins led to the partition of the tissues into two groups: the bulb and the volva that had the highest phallotoxin concentration and the pileus, lamellae, annulus and stipe that were rich in amatoxins. The bulb and volva of all specimens from each collection site had similar phallotoxin percentage. Nevertheless, the toxin content of these two tissues was different, and among the different parts of the carpophore the bulb was the poorest in toxins. The type of soil also affected toxin distribution, and in particular the phallotoxin relative concentration. Predominance of the acidic phallotoxins over the neutral phallotoxins in all tissues characterized the carpophores collected from the Jurassic limestone. In contrast, the main features of all specimens from siliceous soil and clay with chert were the elevated amounts of neutral phallotoxins (phalloidin and phallisin) leading to a higher percentage of phallotoxins over amatoxins. This study has shown that the tissues of *A. phalloides* could be characterized by their toxin distribution. Similarities in toxin composition allowed division of the different parts of the carpophore into two groups. Furthermore, this investigation strongly indicates that the collection site significantly influences toxin composition of the carpophore tissues.

Key Words: amatoxins, carpophore tissues, ecology, phallotoxins

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INTRODUCTION

Amanita phalloides (Vaill.: Fr.) Link (Courtecuisse and Duhem, 1994) contains two classes of hepatotoxic cyclopeptides: amatoxins and phallotoxins (Wieland, 1986, 1987; Wieland and Faulstich, 1991). The amounts of these toxins in the carpophore have been determined by several analytical methods generally using pooled material and most of these investigations have been reviewed (Enjalbert et al., 1993). Variations in the toxin composition of the carpophores have been attributed to the collection site of the material (Andary et al., 1977; Yocum and Simons, 1977; Stijve and Seeger, 1979; Enjalbert et al., 1989). These findings suggest that environmental conditions could determine toxin distribution in the carpophores. Differences in amounts of amatoxins and phallotoxins in different parts of the carpophore have also been noted (Andary et al., 1979; Bodenmuller et al., 1981; Enjalbert et al., 1989).

The specificity and sensitivity of the high-performance liquid chromatography (HPLC) allows for easy assay of the main amatoxins, namely, α-amanitin (α-Ama), β-amanitin (β-Ama) and γ-amanitin (γ-Ama) and the chief phallotoxins, phallisacin (PSC), phallacidin (PCD), phallisin (PHS), phalloidin

(PHD) and phalloin (PHN) using a very small amount of material (Enjalbert et al., 1992). By using HPLC on a small set of specimens we found that the toxic cyclopeptides were not equally distributed throughout the carpophore (Enjalbert et al., 1993). In addition, a fine subdivision of the carpophore revealed marked differences in the toxin composition of the tissues; nevertheless, phallotoxin percentage of the bulb and volva was similar (Enjalbert et al., 1993). To confirm these results, a survey of additional mushrooms from various collections was necessary.

The present study was undertaken to determine the distribution of amatoxins and phallotoxins in the tissues of 27 *A. phalloides* expanded carpophores and 4 buttons collected in different geological sites. Based on the toxin composition, the tissues of individual carpophores were evaluated and similarities in the toxin distribution between the bulb and the volva were confirmed. Also, this investigation demonstrated the influence of the collection site on toxin concentrations and showed that soil characteristics are important in determining toxin diversity, particularly the predominance of either acidic or neutral phallotoxins.

MATERIALS AND METHODS

Collection of carpophores.—*A. phalloides* carpophores were collected from different sites in the Franche-Comté region (France) during 1993 and 1994. Four different geological soils were sampled to determine the role of the collection site on phallotoxin and amatoxin composition. These included a Jurassic limestone soil near Baume-les-Dames a clay with chert soil near Naisey and Osse, both in the Department of Doubs, a siliceous soil in the Massif de la Serre, and a gley-like argillaceous sandy soil near Deschaux, both in the Department of Jura. Fourteen specific areas located within the different sites, characterized by one or two dominant tree species were the source of 27 expanded carpophores and 4 buttons. The thirty-one specimens and the associated plant species are reported in TABLE I. The specimens harvested in each area were close to each other and probably developed from the same mycelium. The carpophores represented two developmental stages: young specimens with pileus totally free of the universal veil but with the lamellae concealed by the partial veil and fully-developed specimens with pileus completely expanded and a typical membranous annulus surrounding the stipe. Voucher specimens are deposited in the MPU herbarium (Holmgren and Keuken, 1974). The carpophore was divided into the following tissues: the pileus, the lamellae, the partial veil in the young specimens or annulus in the fully-developed specimens, the stipe, the volva and the bulb. The stipe tissue was taken from the median section of the stipe proper and the bulb corresponded to the swollen stipe base.

Extraction procedure.—A 2 g portion (wet weight) of the pileus, lamellae, stipe or bulb, or the entire (0.1–1 g, wet weight) annulus or volva was frozen in liquid nitrogen. The frozen tissues were ground in liquid nitrogen with a mortar and pestle. Amatoxins and phallotoxins were extracted with 0.5 −4 mL of extraction medium composed of methanol-water-0.01 M hydrochloric acid (5:4:1, v/v/v). The volume of the extraction medium was adjusted for sample weight. The mixture was sonicated for 2 min with a high intensity ultrasonic processor equipped with a 3 mm diameter sonde, Vibracell model (Bioblock Scientific, Strasbourg, France). During the sonication process the preparation was immersed in an ice bath to avoid heating of the samples. The extracts were incubated overnight at 4 C, then centrifuged at 10 000 g for 10 min at 4 C. The supernatant (E) was poured off and saved. To insure completeness of the extraction, the pellet was rinsed twice. The residue was resuspended in 0.5–1 mL of extraction medium, homogenized in a vortex mixer for 2 min and centrifuged again as above. The surpernatant (R_1) was decanted; then a second supernatant (R_2) was obtained by a second wash with 0.25–0.50 mL of the extraction medium under the same conditions. To evaluate the toxin content throughout the extraction process, supernatants E, R_1, R_2 from the pileus, lamellae, stipe and bulb of eight carpophores were analyzed separately. The combined supernatants were used to determine the phallotoxin and amatoxin composition of the samples. The extract obtained by the combination of E, R_1, R_2 contained 10 to 37% of the tissue (wet weight).

HPLC assays.—The analyses were performed using a reversed-phase HPLC method (Enjalbert et al., 1992) but slightly modified to account for an HPLC system consisting of a Waters multisolvent delivery module, an Interchim Rheodyne Injection valve 7125 fitted with a 20-µL loop, and a Merck-Hitachi variable wavenlength UV diode-array detector, L 4500. Chromatograms were processed with a system Dad Data Manager. Separations were performed on a reversed-phase 5 µm Ultrasphere ODC column (250 × 4.6 mm I.D.) (Beckman Instruments, San Ramon, CA, USA). The mobile phases were (A) 0.02 M aqueous ammonium acetate adjusted to pH 5.0 with glacial acetic acid and (B) acetonitrile. These were prepared with double distilled water, analytical reagent grade chemicals and HPLC grade acetonitrile. All eluents were degassed by sonication prior to use. The elution

TABLE I. Origin and site characteristics of the samples

Sample No.	Date	Location[a]	Associated species[b]	Geological site	Stage of development
1	22 Sep. 1993	Naisey	*Picea abies* (L.) Karsten	Clay with chert	Button
2					1[c]
3					2[d]
4	26 Sep. 1993	Grosse Grange	*Picea abies*		1
5			+ *Populus tremula* L.		2
6		Osse	*Corylus avellana* L.		Button
7			+ *Hedera helix* L.		1
8					2
9		Gros Bois	*Fagus sylvatica* L.		Button
10					1
11					2
12	07 Oct. 1993	Ermitage (La Serre)	*Corylus avellana*	Siliceous soil	1
13					2
14					2
15			*Carpinus betulus* L.		2
16			*Fagus sylvatica*		2
17	06 Oct. 1994	La Serre	*Fagus sylvatica*		1
18					2
19			*Quercus robur* L.		1
20					2
21			*Castanea sativa* Miller		2
22	28 Sep. 1994	Bois Rodolphe	*Fagus sylvatica*	Limestone	Button
23		(Baume-les-Dames)			1
24					2
25			*Carpinus betulus*		1
26					2
27					2
28			*Quercus robur*		1
29					2
30	28 Sep. 1994	Deschaux	*Fagus sylvatica*	Argillaceous sand	2
31					2

[a] Sites located in Franche-Comté region (France).
[b] According to *Flora Europaea* (Tutin et al., 1964).
[c] Young specimen.
[d] Fully-developed specimen.

profile consisted of three isocratic steps over a total duration of 37 min: 1) 10% v/v B for 4 min, 2) 18% v/v B for 18 min and 3) 30% v/v B for 15 min. The re-equilibration time was 15 min with the 10% v/v B eluent. The column temperature was set at 25 C using an Interchim Crococil oven TM 701 (Montluçon, France). The mobile phase flow-rate was 1 mL/min. The absorbance of the eluate was monitored at 290 nm for the phallotoxins and 300 nm for the amatoxins. Identification of the toxins was based on retention time and on line UV spectral data (Enjalbert et al., 1992).

α-Ama, β-Ama, PHD and PCD were purchased from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). γ-Ama, PHS, PHN and a mixture of PCD and PSC, prepared according to previously published procedure (Wieland and Dudensing, 1956; Wieland and Mannes, 1957; Wieland et al., 1967), were kindly provided by Prof. H. Faulstich. A 20 μL aliquot of each standard or crude extract was injected into the column. A four-point calibration curve was prepared with a range of 1–100 μg/mL for α-Ama, β-Ama, PHD and PCD and a range of 1–50 μg/mL for γ-Ama, PHS, PHN and the mixture of PCD and PSC. The regression coefficients of all curves were greater than 0.998. The elution sequence, namely, β-Ama < α-Ama < PSC < γ-Ama < PCD < PHS < PHD< PHN, was similar to that previously obtained (Enjalbert et al., 1992). The average retention time ± SD of the toxins was found to be 10.69 ± 0.06 min, 15.41 ± 0.09 min, 17.51 ± 0.07 min, 18.03 ± 0.11 min, 19.23 ± 0.07 min, 22.13 ± 0.13 min, 26.56 ± 0.16

min, 34.53 ± 0.06 min for β-Ama, α-Ama, PSC, γ-Ama, PCD, PHS, PHD and PHN, respectively, (n = 10). The data are expressed in terms of μg toxin per g of tissue (wet weight). The limit of detection for both groups of toxins was 0.01 μg/mL of extraction medium. The inter-assay precision was verified by assaying the same extract three times. The coefficient of variation calculated for all toxins was found to be in the range of 5 to 7%.

Statistical analysis.—Three complementary analyses using Statgraphics (Uniware, Paris, 1994) were carried out to evaluate the significance of the data from the HPLC assays (Robert, 1989; Tenenhaus, 1994). The variables corresponded to the β-Ama, α-Ama, γ-Ama, PSC, PCD, PHS, PHD and PHN concentrations in the samples together with three ratios calculated from these values, namely, acidic amatoxin (β) / neutral amatoxins (α + γ), acidic phallotoxins (Pa) / neutral phallotoxins (Pn) and phallotoxins (P) / amatoxins (A).

First, the hierarchical clustering analysis (HCA) was used. This method consists in partitioning the large set into more homogeneous groups, by means of Ward's method also known as the Inertia Criteria method (Morrisson, 1990). The sequence of partitions indicated by a numerical index can be shown as a dendrogram. Next, a principal component analysis (PCA) was performed on the 11 variables. This method reduces the number of variables by creating principal components, linear combinations of these variables, that explain most of the variability. All the information is distributed according to these principal components and only the first three representing 80% of the total information were taken into account. The first principal component (horizontal axis) accounts for the maximum variation between samples. The second principal component, represented by the vertical axis, accounts for the maximum of the remaining variation, etc. Each principal plane is described by two types of representation: a plot of individual samples and a correlation circle. Correlations of the original variables with the principal components are represented on the principal planes by lines within a correlation circle with a radius equal to 1; the length and orientation of these lines indicate the levels of their correlation. These two methods (HCA, PCA) were applied to the data obtained from 164 samples corresponding to the five or six tissues dissected from the 27 expanded carpophores and the 4 buttons.

Finally, a discriminant analysis (DA) was carried out. This analysis is used when the data are classified into two or more clusters to find one or more original variables to discriminate between the clusters. It maximizes the "between group" variation over "within group" variation ratio. This method displays the actual and predicted classification results as percentages. The table of classification results presents the percentage of observations in each group that are correctly predicted (well-classified) and the percentage of observations that are predicted as belonging to the other groups. The normal distribution of the response variables was verified for each tissue and each site. DA was used to analyze the data obtained from only the 160 tissues excluding the buttons because they were not found in all four sites. The analysis was validated by removing a set of test samples from the total population. The data for the remaining base population, composed of 130 tissues from the carpophores collected in either 1993 or 1994 (see TABLE I), were used to construct discriminant functions for separating the sites. These functions, then, were used to predict the sites of the test samples taken from the carpophores collected from siliceous soil in 1994. (Samples from siliceous soil were used for this validation because they were the only ones collected in both 1993 and 1994).

RESULTS

Extraction procedure.—The effectiveness of the extraction method was evaluated by measuring the amounts of the major toxins: β-Ama, α-Ama, PCD and PHD in supernatants E, R_1 R_2. These assays were carried out on the pileus, lamellae, stipe and bulb of eight carpophores. The average concentrations of the four toxins extracted from each of the four tissues, expressed as a percentage (± = SD, n = 8), were between 85.37 ± 1.15% and 87.54 ± 1.08% in E, between 9.25 ± 1.25% and 11.38 ± 1.02% in R_1 and between 2.76 ± 0.51% and 3.31 ± 0.55% in R_2. The narrowness of the range of percentages indicates that the characteristics of the tissue and its toxin concentration do not affect the extraction yield of each toxin. This is exemplified by the results of the extraction yield of β-Ama, α-Ama, PCD and PHD in the supernatants from 32 samples, corresponding to the pileus, lamellae, stipe and bulb of eight carpophores. The average concentrations of β-Ama, α-Ama, PCD and PHD, expressed as a percentage, were 86.78 ± 1.60%, 86.53 ± 1.61%, 86.50 ± 1.52% and 86.88 ± 1.49%, respectively, in E; 10.16 ± 1.42%, 10.32 ± 1.50%, 10.30 ± 1.43%, 10.08 ± 1.16%, respectively, in R_1; and 3.02 ± 0.43%, 3.15 ± 0.43%, 3.16 ± 0.39% and 3.07 ± 0.52%, respectively, in R_2 (± SEM, n = 32).

HPLC assays.—The amatoxin and phallotoxin distribution in the tissues of the 27 carpophores together

with the 4 buttons collected from four different sites is presented in TABLE II. The results report the average amounts, expressed as a percentage, of both the amatoxins (β-Ama, α-Ama, γ-Ama) and the phallotoxins (PSC, PCD, PHS, PHD and PHN). The comparison between acidic and neutral compounds in the two groups of toxins as well as the proportion of phallotoxins and amatoxins is expressed as three ratios, namely, β/α + γ, Pa/Pn and P/A. Whatever the tissue analyzed, the percentages of γ-Ama, PHS and PHN were small, the mean values were 4.75 ± 0.31%, 3.40 ± 0.25% and 3.09 ± 0.17% for γ-Ama, PHS and PHN, respectively, (± = SEM, n = 160). The major components of our samples were β-Ama and α-Ama for the amatoxins and PCD, PHD and PSC for the phallotoxins. These findings agree with previously published results (Schäfer and Faulstich, 1977; Yocum and Simons, 1977; Andary et al., 1979; Stijve and Seeger, 1979; Beutler and Der Marderosian, 1981, Bodenmuller et al., 1981; Enjalbert et al., 1989). However the range of the average relative concentrations of the major toxins was particularly wide: 5.20 to 30.33%, 5.17 to 22.58%, 18.04 to 34.33%, 13.49 to 37.37% for β-Ama, α-Ama, PCD and PHD, respectively. The amounts of the acidic and neutral amatoxins and phallotoxins varied from tissue to tissue. The annulus tissue contained the greatest concentration of β-Ama and the bulb and volva presented the lowest Pa/Pn ratio. Important differences related to the amatoxin and phallotoxin concentrations in the carpophore tissues were also observed (TABLE II). The fact that these results show large variations in amatoxin and phallotoxin distribution in the different tissues of the carpophores strongly suggests that toxin concentrations are site determined. Therefore, a multivariate analysis was applied to these data to assess the effect of tissue and site factors on the toxin distribution in different parts of the carpophore.

Hierarchical clustering analysis.—HCA was applied to 164 samples (the 160 tissue samples dissected from 27 carpophores as well as the 4 buttons harvested from the different sites). The dendrogram truncated to 67% of the explained variance is shown in FIG. 1. The first level of the vertical tree differentiates two groups of samples, one is composed of the bulb (B) and volva (V) (node 325), the other is represented by the annulus (A), lamellae (L), pileus (P), stipe (S) and button (Bn) (node 326). The second level of the tree shows that each of the previous groups could be divided into two subgroups that are correlated with the sites. Node 319 corresponds to the samples harvested from the Jurassic limestone (L) site, and node 324 to samples from Jurassic limestone (L) and siliceous soil (S). Nodes 323 and 321 represent the specimens from the other sites, namely, argillaceous sand (A), clay with chert (C) and siliceous soil (S). The percentage of explained variance was found to be 54% for the aggregations into these four clusters (319, 321, 323, 324). The results indicate that both tissue and site factors are involved in the distribution of amatoxins and phallotoxins in the samples.

Amatoxin and phallotoxin composition in the different tissues.—PCA performed on the matrix of 164 samples by 11 variables showed that the first principal component, accounting for 47.2% of total variance, was strongly related to the tissue type. FIGURE 2 illustrates the contrast between the two groups: the first group is composed of B and V whereas the second group consists of A, L, P, S and the 4 buttons. Furthermore, the correlation circle shows that among the 11 variables, seven of them are highly correlated with the first PCA axis and contribute to the partition between these two groups. The P/A ratio and the two neutral phallotoxins, namely, PHS and PHD (r = + 0.93, + 0.83, + 0.79), respectively, were the main variables of the first group, whereas the three amatoxins, namely, β-Ama, α-Ama and γ-Ama, (r = −0.90, −0.89, −0.71), respectively, were the predominant variables of the second group (FIG. 3). The bulb and the volva contained elevated amounts of the two neutral phallotoxins, PHS and PHD, associated with relatively high PCD levels and consequently a large predominance of the phallotoxins over the amatoxins.

Lastly, the data from all the tissues (160 samples) were analyzed using DA relative to the tissue factor. The first and second discriminant functions represented 82.3% and 9.3% of the total variance, respectively. The first discriminant plane shows that two clusters, B, V and A, L, P, S, corresponding to the main groups in HCA and PCA, can be formed (FIG. 4). The two centroids representing the discriminant functions of the bulb and the volva, are markedly separated from the four others, representing those of the annulus (A), lamellae (L), pileus (P) and stipe (S). The distance between the annulus (A) and the stipe (S) underscores the difference in amatoxin composition of these two tissues (TABLE II). The classification results reported in TABLE III are in agreement with this discrimination. Except for the lamellae and pileus which are dispersed, between 64% and 70% of the four other tissues are correctly predicted. The majority of the remaining samples are predicted in the tissues belonging to the same group (TABLE III).

Relationship between the collection site and the amatoxin and phallotoxin composition.—The second principal component was most strongly related to site factor

TABLE II. Amatoxin and phallotoxin concentrations (% and ratios) in the different samples of *A. phalloides* specimens from different geological sites

Sample[a]	Site[b]	n	β-Ama[c]	α-Ama	γ-Ama	PSC	PCD	PHS	PHD	PHN	β/α + γ	Pa/Pn	P/A
A	A	2	30.33 ± 0.07[d]	13.39 ± 1.02	7.80 ± 1.58	4.71 ± 0.68	21.38 ± 0.31	3.95 ± 0.55	13.49 ± 1.58	4.96 ± 0.92	1.43 ± 0.04	1.17 ± 0.01	0.94 ± 0.02
B	A	2	5.20 ± 0.64	5.17 ± 1.55	2.17 ± 0.57	6.87 ± 1.52	33.79 ± 0.46	6.32 ± 0.12	35.61 ± 1.10	4.88 ± 0.46	0.75 ± 0.13	0.87 ± 0.01	7.38 ± 1.84
L	A	2	29.47 ± 0.51	22.58 ± 0.46	6.60 ± 0.44	2.16 ± 0.48	19.49 ± 0.16	2.11 ± 0.04	15.23 ± 0.90	2.37 ± 0.16	1.01 ± 0.01	1.10 ± 0.01	0.71 ± 0.04
P	A	2	29.57 ± 0.62	20.93 ± 0.30	6.41 ± 0.92	2.64 ± 1.07	20.44 ± 0.22	2.67 ± 0.11	14.70 ± 0.12	2.66 ± 0.17	1.08 ± 0.00	1.15 ± 0.07	0.76 ± 0.04
S	A	2	24.51 ± 0.20	20.23 ± 0.57	7.26 ± 0.01	2.56 ± 1.00	23.36 ± 0.58	2.58 ± 0.51	16.56 ± 0.57	2.96 ± 0.10	0.89 ± 0.01	1.18 ± 0.08	0.93 ± 0.02
V	A	2	5.44 ± 0.96	4.71 ± 0.16	3.28 ± 0.21	5.66 ± 0.65	34.33 ± 1.39	5.87 ± 0.34	36.83 ± 0.02	3.89 ± 0.15	0.69 ± 0.15	0.86 ± 0.02	6.46 ± 0.33
A	C	8	20.00 ± 1.94	16.48 ± 1.62	4.19 ± 0.43	7.65 ± 1.27	19.20 ± 1.73	3.32 ± 0.32	26.33 ± 1.76	2.84 ± 0.17	0.98 ± 0.07	0.86 ± 0.12	1.73 ± 0.44
B	C	8	9.91 ± 1.61	11.03 ± 1.95	3.30 ± 0.61	9.17 ± 1.27	21.65 ± 2.14	4.54 ± 0.32	36.61 ± 2.30	3.80 ± 0.27	0.70 ± 0.03	0.70 ± 0.08	3.90 ± 0.66
L	C	8	14.88 ± 1.55	13.59 ± 1.55	3.13 ± 0.48	8.63 ± 1.63	19.36 ± 2.19	3.83 ± 0.37	33.05 ± 1.86	3.54 ± 0.11	0.91 ± 0.05	0.72 ± 0.13	2.47 ± 0.40
P	C	8	17.03 ± 1.61	15.38 ± 1.80	3.65 ± 0.51	8.43 ± 1.75	20.09 ± 2.64	3.51 ± 0.25	28.71 ± 1.00	3.20 ± 0.09	0.96 ± 0.10	0.83 ± 0.14	2.11 ± 0.46
S	C	8	15.97 ± 1.12	17.53 ± 1.07	3.95 ± 0.43	8.57 ± 1.45	21.11 ± 2.02	3.24 ± 0.26	26.36 ± 1.27	3.29 ± 0.12	0.74 ± 0.02	0.94 ± 0.15	1.77 ± 0.21
V	C	8	11.84 ± 0.89	11.51 ± 1.45	4.62 ± 0.67	8.65 ± 1.34	19.94 ± 2.01	4.66 ± 0.46	35.54 ± 1.81	3.26 ± 0.20	0.86 ± 0.19	0.69 ± 0.11	2.79 ± 0.33
A	L	6	22.86 ± 1.50	17.77 ± 1.64	6.38 ± 1.02	11.24 ± 0.82	20.79 ± 0.57	2.59 ± 0.31	15.75 ± 0.94	2.62 ± 0.26	1.02 ± 0.18	1.54 ± 0.07	1.14 ± 0.07
B	L	7	7.21 ± 0.81	9.03 ± 0.72	2.89 ± 0.20	17.66 ± 0.82	29.30 ± 1.55	3.93 ± 0.14	27.09 ± 1.12	2.89 ± 0.25	0.62 ± 0.08	1.40 ± 0.09	4.36 ± 0.37
L	L	7	22.35 ± 1.26	21.95 ± 1.49	5.07 ± 0.42	12.01 ± 0.70	19.81 ± 0.86	1.69 ± 0.04	15.72 ± 0.54	1.40 ± 0.08	0.87 ± 0.11	1.70 ± 0.07	1.03 ± 0.03
P	L	7	21.72 ± 1.32	20.95 ± 1.47	5.41 ± 0.48	12.07 ± 0.71	20.60 ± 0.60	1.99 ± 0.05	15.49 ± 0.50	1.75 ± 0.12	0.88 ± 0.13	1.71 ± 0.05	1.08 ± 0.03
S	L	7	18.48 ± 1.58	19.49 ± 1.40	5.24 ± 0.47	12.76 ± 0.75	23.71 ± 1.43	2.21 ± 0.16	16.09 ± 0.63	2.01 ± 0.13	0.79 ± 0.12	1.80 ± 0.05	1.34 ± 0.11
V	L	7	6.67 ± 1.05	9.02 ± 1.23	3.84 ± 0.52	17.21 ± 1.02	28.82 ± 1.56	4.30 ± 0.35	27.43 ± 1.42	2.71 ± 0.32	0.54 ± 0.06	1.36 ± 0.08	4.79 ± 0.90
A	S	9	23.88 ± 1.10	16.94 ± 1.59	5.67 ± 0.40	6.71 ± 0.65	18.25 ± 1.68	2.39 ± 0.13	23.04 ± 0.81	3.11 ± 0.35	1.14 ± 0.15	0.89 ± 0.08	1.19 ± 0.10
B	S	10	7.30 ± 0.67	10.12 ± 0.77	3.25 ± 0.46	10.33 ± 0.92	25.33 ± 1.51	4.11 ± 0.22	35.65 ± 1.44	3.92 ± 0.35	0.56 ± 0.06	0.83 ± 0.06	4.07 ± 0.35
L	S	10	20.38 ± 1.25	18.33 ± 1.48	5.18 ± 0.37	7.35 ± 0.80	18.04 ± 1.39	2.41 ± 0.21	25.54 ± 1.47	2.78 ± 0.16	0.90 ± 0.08	0.85 ± 0.08	1.34 ± 0.13
P	S	10	20.51 ± 0.90	18.08 ± 1.75	5.84 ± 0.59	6.97 ± 0.69	19.68 ± 1.34	2.43 ± 0.23	23.69 ± 0.98	2.81 ± 0.20	0.90 ± 0.08	0.93 ± 0.07	1.30 ± 0.10
S	S	10	16.75 ± 1.20	17.86 ± 1.33	5.41 ± 0.41	8.44 ± 0.70	22.20 ± 1.39	2.52 ± 0.24	23.74 ± 1.05	3.08 ± 0.22	0.74 ± 0.06	1.06 ± 0.07	1.59 ± 0.16
V	S	10	6.31 ± 0.77	9.55 ± 1.26	3.56 ± 0.75	8.88 ± 0.63	26.38 ± 1.88	4.48 ± 0.25	37.37 ± 1.59	3.48 ± 0.28	0.50 ± 0.05	0.79 ± 0.06	4.89 ± 0.67
Bn	C	3	19.53 ± 0.58	17.93 ± 3.22	4.91 ± 1.47	8.82 ± 3.64	17.23 ± 1.84	3.09 ± 0.50	25.79 ± 0.82	2.70 ± 0.25	0.94 ± 0.20	0.83 ± 0.17	1.44 ± 0.33
Bn	L	1	29.37	14.67	6.82	8.41	17.06	2.05	19.57	2.05	1.37	1.08	0.97

[a] A, annulus; B, bulb; L, lamellae; P, pileus; S, stipe; V, volva; Bn, button.

[b] A, argillaceous sand; C, clay with chert; L, limestone; S, siliceous soil.

[c] β-Ama, β-amanitin; α-Ama, α-amanitin; γ-Ama, γ-amanitin; PSC, phallisacin; PCD, phallacidin; PHS, phallisin; PHD, phalloidin; PHN, phalloin; β/α + γ, acidic amatoxin : neutral amatoxins; Pa/Pn, acidic phallotoxins : neutral phallotoxins; P/A, phallotoxins : amatoxins.

[d] Data are the means of n specimens ± SEM.

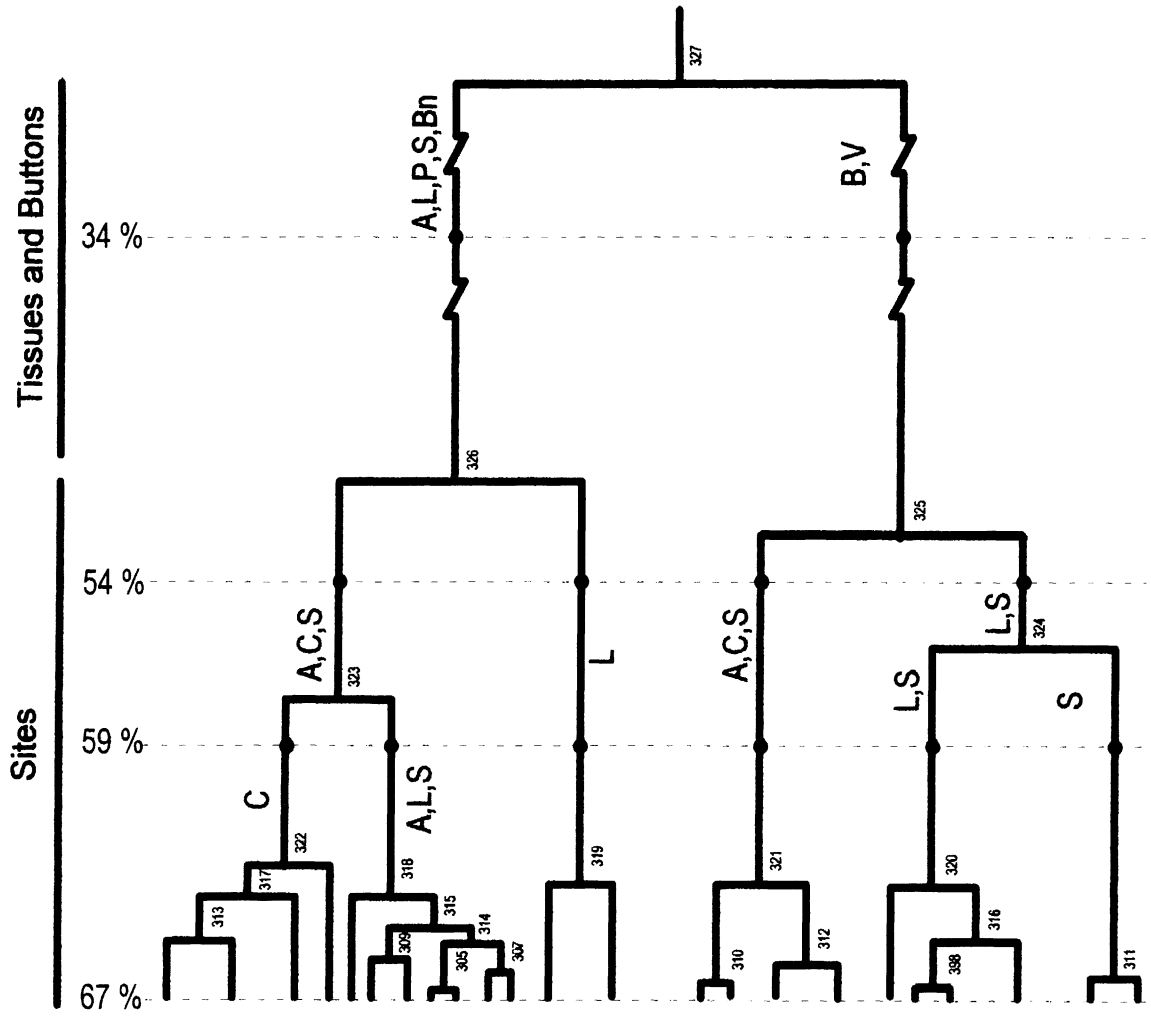

FIG. 1. Vertical tree of hierarchical analysis of the 164 samples collected from different geological sites. At 54% of the explained variance four groups were formed: 323, 319, 321, 324. Node 323 corresponds to the annulus (A), lamellae (L), pileus (P), stipe (S) and buttons (Bn) from the argillaceous sand (A), clay with chert (C) and siliceous soil (S); node 319 corresponds to the same samples A, L, P, S, Bn from limestone (L). Node 321 consists of the bulb (B) and volva (V) from A, C, S; node 324 consists of the same samples B, V from L and S.

and explained 24.2% of total variation. The contrast between the samples collected from limestone (L) and those from clay with chert (C) is displayed in FIG. 5. The samples from siliceous soil (S) and argillaceous sand (A) are situated in the center of the 2 × 3 plane. The correlation circle indicates that the direction of the second axis is strongly determined by the Pa/Pn ratio ($r = -0.95$) and the PSC concentration ($r = -0.80$) (FIG. 6). Analysis of the 2 × 3 principal plane shows that the samples taken from limestone soil were characterized by a high PSC level giving a high Pa/Pn ratio. In contrast, the samples from clay with chert contained high amounts of neutral phallotoxin associated with relatively low PCD levels. This phallotoxin composition resulted in a low Pa/Pn ratio. With regard to the samples from siliceous soil and argillaceous sand, the balance between the neutral and acidic phallotoxins is shown by a Pa/Pn ratio of about 1 (TABLE II).

The first discriminant plane of DA relative to the site factor represented 90% of the total variation among the groups. FIGURE 7 clearly displays the discrimination between three clusters. To the right of the plane, cluster L represents the samples from Jurassic limestone. On the top left of the plane, the samples, collected from clay with chert together with those from siliceous soil, form cluster C, S; their centroids are practically superposed. Finally, cluster A, shown on the bottom left of the plane, is constituted by the argillaceous sand samples.The occurrence of these three clusters was confirmed by the classification results reported in TABLE IV. The diagonal of the matrix shows that the samples taken from argillaceous sand (A), limestone (L) and siliceous soil (S)

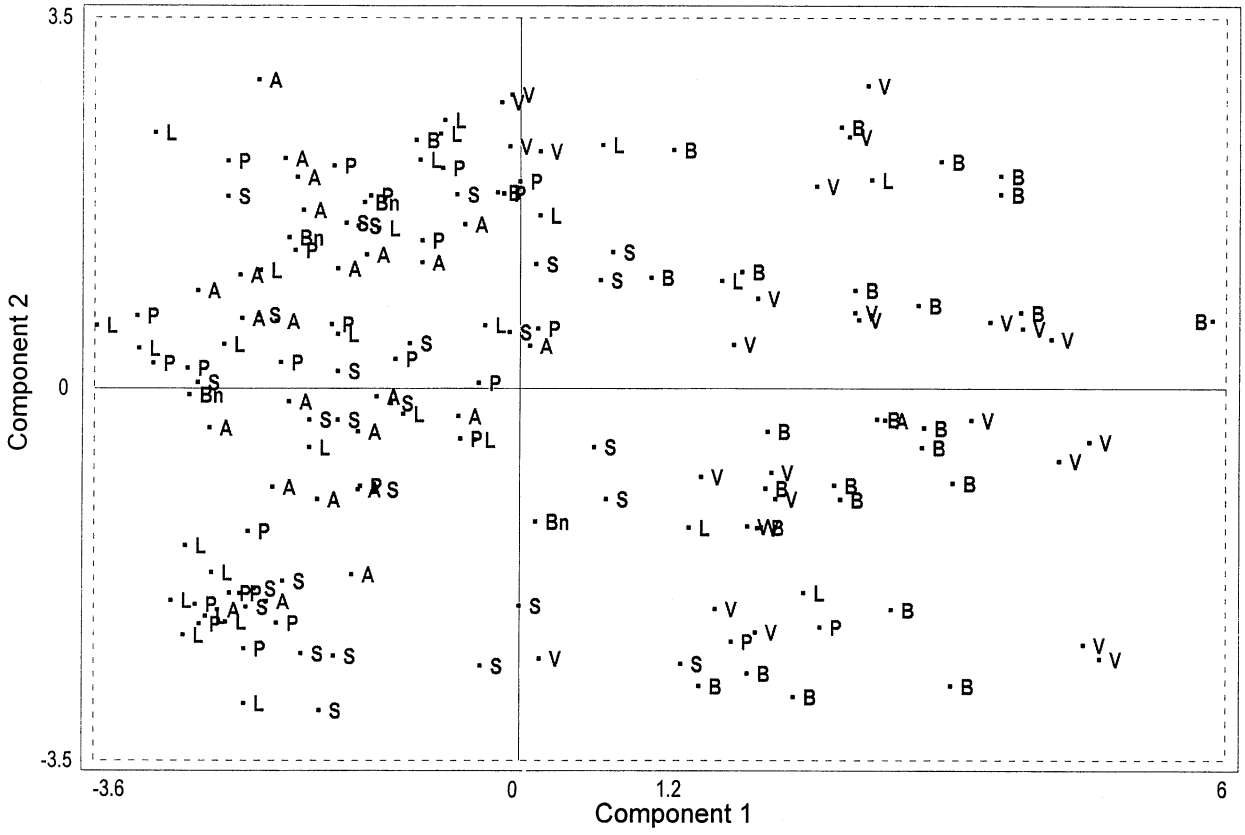

FIG. 2. Plot of the first two principal components of the 164 samples: A, annulus; B, bulb; L, lamellae; P, pileus; S, stipe; V, volva; Bn, button.

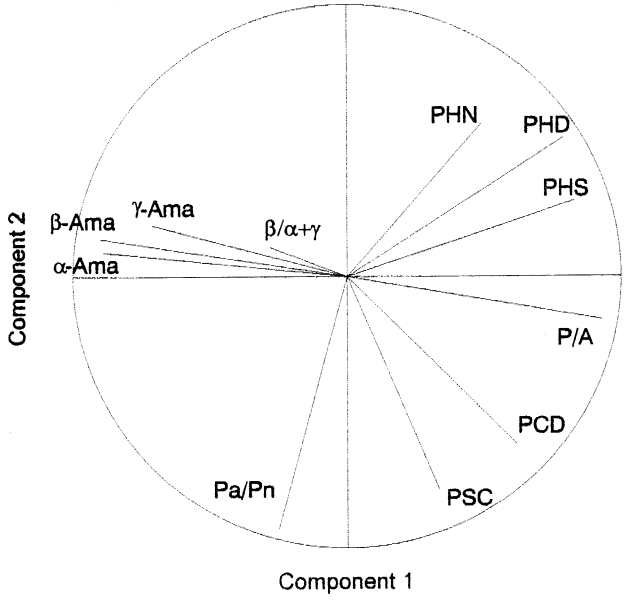

FIG. 3. Correlation circle of 11 variables: P/A, PHS, PHD, β-Ama, α-Ama and γ-Ama are correlated with the first component in FIG. 2. Abbreviations are indicated in TABLE II.

are correctly classified i.e., 100%, 96% and 93%, respectively. The samples collected from the clay with chert are distributed between the three predicted sites: C, 71%; S, 21% and L, 8%. Cluster C, S consisting of the clay with chert and siliceous samples shows 92% (71% + 21%) and 100% (93% + 7%) well-classified samples, respectively. Analysis of the test samples, corresponding to the 30 samples collected from siliceous soil in 1994, indicated that 92% of these samples were classified in the C, S cluster.

DISCUSSION

Efficiency of the extraction procedure.—The advantage of carrying out these assays on fresh material using a 5:4:1 (v/v/v) methanol -water −0.01 M hydrochloric acid extraction medium has been reported (Stijve and Seeger, 1979 ; Enjalbert et al., 1989). The small volumes of extraction medium used in our procedure avoided the inevitable loss of toxins that results from concentrating large initial volumes. A second ultrasonication of tissue and an additional rinse under the same conditions did not recover detectable amounts of residual toxins. The reproducibility of this extraction method was verified by assaying two

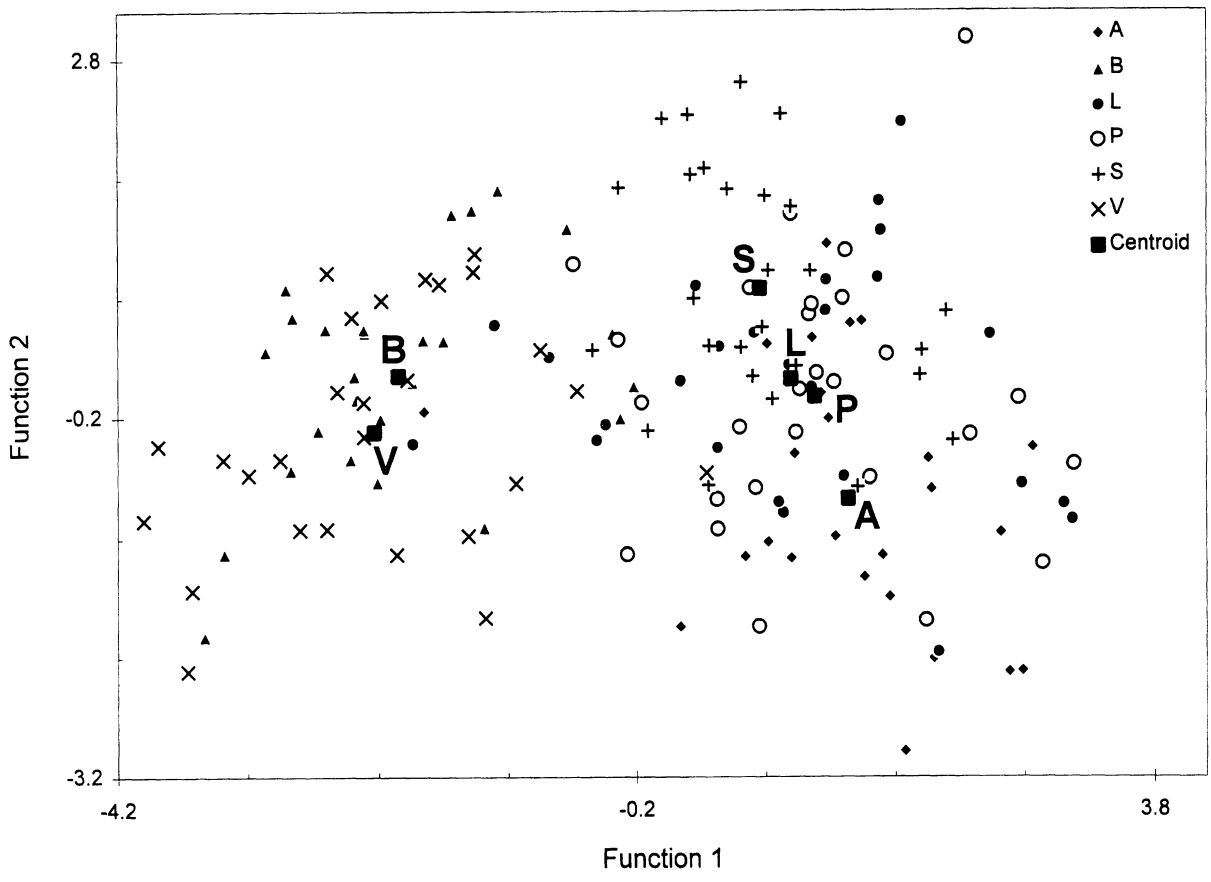

FIG. 4. Discriminant analysis plot for the tissues: A, annulus; B, bulb; L, lamellae; P, pileus; S, stipe; V, volva. Left cluster: B, V; right cluster: A, L, P, S.

extracts of the same sample. The variation coefficient of the toxin amounts was approximatly 4%. These results suggest that this extraction procedure is reliable for histologically different samples presenting, in addition, a large range of phallotoxin and amatoxin concentrations.

Toxin composition of carpophore tissue in relation to collection site.—Statistical analyses of our data support the role of the carpophore tissue in toxin distribution. Based on substantial differences in the amounts of amatoxins and phallotoxins in the carpophore tissues, two groups could be formed: the bulb and volva and the pileus, lamellae, annulus and stipe.

Analyses of the 27 carpophores showed that the bulb and volva contained high amounts of phallotoxins particularly the neutral toxins, i.e., PHD and PHS. The average PHD relative concentrations in the bulb and volva were 33.99 ± 2.31% and 34.29 ± 2.32%, respectively; the mean PHS relative concentrations in the same tissues were 4.73 ± 0.55 and 4.83 ± 0.36%, respectively (± = SEM, n = 27). These findings confirm those reported earlier on the PHD content of the volva (Wieland and Faulstich, 1983; Wieland, 1986; Enjalbert et al., 1989) and the similarities in the distribution of phallotoxins in the volva and the bulb (Enjalbert et al., 1993). However, it should be noted that the average toxin contents of the bulb and volva, accounting for 209.93 ± 10.32 μg/g and 507.17 ± 41.90 μg/g of tissue (wet weight) (± = SEM, n = 27), respectively, were very different. The toxin content in the bulb was two- to threefold lower than that in the volva; in fact, among all the tissues, the bulb was the lowest in toxins. The subdivision of the stipe into two portions, the bulb and the stipe proper, was necessary since the distribution of the toxins in these two parts of the carpophore was clearly different. Moreover, similarities in the toxin composition of the bulb and the volva, in particular the predominance of the neutral phallotoxins over the acidic phallotoxins, were confirmed.

High amatoxin concentration in the pileus, lamellae and stipe are in agreement with previous reports (Bodenmuller et al, 1981; Wieland, 1986; Enjalbert et al., 1993). These three tissues are particularly important to the later stages of the carpophore development and sporulation. With regards to the stipe, more toxin was found in the upper portion than in the lower part of this tissue (data not shown). These

TABLE III. Discriminant analysis: classification results for the tissues

Actual tissues	Predicted tissues: Annulus A	Bulb B	Lamellae L	Pileus P	Stipe S	Volva V	Total
A	64% (16)[a]	4% (1)	12% (3)	4% (1)	16% (4)	—	100% (25)
B	—	67% (18)	7% (2)	—	7% (2)	19% (5)	100% (27)
L	22% (6)	11% (3)	41% (11)	18% (5)	4% (1)	4% (1)	100% (27)
P	26% (7)	4% (1)	29% (8)	22% (6)	15% (4)	4% (1)	100% (27)
S	4% (1)	—	19% (5)	7% (2)	70% (19)	—	100% (27)
V	—	26% (7)	4% (1)	4% (1)	—	66% (18)	100% (27)

[a] Number of samples.

findings are not surprising since the amatoxin concentration in the carpophore varied, with the smallest levels in the bulb and the highest in the pileus and lamellae (TABLE II). Such an amatoxin gradient has also been observed for *A. suballiacea* Murrill specimens (Preston et al., 1982). Furthermore, the stipe was characterized by the nearly equal distribution of β-Ama and α-Ama, i.e., 18.93 ± 1.93% and 18.78 ± 0.65%, respectively (± = SEM, n = 27). Lastly, the large amount of amatoxins and the predominance of β-Ama over α-Ama in the annulus, demonstrated in a previous study (Enjalbert et al., 1993), thus seems to be the rule; the average β-Ama relative concentration represented 24.27 ± 2.18% whereas the mean α-Ama relative concentration accounted for 16.25 ± 0.96% (± = SEM, n = 25).

In addition, the statistical analyses of our data indicate a relationship between the collection site and toxin composition of the tissues. Our collection material could be divided into three clusters depending on the geological site, namely, L, limestone, C, S, clay with chert and siliceous soil and A, argillaceous sand.

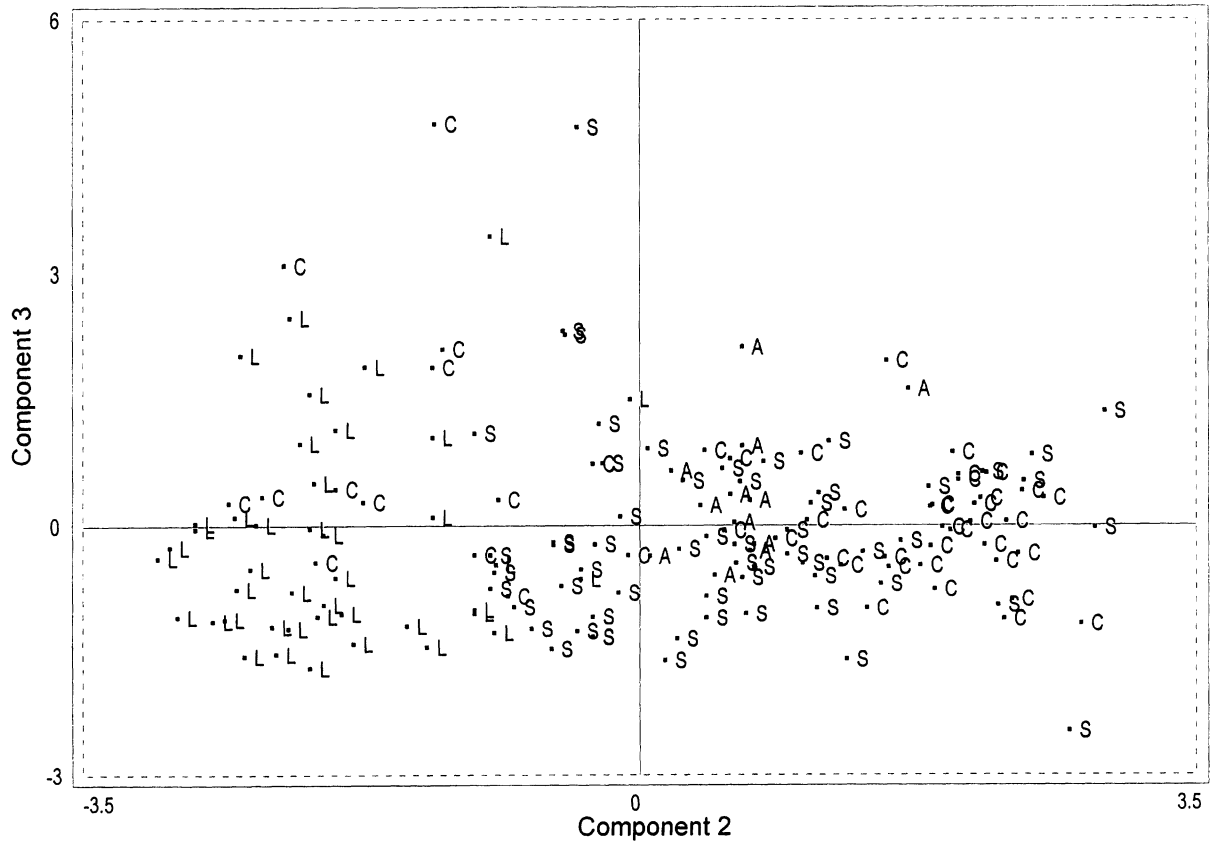

FIG. 5. Plot of the second and third principal components of the 164 samples. Sites: A, argillaceous sand; C, clay with chert; L, limestone; S, siliceous soil.

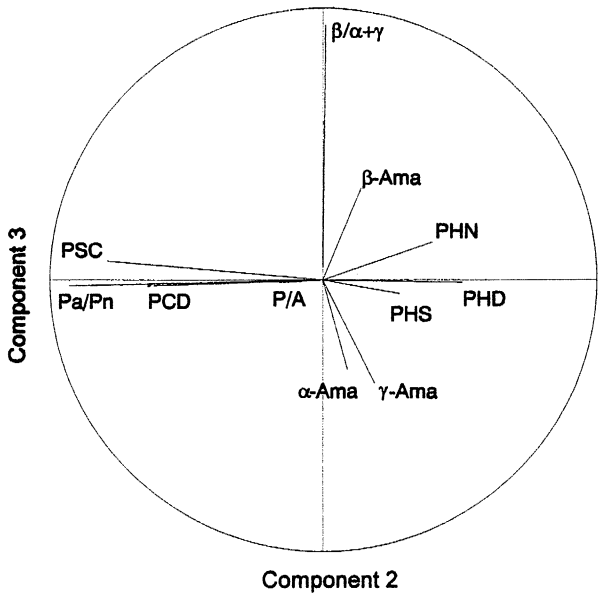

FIG. 6. Correlation circle of 11 variables: Pa/Pn and PSC are correlated with the second component in FIG. 5. Abbreviations are indicated in TABLE II.

The influence of the soil on toxin composition in general and on phallotoxin distribution in particular now seems to be established. Next, we questioned whether the toxin profiles of the tissues were stable. Since the samples were taken from the carpophores collected from siliceous soil in 1993 and in 1994, we were able to analyze the year-to-year stability of the toxin composition for this site. Differences in the carpophore toxin content have been noted as a function of the collection time and the water content (Stijve and Seeger, 1979; Enjalbert et al., 1993). The average values of toxin content of the carpophores harvested in fall of 1993 and 1994 were found to be 326.50 ± 12.07 μg/g and 486.81 ± 24.61 μg/g of tissue (wet weight) (± = SEM, n = 5), respectively. Nevertheless, the results of the DA showed a very similar composition for all tissues from the carpophores collected over this two-year period, since 93% of the tissues taken in 1993 were correctly predicted and 92% of test samples set, corresponding to samples collected in 1994 also belonged to the C,S cluster. Moreover, the main feature of the samples from Jurassic limestone was high amounts of acidic phallotoxins due to elevated PSC levels ranging from 11.24 ± 0.82% to

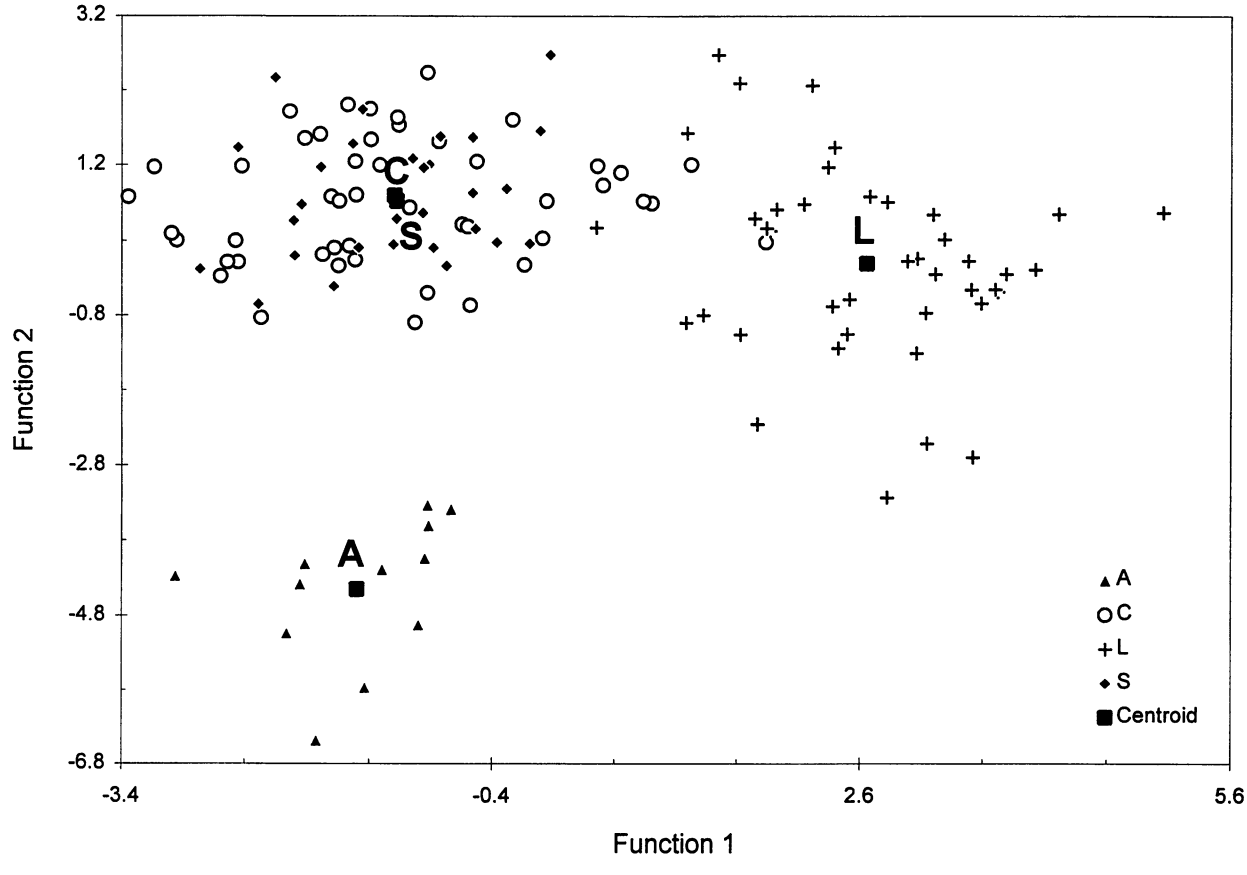

FIG. 7. Discriminant analysis plot for the sites: A, argillaceous sand; C, clay with chert; L, limestone; S, siliceous soil. Top left: cluster C, S; bottom left: cluster A; right: cluster L.

TABLE IV. Discriminant analysis: classification results for the collection sites

Actual sites	Predicted sites: Argillaceous sand A	Clay with chert C	Limestone L	Siliceous soil S	Total
A	100% (12)[a]	—	—	—	100% (12)
C	—	71% (34)	8% (4)	21% (10)	100% (48)
L	—	2% (1)	96% (39)	2% (1)	100% (41)
S	—	7% (2)	—	93% (27)	100% (29)[b]

[a] Number of samples.
[b] Reduced data set with only the samples taken in 1993.

17.66 ± 0.82%. In a previous study of the carpophores collected from the Causse du Larzac, limestone plateau, (Department of Aveyron, France), the prevalence of the acidic phallotoxins over the neutral phallotoxins was also noted (Enjalbert et al., 1989). Besides, the pileus and lamellae taken from the carpophores from the Jurassic limestone presented high α-Ama concentrations i.e., 20.95 ± 1.47% and 21.95 ± 1.49%, respectively (± = SEM, n = 7). Similarities in the toxin distribution between the clay with chert samples and the siliceous soil samples were expected. As a matter a fact, these clayey soils were distinguishable by the presence of the siliceous concretions (chert). The occurrence of this particular element explains the similarity in toxin composition of the samples from this site and those from the siliceous soil. The pileus, lamellae, annulus, and stipe of the C, S cluster were individualized by a high PHD relative concentration (average value 26.09 ± 0.56%) associated with moderate α-Ama amounts (average value 16.89 ± 0.55% (± = SEM, n = 71). This toxin composition led to the substantial predominance of phallotoxins over amatoxins. Finally, the effect of argillaceous sand on toxin composition, only examined in a few carpophores, should be confirmed by investigating additional material before drawing definitive conclusions. Nevertheless, the clear individuality of this cluster A seems to reflect the influence of this type of soil on toxin distribution.

This study suggests furthermore that associated woody plants such as *Corylus avellana, Fagus sylvatica* and *Quercus robur* do not seem to have an effect on the toxin distribution of *A. phalloides* since these species were present in several collection sites. An investigation on the mycorrhizal relationship between *A. phalloides* and some trees such as *Q. ilex* L. and *Pinus pinea* L. has indicated that these hosts do not have an effect on amatoxin concentrations (Andary et al., 1979). Our analyses are in agreement with this report.

Overall, our results indicate that the histologically different parts of the carpophore are characterized by variations in the distribution of the amatoxins and phallotoxins. These differences are likely related, at least in part, to carpophore development and biochemical characteristics of the tissues. The present study also clearly indicates that the geological site influences the distibution of the toxins in the carpophore tissues. Nevertheless, the characteristics of the soil appear to have a greater influence on phallotoxin composition than on amatoxin composition. The variability of the distribution of the toxins in the tissues could also be related to genetic differences in strains of *A. phalloides,* but the relatively small size of the samples collected from each site did not allow us to evaluate the role of this factor. The fact remains that the partitioning of the samples from different geological sites into three distinct clusters; namely, clay with chert and siliceous soil, limestone and argillaceous sand, and the year-to-year stability of the toxin profiles in two of the geological sites (siliceous soil and limestone) are arguments in favor of a determinant role of the soil in the distribution of amatoxins and phallotoxins in *A. phalloides* tissues.

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