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# Toxin Production in *Pyrenophora teres*, the Ascomycete Causing the Net-spot Blotch Disease of Barley (*Hordeum vulgare* L.)*

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**Toxin production in a large number of *Pyrenophora teres* isolates have been investigated. During fungal growth, the pH of the medium decreases from 6.5 to about 3.0. Aspergillomarasmine A is the major toxin excreted into the culture medium. Nonenzymatic acid-catalyzed conversion of aspergillomarasmine A to anhydroaspergillomarasmine A proceeds at low pH and is prevented by repeated titration of the culture medium to pH 6.5. The four possible stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid have been chemically synthesized and their absolute configuration determined. From circular dichroism and NMR spectroscopy and by measurements of specific optical rotation, the LL-form is identified as the stereoisomer produced by *P. teres*. Biosynthetic experiments using radioisotopes demonstrate that the LL-isomer of *N*-(2-amino-2-carboxyethyl)aspartic acid is a direct precursor of aspergillomarasmine A. Consequently, L-configuration is assigned to the two corresponding asymmetric carbon atoms of aspergillomarasmine A. This is in contrast to earlier reports which had indicated D configuration. The phytotoxicity of anhydroaspergillomarasmine A is comparable with that of L-aspartic acid, whereas LL-*N*-(2-amino-2-carboxyethyl)aspartic acid exerts strong phytotoxicity in the bioassays as shown previously for aspergillomarasmine A. The amount of LL-*N*-(2-amino-2-carboxyethyl)aspartic acid which accumulates in the *P. teres* cultures is low, indicating that aspergillomarasmine A is the toxin which plays the major role in the pathological changes associated with the barley net-spot blotch disease.**

Barley (*Hordeum vulgare* L.) grown in temperate humid regions is liable to attack by the fungus *Pyrenophora teres* Drechs. which causes the net-spot blotch disease in barley (1). Infested crop residues and volunteer plants carry over the pathogen from one season to the next (2, 3). *P. teres* produces phytotoxic compounds which are reported to be partly or wholly responsible for the pathological changes following infection (4). Previously, three compounds designated Toxins A, B, and C have been isolated from culture filtrates of the fungus (Fig. 1) (5). Toxin A is *N*-(2-amino-2-carboxyethyl)aspartic acid and has not been isolated from other natural sources. Toxin C is *N*-[2-(2-amino-2-carboxyethylamino)-2-carboxyethyl]aspartic acid and is identical with aspergillomarasmine A isolated from cultures of *Aspergillus flavus oryzae* (6-8), *Colletotrichum gloeosporioides* (9), and *Fusarium oxysporum f. sp. melonis* (10). Toxin B is 1-(2-amino-2-carboxyethyl)-6-carboxy-3-carboxymethyl-2-piperazinone and identical to anhydroaspergillomarasmine A, a lactam of aspergillomarasmine A. The phytotoxin lycomarasmine (11, 12) produced by various fusarium strains (13) is structurally related to aspergillomarasmine A (Fig. 1). Aspergillomarasmines A and B perturb the water balance of the plant cell, and the activity is enhanced by the presence of metal ions, especially ferric ions (14).

Aspergillomarasmines A and B have been suggested as lycomarasmine precursors (Fig. 1) (7). Biosynthetic studies have indicated direct incorporation of aspartic acid into the C4 moiety of lycomarasmine (15). Serine is a likely precursor for the C3 moieties. Upon binding to the active site of the enzyme catalyzing the condensation reaction, the serine may be transformed into dehydroalanine (16, 17). *N*-(2-Amino-2-carboxyethyl)aspartic acid is then envisioned formed by a nucleophilic addition reaction with aspartic acid in analogy with the biosynthesis of *e.g.* L-mimosine (18) and willardine (19).

In the present paper we have analyzed toxin production in a large number of *P. teres* isolates grown under different physiological conditions. The toxins are sequentially produced in the *P. teres* cultures indicating a possible biosynthetic route for their formation. This route has been verified by tracer experiments. The absolute configuration of the *N*-(2-amino-2-carboxyethyl)aspartic acid produced by the fungus and of the corresponding asymmetric carbon atoms in the derived aspergillomarasmine A has been determined and differs from the configurations previously deduced from enzymatic degradation experiments.

## MATERIALS AND METHODS

*Fungal Isolates and Maintenance*—In October 1984 and 1985, 26 monosporial isolates of *P. teres* were obtained from leaf-spots on volunteer barley plants collected on different stubble fields on Sealand and in Jutland. The isolates were numbered 1 to 26. The identity of the isolates was confirmed by microscopic examination. Two forms of *P. teres* may be distinguished dependent on the morphological appearance of the symptoms produced on the infected barley leaves (4). All isolates used in the present study belong to the spot-type. The monosporial isolates were grown in Petri dishes on 4.4% potato-dextrose agar at room temperature under a 12-h near-ultraviolet and 12-h dark cycle and stored at 4 °C in the dark. Small mycelia-covered agar blocks were transferred to fresh agar at appropriate intervals.

The *P. teres* isolate No. 12 have been submitted to the American Type Culture Collection, Rockville, MD and to C. A. B. International, Mycological Institute, Kew, Great Britain and can be acquired under acquisition numbers 76105 and 341970, respectively.

*Toxin Production Using Different Isolates*—Six days after inoculation with *P. teres*, the mycelium developed on a single agar plate was homogenized in 30 ml of water. Aliquots (5 ml) were transferred to Roux bottles each containing 100 ml of modified Fries medium (15) except that the medium contained 30 g of glucose/liter instead

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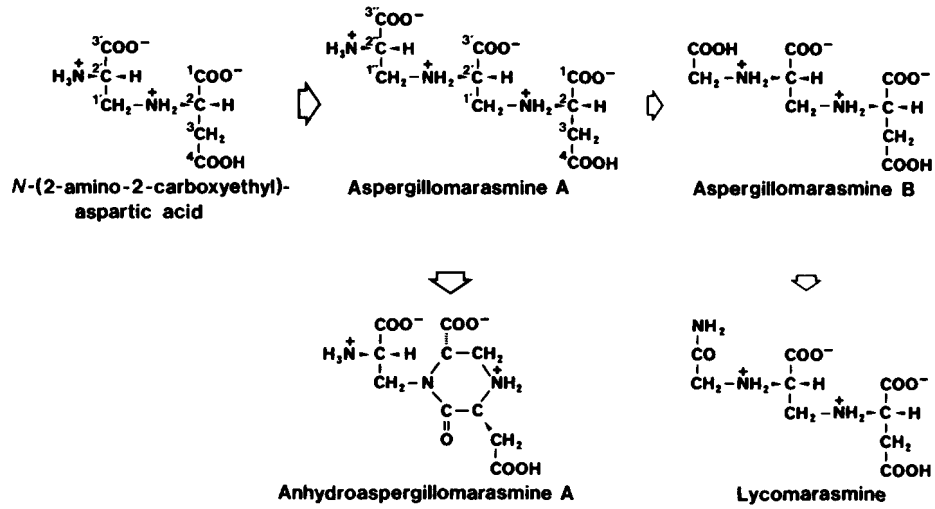


FIG. 1. **The biosynthetic pathway for *P. teres* toxins and related phytotoxins.** Conversions marked with *heavy arrows* are shown in the present study to proceed in cultures of *P. teres*.

TABLE I

*Electrophoretic mobility of P. teres toxins as analyzed by high voltage paper electrophoresis*

All electrophoretic mobilities are indicated relative to aspartic acid.

| | pH 6.5 | pH 3.6 | pH 1.9 |
|---|---|---|---|
| *N*-(2-Amino-2-carboxyethyl)-aspartic acid | 0.95 | 1.10 | 0.60 |
| Aspergillomarasmine A | 1.20 | 1.80 | 0.60 |
| Anhydroaspergillomarasmine A | 1.10 | 1.45 | 0.85 |
| Glutamic acid | 0.90 | 0.30 | 1.10 |

of 30 g of sucrose/liter. The Roux bottles were kept at room temperature without shaking. Aliquots (2 ml) were withdrawn at appropriate time intervals. Each aliquot was centrifuged for 30 min at 165.000 × *g* and the mycelia-free supernatant collected. After measurement of the pH, the supernatant was lyophilized to dryness. The residue was redissolved in 100 μl of $H_2O$ with adequate amounts of $NH_3$ being added to retain neutral pH. Aliquots (5–10 μl) were analyzed by high voltage paper electrophoresis to determine the toxin content.

In addition, *P. teres* was grown in Erlenmeyer flasks containing 100 ml of medium. In parallel experiments, the pH of the culture medium was maintained at pH 6.5 by titration with 1 N NaOH every 2nd day.

*Toxin Analysis*—The toxin composition of the culture filtrates was analyzed by high voltage paper electrophoresis at pH 6.5 (pyridine:acetic acid:water, 60/1/939) and at pH 3.6 (pyridine:acetic acid:water, 1/10/189) as described previously (5). The use of an additional electrophoretic system at pH 1.9 (pyridine:acetic acid:water, 1/4/45) ensured unambiguous identification of the toxins. Toxins and other amino acids were visualized by spraying of the paper electrophoretograms with 0.2% ninhydrin in acetone and heating. The electrophoretic mobilities observed in each system relative to that of aspartic acid is shown in Table I.

*Chemical Synthesis and Isolation of the Four Stereoisomers of N-(2-Amino-2-carboxyethyl)aspartic Acid*—Maleic acid disodiumsalt monohydrate (two times molar excess) and L- or D-diaminopropionic acid hydrobromide were dissolved in $H_2O$ and the pH adjusted to pH 6 with NaOH. The reaction mixture was stirred for 6 h at 80 °C and then applied to an Amberlite IR-120 cation exchange resin ($H^+$ form). Unreacted maleic acid was removed with $H_2O$ and elution of the total amino acids performed with 2 M $NH_3$. The eluate was evaporated to dryness, dissolved in $H_2O$, and applied to a Dowex 1 × 8 anion exchange resin ($OAc^-$ form). Unreacted diaminopropionic acid was removed with $H_2O$ and the diastereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid eluted with a gradient of 0–1 M HOAc. Ninhydrin-positive fractions were monitored by high voltage paper electrophoresis (pH 3.6), and fractions containing pure *N*-(2-amino-2-carboxyethyl)aspartic acid were combined and evaporated to dryness. The two diastereoisomers produced in each experiment using either L- or D-diaminopropionic acid as the starting material were resolved by repeated recrystallization from hot water as monitored by measurements of optical rotation and by $^1H$ NMR spectroscopy. According to the nomenclature system used, the LL- and DL-diastereoisomers differ in configuration at the C4 moiety of the molecule.

A mixture of the $^{14}C$-labeled LL- and DL-diastereoisomers was synthesized by reacting maleic acid disodiumsalt monohydrate (250 μCi, 16 mg) and L-diaminopropionic acid hydrochloride (60 mg) dissolved in 0.7 ml of $H_2O$ for 16 h at 60 °C in a closed vial. The components of the reaction mixture were separated by preparative high voltage paper electrophoresis (pH 3.6) and their location on the electrophoretogram established by autoradiography and by ninhydrin staining of a guide strip. The area containing the mixture of the LL- and DL-stereoisomers was cut out and the isomers eluted with 0.5 M $NH_3$.

*Absolute Configuration Determinations by Circular Dichroism*—The circular dichroism spectra (210–250 nm) of each of the two isomers of *N*-(2-amino-2-carboxyethyl)aspartic acid chemically synthesized from L-diaminopropionic acid were recorded. Each isomer (4 mg) was hydrolyzed in 6 N HCl for 4 h at 120 °C in sealed ampules in an argon atmosphere. The brown clear hydrolyzates were evaporated to dryness. $^1H$ NMR spectroscopy demonstrated the formation of aspartic acid and residual amounts (<5%) of unconverted *N*-(2-amino-2-carboxyethyl)aspartic acid. After evaporation to dryness, the residue was dissolved in $H_2O$ and applied to Dowex 1 × 8 ($OAc^-$ form). After washing with $H_2O$, aspartic acid was eluted with 1 N HOAc. The yield of aspartic acid was 40% as determined by $^1H$ NMR spectroscopy using acetic acid as an internal standard. The circular dichroism spectra of the isolated aspartic acid were recorded and compared with that of authentic L-aspartic acid.

*Biosynthetic Studies Using Stereoisomers of N-(2-Amino-2-carboxyethyl)aspartic Acid*—Fourteen days after inoculation with *P. teres* (isolate No. 12), the mycelium developed on a single agar plate was homogenized in 45 ml of $H_2O$. Aliquots (2 ml) of the homogenate were transfered to sterile Petri dishes each containing 13 ml of Fries medium and one of the stereoisomers (3.6 μmol, 1 ml of sterile filtrated solution). The toxin content was analyzed after a 4-day growth period. Petri dishes inoculated with boiled (10 min) mycelium or without stereoisomer addition served as controls.

Ten days after inoculation with *P. teres* (isolate No. 12), the mycelium developed on two agar plates was homogenized in 45 ml of $H_2O$. Aliquots (7.5 ml) of the homogenate were transferred to sterile Erlenmeyer flasks each containing 50 ml of Fries medium and the $^{14}C$-labeled LL- and DL-diastereoisomers (5.4 μmol, 15 μCi). The pH was maintained at 6.5 by titration with 1 N NaOH. Aliquots (2–5 ml) were made at different time intervals and their toxin content analyzed as indicated above, except that the electrophoretograms were subjected to x-ray autoradiography before development with ninhydrin and that differently sized aliquots were applied to the electrophoretograms to permit optimal detection of the relative toxin content.

*Phytotoxicity Assays*—Five-day-old barley leaves were excised under water and positioned in 1 mM solutions (1 ml) of the four chemically synthesized stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid, in aspergillomarasmine A or in anhydroaspergillomarasmine A for 3 days in a 12-h light/12-h dark regime. The solutions were buffered to pH 7 by addition of ammonia and leaves positioned in water or 1 mM L-aspartic acid served as controls. During

the experiment, deionized water was added when appropriate. Phytotoxicity was monitored visually as the degree of chlorosis and necrosis induced. Two resistant barley cultivars (Nordal and CI 2750) and one sensitive variety (Welam) were used.

## RESULTS

The different isolates of *P. teres* showed a great variation with respect to growth rate on the Fries medium. During growth, the pH of the growth medium decreased. The minimum pH value reached by the isolates varied between 2.8 and 4.5. The isolates which produced the low pH values were found to be those which produced the highest amounts of toxins. Glucose was used as carbon source instead of sucrose because this resulted in a general increase of toxin production. Whereas the total amount of toxin produced varied from one isolate to the other, the variation in the relative content of the different toxins as a function of the growth time appeared the same from one isolate to the other. The *P. teres* isolate denoted No. 12 showed fast growth and produced high amounts of toxins. The pH profile of the growth medium and the toxin content obtained using isolate No. 12 are shown in Fig. 2 and 3, respectively. Aspergillomarasmine A is the first detectable toxin produced. It accumulates between 10 and 16 days after inoculation (Fig. 3*A*), a period where the pH of the culture medium decreases from 3.8 to 2.8 (Fig. 2). A steady accumulation of anhydroaspergillomarasmine A is observed after a culture period of 12 days, and anhydroaspergillomarasmine A is the major toxin produced. A ninhydrin positive component migrating at the position of *N*-(2-amino-2-carboxyethyl)aspartic acid is detectable after 16 days (Fig. 3*A*). However, aspartic acid and *N*-(2-amino-2-carboxyethyl)aspartic acid are not separated using electrophoretic separation at pH 3.6. Analyses using electrophoretic separation at pH 1.9 demonstrate the presence of aspartic acid but only minute amounts of *N*-(2-amino-2-carboxyethyl)aspartic acid (data not shown). This is in contrast to a previous report (5) where *N*-(2-amino-2-carboxyethyl)aspartic acid was found as a major component in the growth medium of *P. teres*. None of the other 25 isolates tested in the present study gave rise to accumulation of *N*-(2-amino-2-carboxyethyl)aspartic acid. Unfortunately, the isolates of *P. teres* originally reported to accumulate *N*-(2-amino-2-carboxyethyl)aspartic acid (5) have all been lost.[1] However, a small voucher sample of the originally isolated *N*-(2-amino-2-carboxyethyl)aspartic acid has been obtained and its identity confirmed by $^1$H NMR analysis.

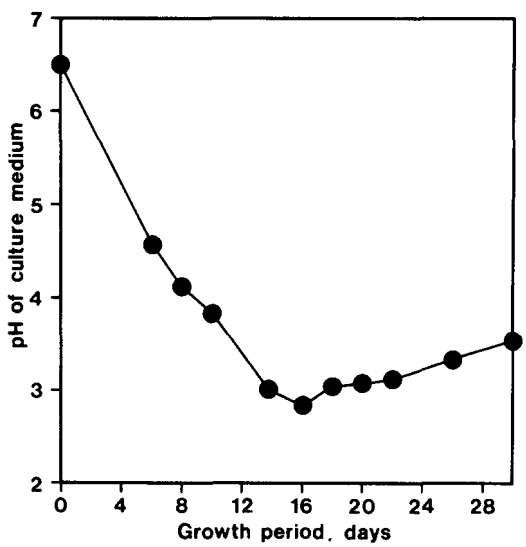


FIG. 2. **The pH profile of the *P. teres* culture medium as a function of the growth period.**

In the earlier studies (5), anhydroaspergillomarasmine A was obtained only in trace amounts and was thought of as an artifact mainly produced during the isolation procedure. Anhydroaspergillomarasmine A may be formed non-enzymatically from aspergillomarasmine A by ring closure in dilute acid (Fig. 1). In the present study, the accumulation of anhydroaspergillomarasmine A is observed in the *P. teres* cultures at pH values below 3.5. The low pH of the culture medium may catalyze a chemical conversion. To test this possibility, a parallel experiment was carried out where the pH of the culture medium was adjusted to pH 6.5 every second day by addition of 1 N NaOH under sterile conditions (Fig. 3*B*). When maintained at constant pH, aspergillomarasmine A is the major toxin produced. Only low amounts of anhydroaspergillomarasmine A accumulate. This experiment demonstrates that the low pH of the growth medium results in a non-enzymatic conversion of aspergillomarasmine A into anhydroaspergillomarasmine A. The ninhydrin-positive spot at the position of *N*-(2-amino-2-carboxyethyl)aspartic acid was due to aspartic acid as demonstrated by electrophoretic analyses at pH 3.6 and 1.9 (data not shown).

The chemical structure of *N*-(2-amino-2-carboxyethyl)aspartic acid indicates that this could be a precursor of aspergillomarasmine A. The failure to detect the accumulation of *N*-(2-amino-2-carboxyethyl)aspartic acid in the culture medium in the present study could indicate a more efficient conversion of *N*-(2-amino-2-carboxyethyl)aspartic acid to aspergillomarasmine A when compared with the isolates used by Bach *et al.* (5). To test this hypothesis, *N*-(2-

[1] E. Bach, personal communication.

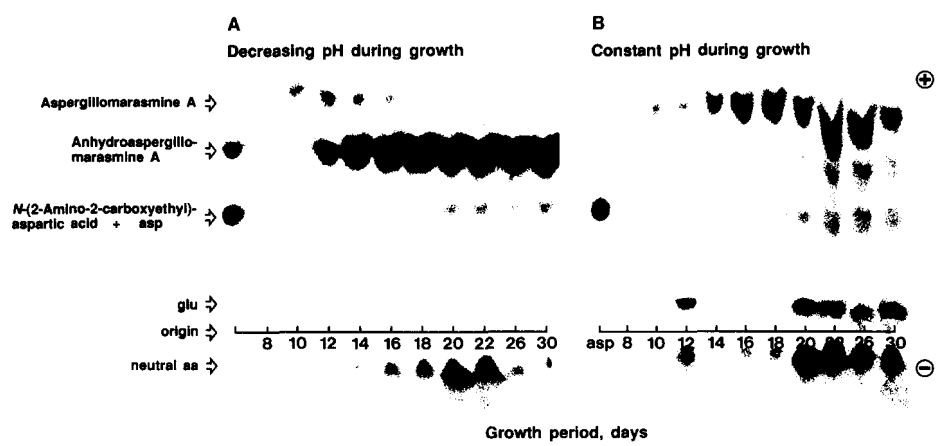


FIG. 3. **Toxin content of the culture medium of *P. teres* as a function of the growth period when analyzed in cultures at variable pH (Fig. 2) (*A*) and maintained at constant pH (*B*).** High-voltage electrophoresis at pH 3.6.

amino-2-carboxyethyl)aspartic acid was chemically synthesized from maleic acid and diaminopropionic acid. At the experimental conditions used, the β-amino group of diaminopropionic acid reacts with maleic acid as demonstrated by $^{13}C$ NMR spectra recorded in NaOD. Thus the signals of the $CH_2$ and CH groups in the diaminopropionic acid moiety of the products are shifted relatively to those in diaminopropionic acid by +6 and −2 ppm, respectively. In the aspartic acid moiety, the $CH_2$ and CH groups are correspondingly shifted relatively to those of aspartic acid by −2 and +8 ppm, respectively. *N*-(2-Amino-2-carboxyethyl)aspartic acid has two chiral carbon atoms (carbon atoms 2 and 2′ (Fig. 1)), and the absolute configuration of the stereoisomer produced by *P. teres* has not previously been determined directly. Reaction of maleic acid with L- and D-diaminopropionic acid results in the formation of the LL and DL and of the LD and DD stereoisomers, respectively, where the first prefix indicates the configuration of carbon atom 2, whereas the second prefix indicates the configuration of carbon atom 2′. The absolute configuration at carbon atom 2′ is defined by the L- or D-diaminopropionic acid used as starting material for the chemical synthesis. Different solubilities of the two stereoisomers produced in each reaction mixture permitted their separation by repeated recrystallization from water. The separation was monitored by measurements of optical rotation and by $^1H$ NMR spectroscopy.

The $^1H$ NMR spectra of the LL- and DL-isomers both exhibit two partially overlapping ABX spin systems. In spite of very similar chemical shifts and coupling constants for the two isomers, the 250 MHz spectra are sufficiently different to allow the determination of the ratios between them in mixture (Fig. 4). Detailed analysis (simulation) of the spectrum of the LL-isomer (Fig. 4*A*) gave chemical shifts of 2.31, 2.51, and 3.39 ppm for the ABX system of the aspartic acid moiety and 2.57, 2.75, and 3.35 ppm for the ABX system of the diaminopropionic acid moiety. The corresponding chemical shifts for the DL-isomer (Fig. 4*B*) were 2.31, 2.51, and 3.37 ppm and 2.52, 2.81, and 3.31 ppm, respectively. To determine the absolute configuration at carbon atom 3, the two stereoisomers were subjected to acid hydrolysis (8). The absolute configuration of the aspartic acid generated from each stereoisomer was determined from the circular dichroism spectra (Fig. 5). Because the LL- and DL-isomers give rise to L- and D-aspartic acid, respectively, this establishes the absolute configuration of each stereoisomer. The yield of aspartic acid reisolated from the hydrolysate was 40%. A major part of the L- and D-aspartic acid generated in the two hydrolysis experiments had racemized as indicated by a smaller mmolar amplitude of the circular dichroism spectra compared with that obtained with authentic L-aspartic acid (Fig. 5). The specific optical rotation of the LL- and DD-stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid were determined to be −29 and +26 °, respectively, when measured in phosphate buffer at pH 7.0. When measured in 1 N HCl, the specific optical rotations were +27 ° and −23 °, respectively. These experimentally determined values agree well with calculated values of +30 ° and −30 ° in 1 N HCl based on the specific optical rotation of the L- and D-isomers of diaminopropionic acid and aspartic acid. According to the Lutz-Jirgenson rule (20), amino acids with L-configuration are more dextrorotatory at lower pH (20, 21). Although the rule is normally applied to amino acids with a single asymmetic carbon atom, the measured changes in the optical rotations upon lowering the pH are in accordance with the rule and thus provides independent evidence for the assignments.

The $^1H$ NMR spectrum of *N*-(2-amino-2-carboxyethyl)aspartic acid previously isolated from the culture filtrates of *P. teres* corresponds to the $^1H$ NMR-spectrum recorded for the LL-stereoisomer (Fig. 4). The spectra of the

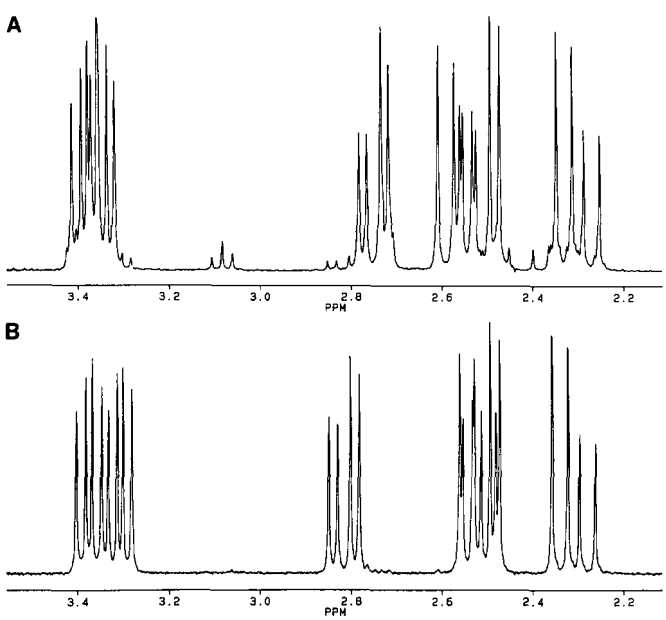


FIG. 4. **$^1H$ NMR spectrum in 0.2 N NaOD of LL-*N*-(2-amino-2-carboxyethyl)aspartic acid (*A*) and of DL-*N*-(2-amino-2-carboxyethyl)aspartic acid (*B*).** The LL-isomer contains a residual amount of the DL-isomer and minor amounts of one or more unknown impurities.

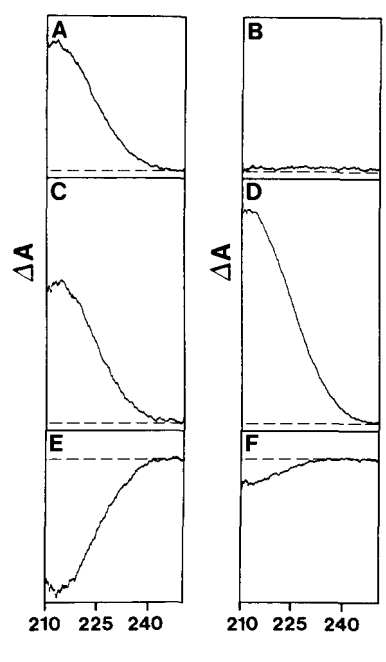


FIG. 5. **Circular dichroism spectra of the chemically synthesized LL- and DL-diastereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid and of the aspartic acid generated by acid hydrolysis.** *A*, authentic L-Asp; *B*, instrument blank; *C*, L-Asp derived from hydrolysis of LL-diastereoisomer; *D*, LL-diastereoisomer; *E*, D-Asp derived from hydrolysis of DL-diastereoisomer; *F*, DL-diastereoisomer. The circular dichroism spectra of the LL- and DL-diastereoisomers and of authentic L-aspartic acid were recorded using 0.5 mM solutions in 1 N HCl. The solutions of the aspartic acids generated by acid hydrolysis were 2.3 mM as determined by $^1H$ NMR spectroscopy.

LL- and DD-stereoisomers will be identical. The specific optical rotation of *N*-(2-amino-2-carboxyethyl)aspartic acid isolated from *P. teres* is −19° when measured in phosphate buffer at pH 7.0 (5). A comparison with the corresponding values indicated above for the LL- and DD-stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid demonstrates that *N*-(2-amino-2-carboxyethyl)aspartic acid produced by *P. teres* must be of LL-configuration.

All four stereoisomers of *N*-(2-amino-2-carboxyethyl) aspartic acid were administered to cultures of *P. teres* in amounts comparable with the amount of anhydroaspergillomarasmine A endogenously formed during a 4-day growth period (Fig. 6). Only the LL-stereoisomer of *N*-(2-amino-2-carboxyethyl)aspartic acid is completely converted to anhydroaspergillomarasmine A. Similar amounts of anhydroaspergillomarasmine A are formed in control cultures to which no stereoisomer is added and in those cultures to which the three other stereoisomers are added. Two possible explanations can be given for the lack of conversion of the LD-, DL-, and DD-stereoisomers. The enzyme system may be specific for the LL-stereoisomer thus preventing the conversion of other stereoisomers. Alternatively, the LD-, DL-, and DD-stereoisomers are not taken up by the fungal cells. Direct uptake is difficult to demonstrate experimentally since growth of *P. teres* results in the formation of a gelatinous mycelial pad. Even after washes and disintegration, it is difficult to evaluate which part of the toxin is located in the fungal cells and which part is absorbed on the surface of the cell wall or entrapped in the gelatinous material. Each eucaryotic microorganism appears to possess two types of transport systems for amino acids: systems that are specific for only one amino acid or a family of structurally related amino acids and general systems shared by a large number of amino acids including amino acid analogues as well as the D-isomers (22). The combined action of these two systems is able to mediate the uptake of a range of different amino acids and their derivatives and is experimentally supported by the detailed studies on the uptake of amino acids in *Saccharomyces cerevisiae* (23). The data presented in Fig. 6 demonstrate that *P. teres* contains an uptake mechanism permitting the uptake of the LL-stereoisomer of *N*-(2-amino-2-carboxyethyl)aspartic acid from the medium. *In vivo*, this stereoisomer would be synthesized by enzymes localized inside the fungal cell. Although not experimentally demonstrated, we consider it likely that *P. teres* also contains an uptake system for the other stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid. All four stereoisomers were recovered unchanged when administered to boiled fungal cultures.

The disappearance of the LL-stereoisomer administered could either indicate its direct incorporation into anhydroaspergillomarasmine A or reflect metabolic conversions involving L-amino acid oxidases, decarboxylases, or transaminases. To discriminate between these two possibilities, the $^{14}C$-labeled LL- and DL-diastereoisomers were chemically synthesized from $^{14}C$-labeled maleic acid and L-diaminopropionic acid. The amount of LL- and DL-diastereoisomers added to the *P. teres* culture (5.4 μmol, 15 μCi) is detectable by autoradiography but not by ninhydrin staining (Fig. 7). After 8 days of growth, efficient incorporation of $^{14}C$ label into aspergillomarasmine A is observed. Because neither aspartic acid, glutamic acid, nor the neutral amino acids are radioactively labeled, the LL-stereoisomer must have been incorporated directly into aspergillomarasmine A. The radioisotope administered is a mixture of two diastereoisomers of which only the LL-form is metabolized (Fig. 6). In agreement with the data presented in Fig. 3*B*, ninhydrin staining of the electrophoretogram indicates accumulation of aspergillomarasmine A after prolonged growth (14 days). Low amounts of anhydroaspergillomarasmine A are also formed. Strong ninhydrin-positive spots corresponding to neutral amino acids and glutamic acid are seen in the later growth stages (28 days). The free amino acids are supposedly liberated into the growth medium by lysis of the mycelial cells. Low amounts of aspartic acid are also formed as indicated by the ninhydrin-positive spot migrating slightly slower than the tracer. An as yet unidentified ninhydrin-positive component is formed at the later growth stages (28 days) (Fig. 7). In some experiments even longer growth periods are needed before this component is accumulated. The component is strongly labeled which suggests a structural relationship to *N*-(2-amino-2-carboxyethyl) aspartic acid or aspergillomarasmine A. The formation of the component was also observed when the *P. teres* cultures were grown in the presence of [$^{14}C$]glucose (Fig. 8). The high electrophoretic mobility indicates that the component is more negatively charged than aspergillomarasmine A at the electrophoretic conditions used. The component does not co-electrophorese with lycomarasmine or lycomarasmic acid.

LL-*N*-(2-Amino-2-carboxyethyl)aspartic acid routinely exerts a stronger phytotoxic effect compared to that of the three other stereoisomers (Fig. 9). The phytotoxic effect of LL-*N*-(2-amino-2-carboxyethyl)aspartic acid is comparable with that of aspergillomarasmine A. The phytotoxic effect of anhydroaspergillomarasmine A is minimal and in most experiments the plants treated with anhydroaspergillomarasmine A

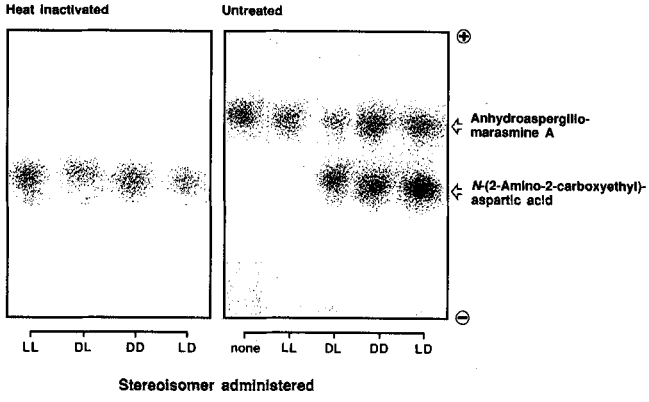


FIG. 6. **Toxin content of the culture medium after administration of the four stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid to heat-inactivated and untreated cultures of *P. teres*.** High-voltage paper electrophoresis at pH 3.6.

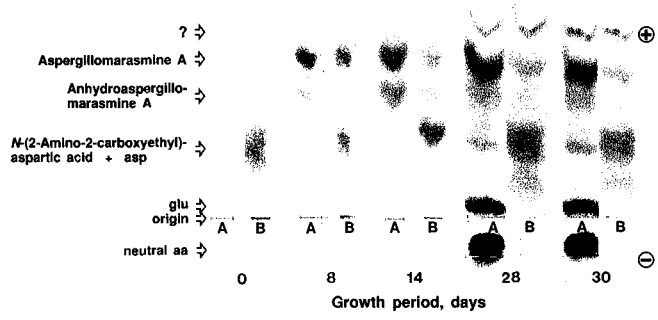


FIG. 7. **Toxin content of the culture medium of *P. teres* as a function of the growth period after administration of $^{14}C$-labeled LL- and DL-*N*-(2-amino-2-carboxyethyl)aspartic acid.** The toxins were separated by paper electrophoresis and visualized by ninhydrin staining (*A*) and autoradiography (*B*). High-voltage paper electrophoresis at pH 3.6.

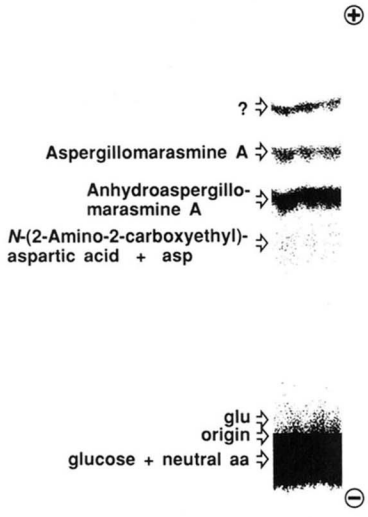


FIG. 8. **Toxin content of the culture medium of *P. teres* after a growth period of 34 days in the presence of $^{14}$C-labeled glucose.** The toxins were separated by paper electrophoresis and visualized by autoradiography. High voltage paper electrophoresis at pH 3.6.

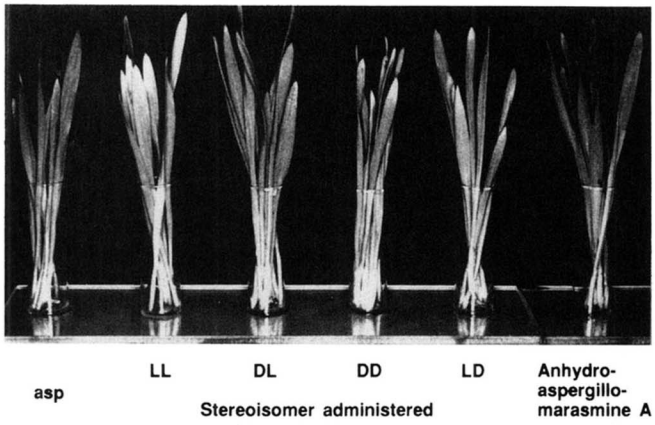


FIG. 9. **The phytotoxicity of stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid and anhydroaspergillomarasmine A on excised leaves of barley cv. Nordal.**

are not distinguishable from the plants positioned in 1 mM aspartic acid or water.

## DISCUSSION

Studies on the sequential *in vitro* production of toxins in *F. oxysporum f. sp. melonis* have suggested that aspergillomarasmine A and aspergillomarasmine B are lycomarasmine precursors as indicated on Fig. 1 (23). The results here presented demonstrate that *N*-(2-amino-2-carboxyethyl)aspartic acid is a direct precursor for aspergillomarasmine A in cultures of *P. teres*. Only the LL-form of the four possible stereoisomers of *N*-(2-amino-2-carboxyethyl)aspartic acid was metabolized by the cultures and the use of the $^{14}$C-labeled stereoisomer demonstrated direct incorporation into aspergillomarasmine A. L-Configuration of carbon atom 2 had earlier been suggested from the isolation of L-aspartic acid upon acid hydrolysis of aspergillomarasmine A (8). In contrast to the results of the present study, the two additional chiral carbon atoms of aspergillomarasmine A were previously deduced to have D-configuration. This conclusion was based on the unreactivity of the 2′ and 2″ amino groups of aspergillomarasmine A with *Crotalus adamantus* L-amino acid oxidase. However, only one of the three tested D-amino acid oxidases were able to attack these amino groups (8). The results of the chemical and biosynthetic studies here presented demonstrate that the asymmetric carbon atoms 2 and 2′ of *N*-(2-amino-2-carboxyethyl)aspartic acid and aspergillomarasmine A and in analogy also of aspergillomarasmine B and lycomarasmine have the L-configuration.

In contrast to the result of a previous study (5), none of the isolates of *P. teres* tested in the present study were found to accumulate significant amounts of *N*-(2-amino-2-carboxyethyl)aspartic acid in the growth medium. All the isolates tested in the present study belongs to the spot-type. The previous study was carried out using spot- as well as net-type isolates but no differences in their ability to produce *N*-(2-amino-2-carboxyethyl)aspartic acid were reported (5). The presently used isolates are apparently more efficient with respect to conversion of *N*-(2-amino-2-carboxyethyl)aspartic acid into aspergillomarasmine A compared with the isolates previously tested. Differences in toxin accumulation have also been reported with isolates of *F. oxysporum* of which one type of isolates produces aspergillomarasmine A whereas a different type further converts aspergillomarasmine A into aspergillomarasmine B and lycomarasmine (24).

LL-*N*-(2-Amino-2-carboxyethyl)aspartic acid and aspergillomarasmine A were found to have similar potencies as phytotoxins, whereas anhydroaspergillomarasmine A was found to have only a marginal effect. From the low amount of *N*-(2-amino-2-carboxyethyl)aspartic acid observed to accumulate in the present study, aspergillomarasmine A is concluded to constitute the most important toxin of *P. teres*. However, the relative amounts of toxins produced in the infected barley leaf may be quite different from those seen in *in vitro* cultures of the fungus. Analysis of toxin production in the infected plant is difficult due to the presence of numerous additional ninhydrin-positive components which comigrate with the toxins. Furthermore, the inhibitory effect of the toxins on the infected plant tissue may be associated with chemical modification, covalent binding, or chelation of the toxins at their active site. This may explain why it has not been possible to re-isolate lycomarasmine from infected plant material.

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