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Transformation of Major Peanut (Arachis hypogaea) Stilbenoid Phytoalexins Caused by Selected Microorganisms

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Article

Transformation of Major Peanut (*Arachis hypogaea*) Stilbenoid Phytoalexins Caused by Selected Microorganisms

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ABSTRACT: The peanut plant accumulates defensive stilbenoid phytoalexins in response to the presence of soil fungi, which in turn produce phytoalexin-detoxifying enzymes for successfully invading the plant host. *Aspergillus* spp. are opportunistic pathogens that invade peanut seeds; most common fungal species often produce highly carcinogenic aflatoxins. The purpose of the present research was to evaluate the in vitro dynamics of peanut phytoalexin transformation/detoxification by important fungal species. This work revealed that in feeding experiments, *Aspergillus* spp. from section *Flavi* were capable of degrading the major peanut phytoalexin, arachidin-3, into its hydroxylated homolog, arachidin-1, and a benzenoid, SB-1. However, *Aspergillus niger* from section *Nigri* as well as other fungal and bacterial species tested, which are not known to be involved in the infection of the peanut plant, were incapable of changing the structure of arachidin-3. The results of feeding experiments with arachidin-1 and resveratrol are also reported. The research provided new knowledge on the dynamics of peanut stilbenoid transformations by essential fungi. These findings may contribute to the elucidation of the phytoalexin detoxification mechanism involved in the infection of peanut by important toxigenic *Aspergillus* spp.

KEYWORDS: *groundnut, detoxification, degradation, prenylated stilbenoid, phytoalexin, Aspergillus*

INTRODUCTION

Interaction of the pathogenic and opportunistic fungi with the plant host is a complex interchange involving pathogenicity of the fungus and resistance by the plant. Phytoalexins are low-molecular-weight antimicrobial compounds that are produced by plants as a response to exogenous stimuli, including fungal invasion. As such, they are involved in an intricate defense system that enables plants to control invading microorganisms.[1] During the initial stages of infection, the fungal pathogen produces proteins and carbohydrates that can elicit plant response[2−4] and may involve the production and accumulation of phytoalexins.[5−7] The fungi, in turn, counteract the presence of phytoalexins by producing enzymes to degrade them.[8,9] A strong correlation between the tolerance of fungal strains to phytoalexins and their pathogenicity on the plant host was observed over 50 years.[10] It was demonstrated that detoxification of phytoalexins by fungal pathogens is crucial for pathogenicity[11] and that the transfer of a single gene encoding a phytoalexin-detoxification enzyme is sufficient to make a pathogen virulent on a new host plant.[12] Genetic evidence for the role of phytoalexins in plant resistance has been reviewed.[11] The metabolism and detoxification of a number of phytoalexins by phytopathogenic fungi have been elucidated for various host plant families, including Cruciferae, Solanaceae, Gramineaceae and Leguminosae.[9] Stilbene phytoalexins are also subjects to biological degradation by plant pathogens. For example, resveratrol, which protects grape plants from pathogens by accumulating at high concentrations,[13] is degraded by *Botrytis cinerea*;[14,15] this fungus produces a hydroxystilbene-degrading enzyme, stilbene oxidase, and has been identified as a laccase, which oxidizes both pterostilbene and resveratrol to produce non-toxic products. Two laccase enzymes of this pathogen modify resveratrol, with one enzyme making it less toxic for the fungus and the other enzyme making it more toxic.[16] Degradation of phytoalexins in peanuts by *Aspergillus flavus* laccases was described by Arias et al.[17] A study of the ability of eight isolates of *B. cinerea* to degrade the stilbene phytoalexins resveratrol and its derivative, pterosilbene, showed a high correlation between phytoalexin degradation and pathogenicity on grapevines.[13] Since the enzymes involved in phytoalexin detoxification are encoded by only a handful of genes, the inactivation of pathogen detoxification genes is a feasible strategy for preventing plant infection.

Peanut, *Arachis hypogaea L.*, is known to produce resveratrol and other stilbene-related phytoalexins, when attacked by a pathogenic fungus.[18,19] Mechanisms of detoxification of stilbenoids by peanut fungi have not been explored despite observations that stilbenoids show antifungal activity under laboratory conditions against *A. flavus*,[20−22] *Aspergillus parasiticus*,[23] *Phomopsis obscurans*, and *Plasmopara viticola*[24] and are capable of inhibiting aflatoxin formation.[25,26] Stilbenoids were

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demonstrated to protect plants from pathogens in a set of experiments, in which expression of resveratrol synthase from grape in several other plants increased their level of disease resistance.[14] In addition to the common stilbenoid detoxification pathway, the hydroxylation of stilbenoid benzene rings or the prenyl moiety,[9] stilbenoids also undergo oligomerization. For example, a study of the metabolism of resveratrol by *B. cinerea* led to the isolation of its dehydrodimer.[14,27] Overcoming the phytoalexin barrier is a crucial step for pathogenicity; in known pathosystems, phytoalexin detoxification is determined by one to five genes. Complete genome sequences for several *Aspergillus* species are available.[28] These sequenced genomes are a valuable resource for identifying homologous and orthologous genes related to phytoalexin detoxification. Elucidation of the biosynthesis of phytoalexins permitted the use of molecular biology tools for the discovery of the genes encoding enzymes of their synthesis pathways and their regulators.[1] Future research is likely to use advanced biotechnology to reveal the mechanisms of plant resistance to fungal invasion and use the information in breeding programs for crop improvement and preharvest aflatoxin contamination control.[29,30] Thus, a logical strategy for reducing or eliminating aflatoxins in peanut and other crops is to prevent *Aspergillus* from invading the plant. However, before this strategy can be tested, a systematic study of the enzymatic detoxification of phytoalexins by important microorganisms needs to be performed. The results of such research may help to better understand the pathway and enzymes involved in the stilbenoid detoxification process.[26,31,32] Knowledge and potential regulation of the action of phytoalexin on invasive toxigenic *Aspergillus* are particularly important in the light of aflatoxin contamination of various agricultural commodities worldwide. The first step for the elucidation of this mechanism is the study of the peanut primary stilbenoid transformations caused by the affiliated soil fungi, such as *A. flavus* and *A. parasiticus*. The dynamics of transformation of major peanut prenylated stilbenoid phytoalexins by common soil fungi has not been reported. The objective of the present research was to evaluate the ability of selected important microorganisms that interact with peanut to detoxify/degrade prenylated stilbenoid phytoalexins and resveratrol.

MATERIALS AND METHODS

Reagents, Materials, and Basic Apparatus. HPLC-grade solvents used in the preparation of the mobile phase were obtained from Fisher (Suwanee, GA). HPLC-grade H_2O was prepared with a ZD20 four-bowl Milli-Q water system (Millipore). HPLC-grade methanol used for media extraction was purchased from VWR (Suwanee, GA). Nunc 48-cell culture wells (Roskilde, Denmark; cat. # 150787) were used in all feeding experiments. Micro weigh boats (25.0 × 8.9 × 7.5 mm) were purchased from VWR (Atlanta, GA; cat. # 76470-181). Soft glass stirring rods (3.17 × 127 mm) were obtained from Fisher (Atlanta, GA).

Reference Compounds. Pure individual stilbenoids, *trans*-arachidin-3 (**1**) and *trans*-arachidin-1 (**2**) (Figure 1), were obtained as described[33] except that preparative HPLC was used as a final purification step rather than preparative TLC. Based on area percent, purity of arachidin-3 and arachidin-1 were 99.2 and 98.1% at 334 and 340 nm, respectively. HPLC separation was achieved by using isocratic mobile phase 3.[19] SB-1, 3-(3,5-dihydroxy-4-[2-isopentenyl]styryl)-4-carbonylmethylbut-2-en-4-olide (**3**), was obtained as described.[34] SB-1 showed 99.7% purity (area percent at 364 nm). Standard of *trans*-resveratrol (**4**) (99% declared purity) was purchased from Sigma. Arahypin-7 (**5**) was made available through the procedure described;[32] its purity was estimated to be at 96.8% (area percent at 347 nm).

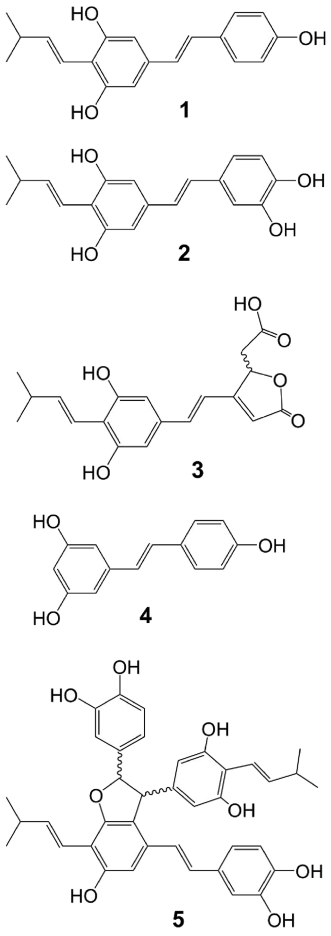

Figure 1. Structures of major peanut stilbenoids: **1**, *trans*-arachidin-3; **2**, *trans*-arachidin-1; **3**, SB-1; **4**, *trans*-resveratrol; and **5**, arahypin-7.

Microorganisms. Selected microorganisms were obtained from the Agricultural Research Service Culture Collection (NRRL; Peoria, IL), NPRL Collection (Dawson, GA), or purchased as indicated below: fungi, *A. flavus* (NRRL 3357; a moderate producer of aflatoxins B_1 and B_2, isolated from moldy peanuts), *A. flavus* (NRRL 29487, L strain; an aggressive high producer of aflatoxins B_1 and B_2, isolated from soil in GA, USA), *A. flavus* (NRRL 21882; a non-producer of aflatoxins, the principal component of the biocontrol product "Aflaguard", isolated from a peanut seed in GA, USA), *A. flavus* (NRRL 18543; a non-producer of aflatoxins, the principal component of the biocontrol product "AF36 Prevail", isolated in AZ, USA), *A. parasiticus* (NRRL 29602; a producer of *O*-methylsterigmatocystin and traces of aflatoxins, isolated from soil in GA, USA), *A. parasiticus* (NRRL 29580; a very high producer of aflatoxins B_1, B_2, G_1, and G_2, isolated from soil in GA, USA), *A. nomius* (NRRL 13137; a high producer of aflatoxins B_1, B_2, G_1, and G_2, isolated from moldy wheat, USA), *A. nomius* (NRRL 26451; a producer of aflatoxins B_1, B_2, G_1, and G_2 with significantly higher G_1 vs B_1 production, isolated from cotton field soil in LA, USA), *Aspergillus tamarii* (NRRL 26090; isolated from soil in GA, USA), *A. tamarii* (NRRL 26071; isolated from soil in GA, USA), *A. niger* (NRRL 326; used in tannic−gallic acid fermentation, CT, USA), *Aspergillus tubingensis* (NRRL 4875; unknown source), *A. caelatus* (NRRL 25528; isolated from peanut field soil in GA, USA), *A. terreus* (NRRL 31139; isolated from a peanut seed in GA, USA), and *Cladosporium* sp. (isolated from a peanut seed, identified to the genus level by Dr. B.W. Horn of the NPRL, ARS, USDA); yeasts, *Saccharomyces cerevisiae* (NRRL Y-12632) and *Candida lipolytica* (NRRL Y-1095); and bacteria, *Bacillus subtilis* (ATCC 6051; purchased from Fisher) and *Rhizobium leguminosarum* (WL 23711, purchased from VWR).

Feeding Technique. Experiments comprised a total of 1728 0.5 mL cell culture wells (0.5 mL working capacity, 2.0 mL total single-cell

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volume) containing essential stilbenoids arachidin-3 (**1**), arachidin-1 (**2**), SB-1 (**3**), and resveratrol (**4**) at 0.3 mM concentrations in 0.5 mL of potato dextrose broth or agar (each experimental well received 44.5 μg of arachidin-3, 46.8 μg of arachidin-1, 51.5 μg of SB-1, or 34.2 μg of resveratrol). To prepare spiked potato dextrose agar (PDA), the stilbenoids were dissolved in 96% EtOH and added to an appropriate amount of molten agar and then cooled to 45−50 °C PDA agar followed by intensive mixing with a magnetic stirrer. The concentration of EtOH in the media did not exceed 1%. Twenty microliters of fungal spores (10^6/mL) were applied to each experimental well and thoroughly distributed on the surface of the agar by a circular motion with a sterile glass rod. Bacteria and yeast cultures were grown on PDA at 30 °C for 5 days in Petri dishes. Suspensions were prepared as follows. Five to twenty milligrams of the cultures were transferred from Petri dishes to weighing boats and weighted to 0.1 mg accuracy. Then, the cultures together with the weighing boats were transferred to glass containers with appropriate volumes of sterile water in a ratio of 1:10,000 and thoroughly mixed by hand shaking; 20 μL of each suspension was added to potato dextrose broth, mixed with a glass rod, and the culture wells were incubated together with PDA and control wells at 30 °C for 5 or 7 days. For comparison, a set of controls containing agar/broth alone, agar/broth with individual phytoalexins but without microorganisms, and agar/broth with individual microorganisms but without phytoalexins, were added to the experimental setup. All the experiments were performed in triplicate. Samples were collected every 24 h and kept frozen at −28 °C up to 2 weeks. The extraction of each sample was performed with 6 mL of MeOH at 22 °C for 18 h without agitation in the dark. Filtered extracts were evaporated to dryness in the stream of N_2, redissolved in 300 μL of MeOH, and filtered again through a glass-fiber filter. From 6 to 8 μL of the filtrates were analyzed by HPLC/MS.

HPLC-Diode-Array Detection (DAD)-MS Analyses. Separations of plant material extracts were performed using a tandem HPLC-MS Surveyor system equipped with MS Pump Plus, Autosampler Plus, a PDA Plus detector (Thermo Electron Corporation, San Jose, CA), and an XSELECT HSS 100 × 4.6 mm i.d., 3.5 μm C18 analytical column (Thermo Electron Corporation). H_2O (A), MeOH (B), 1% of HCOOH in H_2O (C), and 60% of MeOH in H_2O (D) were used in the following gradient: initial conditions, 39% A/0% B/1% C/60% D, changed linearly to 0% A/0% B/1% C/99% D in 18 min, changed linearly to 0% A/99% B/1% C/0% D in 7 min, held isocratic for 4 min, then changed to initial conditions in 0.01 min, and held for 3 min before next injection. The flow rate was 0.7 mL/min. The column was maintained at 40 °C.

MS analyses were performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with an ESI interface and operated with Xcalibur version 1.4 software (Thermo Electron Corporation, San Jose, CA). The data were acquired in the full-scan mode (MS) from m/z 100 to 2000. The heated capillary temperature was 160 °C, APCI vaporizer temperature was 380 °C, sheath gas flow was 60 units, auxiliary gas flow was 5 units, capillary voltage was 10 V, and source voltage was 4.5 kV. In MS^2 analyses, the $[M + H]^+$ ions observed for each chromatographic peak in full-scan analyses were isolated and subjected to source collision-induced dissociation (CID) using He buffer gas. In all CID analyses, the isolation width, relative fragmentation energy, relative activation Q, and activation time were 1.2, 30 or 35%, 0.25, and 30 ms, respectively. Concentrations of *trans*-resveratrol, *trans*-arachidin-1, *trans*-arachidin-3, SB-1, and arahypin-7 in the extracts were determined by reference to peak areas of corresponding pure standards at 307, 339, 335, 362, and 347 nm, respectively.

Data Analysis. The significance of the difference between experimental and control groups was tested by one-way analysis of variance and t test using R.[35] A difference was considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Aspergillus species, the opportunistic peanut pathogens, invade peanut seeds from their surface and grow inside at a certain rate triggering, at the same time, *de novo* phytoalexin production by the seeds[36] and degrading them to less toxic metabolites.[9,11,15,29,37] Within the present research, for the clarity of the experiments, the dynamics of phytoalexin transformation by selected fungal species was studied *in vitro* because the major limitation in our knowledge of phytoalexins is the difficulty in analyzing the events occurring between the plant and the pathogen under natural conditions.[1] The most reasonable experimental setup that, to some degree, resembled the natural fungal invasion was the one described in the Materials and Methods section, when fungal spores were applied on the top of agar with a test stilbenoid evenly distributed in the entire volume of the substrate. Despite the use of modern methods and substantial progress in the study of mechanisms of plant−fungus interactions,[14,17,25,26,37−43] only direct feeding experiments can provide experimental evidence of the dynamics of phytoalexin transformation and the products of their degradation/detoxification. Such a simple strategy is often limited due to the lack of substantial amounts of pure stilbenoids. The significance of stilbene metabolism by fungi is questionable as it is difficult to ascertain whether the degradation of stilbene phytoalexins does correspond to a detoxification process or simply provides fungal pathogens with a carbon source.[14] There are no reliable systematic data on antifungal toxicity of peanut stilbenoid phytoalexins that would allow the description of the stilbenoid degradation pathway as the detoxification pathway.[6,14,17,24,31,40] We note that the correct use of terms "degradation" and/or "detoxification" can be verified only after bioactivity tests of degradation products on the same microbial species.

To study the dynamics of phytoalexin transformation, feeding experiments were conducted, in which microorganisms were fed essential prenylated peanut stilbenoids, arachidin-3 (**1**), arachidin-1 (**2**), and SB-1 (**3**), as well as resveratrol (**4**) (Figure 1). The toxicity of these compounds to the selected microorganisms used in the feeding experiments was estimated before the systematic study was performed. It was done by feeding the microorganisms with increasing concentrations (0.05, 0.1, 0.2, and 0.3 mM) of the test compounds. The highest 0.3 mM concentrations used in most feeding experiments, were visually biologically inactive toward fungal strains tested with the exception of the *Cladosporium* sp. strain, whose growth was inhibited by arachidin-3, and *A. nomius*, whose sporulation was compromised by the same stilbenoid as discussed in the text below. The 0.3 mM concentration was selected since that was the highest concentration available without the test compounds' precipitation in the nutritious medium used.

The microorganisms tested in the course of present research are important to the peanut plant. The *Aspergillus* species differed from each other by their toxigenic potential as it is indicated in the Materials and Methods section. *Rhizobium* species are important nitrogen-fixing bacteria of peanuts,[44] while *Bacillus subtilis* was identified as the factor of internal seed infection.[45] *Cladosporium* and yeast species are not affiliated with peanuts and were tested for comparison. However, in context of the present research, *Cladosporium* spp. are interesting objects for testing since some stilbenoids were demonstrated to inhibit growth of a *Cladosporium* sp.[46]

Our previous research[31] demonstrated that when the same fungal and bacterial species (listed in the Materials and Methods section) were used as phytoalexin elicitors in peanut seeds, the production of major peanut stilbenoids, resveratrol, arachidin-1, and arachidin-3, was detected at high levels. However, the

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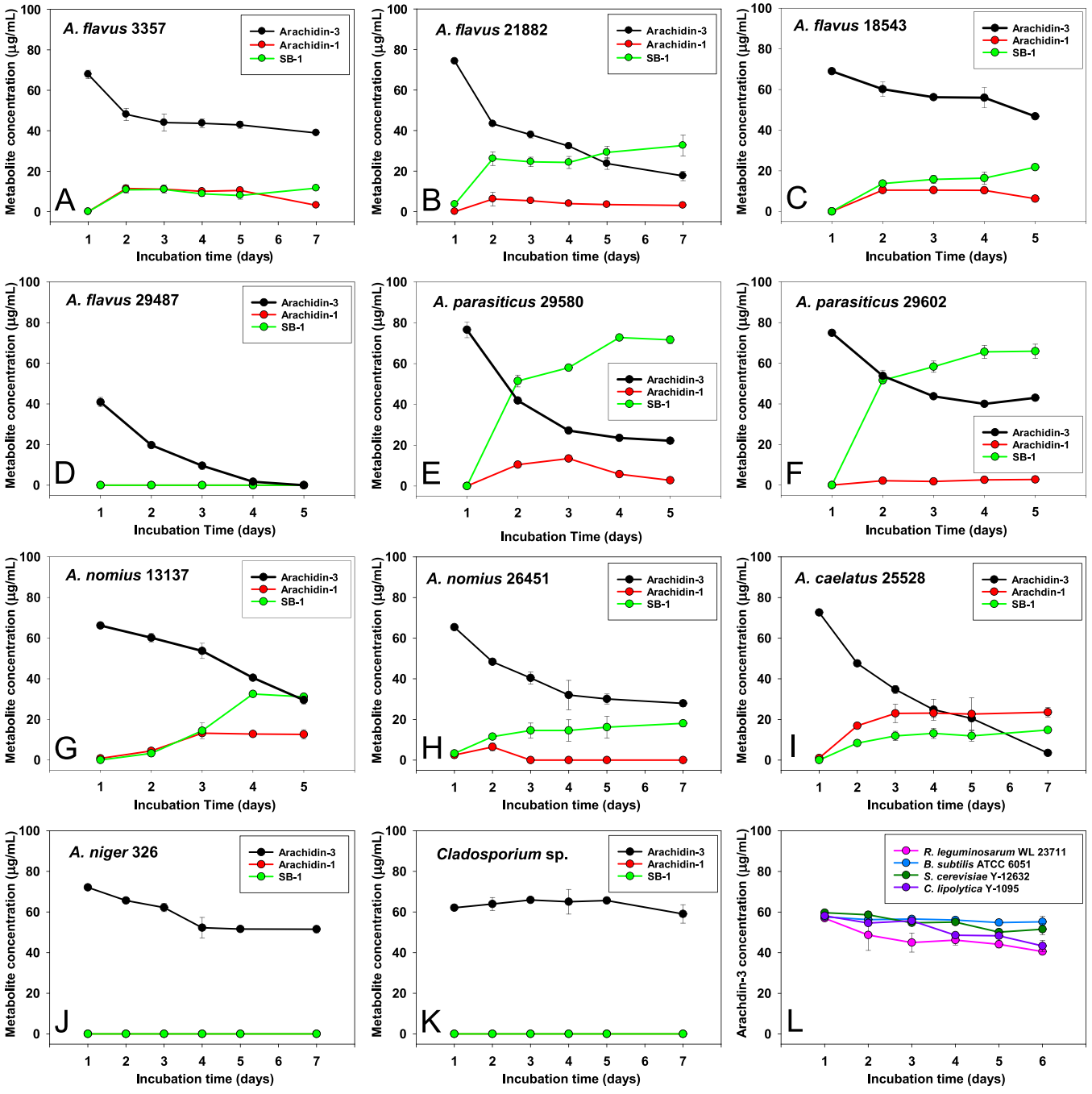

Figure 2. (A–L) Dynamics of transformation of arachidin-3 (**1**) by selected microorganisms. The control graph is not shown since the concentrations of arachidin-3 in the course of incubation were not significantly different from the initial level. Numbers that follow the fungal species names are the Culture Collections numbers listed in the Materials and Methods section.

accumulation of SB-1 was observed only in the presence of *Aspergillus* spp. (*A. caelatus*, *A. niger*, *A. flavus*, and *A. nomius*). These facts suggest that *Aspergillus* spp., compared to other microorganisms tested, may have a different pathway to degrade phytoalexins and that SB-1 may be a product of arachidin-3 and/or presumably intermediate arachidin-1.[34]

Present research demonstrated that *Aspergillus* spp. from section *Flavi* (*A. flavus*, *A. parasiticus*, *A. nomius*, and *A. caelatus*), were capable of degrading one of the key peanut phytoalexin, arachidin-3, into its hydroxylated derivative, arachidin-1, and a benzenoid, SB-1 (Figure 2A–I); all de-novo-formed stilbenoids retained their *trans* configuration in all the experiments, which was evident from the UV absorption spectra and times of their chromatographic retention. *A. flavus* NRRL 3357, a moderate aflatoxin producer, had a mediocre capacity to degrade arachidin-3 as seen from Figure 2A. The most rapid change in concentrations of **1** and **3** occurred within 48 h of incubation, which is likely associated with the fungal colony formation and the excretion of digestive enzymes. About equal low concentrations of **2** and **3** were determined throughout the incubation time. Also, a noticeable concomitant increase of the **3** concentration at the expense of the **2** concentration was observed between 5 and 7 days (Figure 2A). Another two *A. flavus* strains (Figure 2B,C) demonstrated similar results,

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although the conversion degree of **1** into **3** was significantly higher with more aggressive *A. flavus* NRRL 21882; the most significant change in concentrations of **1** and **3** occurred after 48 h of incubation, and concomitant accumulation of **3** with virtually unchanged, very low level of **2** occurred, which is reflected in Figure 2B. However, *A. flavus* NRRL 29487, a highly aggressive producer of aflatoxins (Figure 2D), almost completely degraded **1** within 96 h; this was the only strain of all tested that did not leave any detectable traces of **2** and/or **3** at any time during its incubation.

Toxin-producing *A. parasiticus* strains tested (Figure 2E,F) differed from *A. flavus* strains (Figure 2A−C) by significantly higher rates and levels of compound **3** formation. Also, a high aflatoxin producer, *A. parasiticus* NRRL 29580, showed a substantially higher rate of **1** degradation compared to the *O*-methylsterigmatocystin producer, *A. parasiticus* NRRL 29602. In experiments with the latter strain, compared to the former strain, only small quantities of **2** were observed at all sampling times.

A. nomius is commonly isolated from insects and is a known entomopathogen; it is also a producer of B- and G-type aflatoxins.[47,48] Two strains of *A. nomius* (Figure 2G,H) demonstrated similar patterns of **1** degradation compared with *A. flavus* (Figure 2A−C). After 72 h of incubation, **2** was detected at almost unchanged concentrations for the next 48 h (Figure 2G). At the same time, the concentration of **3** doubled.

An example of the dynamics of degradation/detoxification of **1** by *A. caelatus* is shown in Figure 3. The fragments of typical chromatograms demonstrate the gradual decrease of the concentration of **1** in the course of the experiment and the formation of compounds **2** and **3** (Figure 3). The positive identification of these stilbenoids was verified by a comparison of their chromatographic retention times, UV, and MS data with corresponding data obtained for authentic stilbenoid standards. Compounds **2** and **3** emerged simultaneously as seen from Figures 2I and 3, and the ratios of concentrations of these stilbenoids remained virtually constant throughout the incubation time. One may infer parallel mechanisms of enzymatic conversion of **1** into **2** and **3**. At the end of incubation, the concentration of **1** dropped from an initial value of 89 μg/mL to almost zero. Therefore, compared with other species tested, *A. caelatus* seems to be the most potent species on a par with *A. flavus* NRRL 29487 in terms of stilbenoid degradation (Figure 2D,I). The concomitant increase of levels of **2** and **3** was observed. However, probably due to a non-enzymatic degradation, the combined concentrations of **2** and **3** were significantly lower than the predicted theoretical yield. *A. caelatus* was also the only species among tested that at any given time produced higher concentrations of **2** compared to **3**.

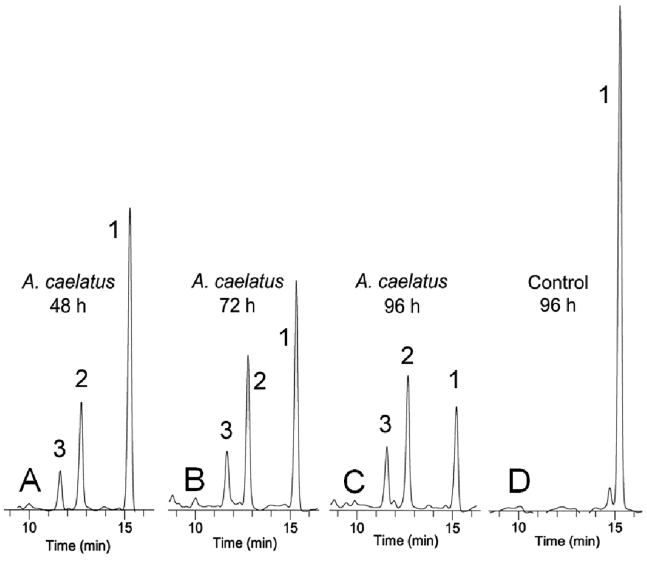

Figure 3. HPLC of extracts of the PDA medium spiked with arachidin-3 (**1**) and incubated with *A. caelatus* NRRL 25528. Plates A−C show gradual degradation of arachidin-3 with the formation of arachidin-1 (**2**) and SB-1 (**3**). Plate D represents control, the spiked medium without the fungus. The concentration of arachidin-3 in control is 67 μg/mL of the medium. The peaks are represented by total light absorbance from 200 to 800 nm. The chromatograms are scaled for direct comparison of stilbenoid concentrations. *A. caelatus* means *A. caelatus* NRRL 25528.

In contrast to the above species, *A. niger* from section *Nigri* was incapable of changing the structure of **1** (Figure 2J); **2** and/or **3** were not detected at any time during the experiments. The inability of *A. niger* to degrade **1** (Figure 2J), compared to other *Aspergillus* spp. tested, was surprising since in preliminary tests, this strain was on a par with other species as a potent elicitor of all major stilbenoids in peanut seeds, including SB-1 (Sobolev, 2008). Apparently, *A. niger* was unable to convert **1** into compounds **2** or **3**. One may suggest that **1** may be degraded to other, yet unidentified stilbenoid metabolites. However, this is an unlikely scenario since the reduction of the initial concentration of **1** was only about 22% after 7 days of incubation. In addition, no extra chromatographic peaks were detected in the experimental extracts compared to the control (*A. niger* grown on PDA agar without **1**). It is reasonable to suggest that compared to other strains of *Aspergillus* from section *Flavi*, the conversion of **1** to **3** is host-dependent in the *A. niger* strain tested. One may also suggest that peanut seeds provide coenzymes to the *A. niger* enzymatic system making it functional in terms of **1** to **3** conversion. Because the structure of **3** is closely related to but-2-en-4-olide synthesized de novo by the legume *Pericopsis elata*,[49] a logical suggestion is that **3** is not only a possible product of degradation of **1** by *Aspergillus* spp. from section *Flavi* but also a peanut metabolite, whose production in seeds is elicited by all *Aspergillus* species tested, including *A. niger*. However, this suggestion is unlikely founded since **3** was detected in our previous experiments only when *Aspegillus* fungi were present.[31,36] A detailed study of the "unusual" behavior of **3** was outside the scope of this study, but such a study is planned and may be helpful for a better understanding of the mechanism of plant−fungus interactions.

Other fungal and bacterial species tested also were incapable of changing the structure of **1** (Figure 2K,L); **2** and/or **3** were not detected during the experiments. The initial concentration of **1** did not significantly change during the incubation with the *Cladosporium*, *S. cerevisiae*, *C. lipolytica*, and *B. subtilis* strains that are not associated with peanut diseases (Figure 2K,L). Similar results were obtained with *R. leguminosarum*, a mutualistic symbiotic bacterium, which is affiliated with the peanut plant and has the ability to fix free nitrogen from the air (Figure 2L). Such results were expected since, by its nature, a symbiotic organism should not compromise the host's defensive system, which in peanuts, is associated with the production of prenylated stilbenoids.

Arachidin-1 was tested against the same fungal strains (Figure 4); for a comparison with *A. niger* NRRL 326, the *A. tubingensis* NRRL 4875 strain from section *Nigri* was also used in the

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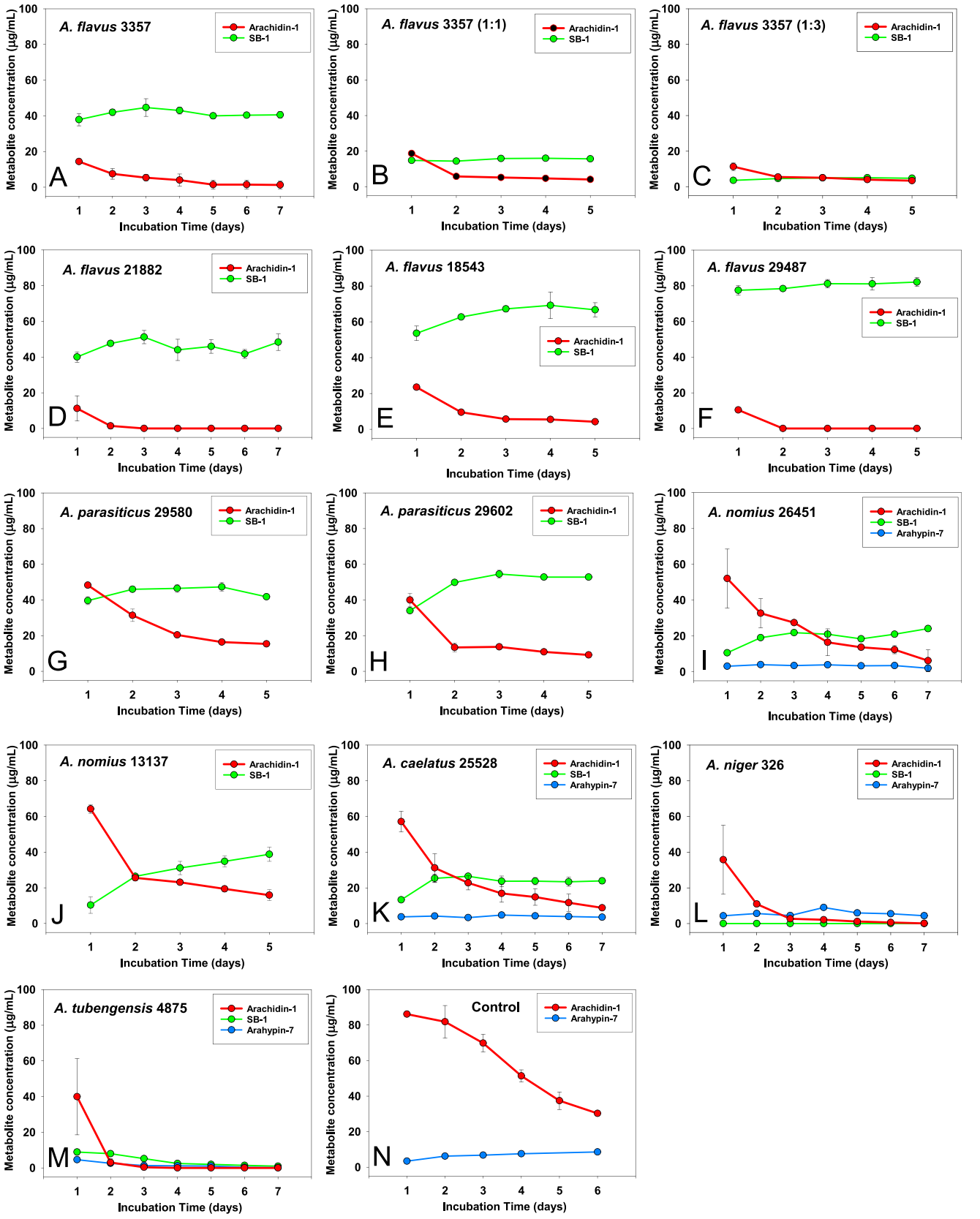

Figure 4. (A–N) Dynamics of transformation of arachidin-1 (**2**) by selected microorganisms. (1:1) and (1:3) marks in plates B and C mean dilution of 1 part of spiked (0.3 mM of arachidin-1) molten agar with 1 or 3 parts of control (blank) molten agar, respectively. Numbers that follow the fungal species names are the NRRL Collection numbers.

feeding experiments. Compared with **1**, fed to *A. flavus* (Figure 2A–D), compound **2**, at the same concentration of 0.3 mM, was easier to convert to **3** by the same fungi (Figure 4A,D–F) as evidenced from prompt significant reduction of **2** and

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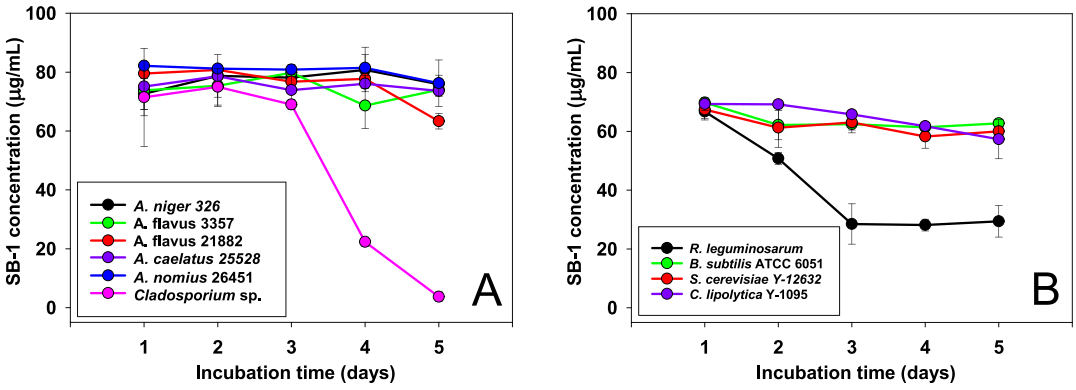

Figure 5. Dynamics of transformation of SB-1 (**3**) by selected microorganisms. The Control graph is not shown since the concentrations of SB-1 in the course of incubation were not significantly different from the initial level. Numbers that follow the fungal species names are the Culture Collections numbers listed in the Materials and Methods section. *R. leguminosarum* means *R. leguminosarum* WL 23711.

concomitant growth of **3** values at early times of incubation. Similar conversion patterns were observed at lower initial concentrations of **2** (Figure 4B,C), at 0.15 and 0.075 mM levels, respectively. Both *A. parasiticus* strains (Figure 4G,H) differed from *A. flavus* basically by a slower initial conversion rate of **2**. *A. nomius* and *A. caelatus* strains (Figure 4I−K) also demonstrated gradual conversion of **2** within the incubation time with the concomitant formation of **3**. A proposed sequence of **3** formation from **2** and the enzymes involved was suggested earlier: the *o*-dihydroxybenzene moiety in **2** (Figure 1) might be oxidatively cleaved by catechol 1,2-dioxygenase to produce a *cis*,*cis*-muconic acid derivative followed by the muconate cycloisomerase enzymatic lactonization of the muconate and final protonation to form **3**.[34]

A. niger and *A. tubingensis* (Figure 4L,M) demonstrated similar patterns of prompt disappearance of **2** added to the growth medium. While **3** was present in all the experimental wells at all sampling times with *A. tubingensis* (Figure 4M), this compound was not detected at any time during the incubation with *A. niger* (Figure 4L). Prompt disappearance of **2** cannot be explained by natural degradation of this compound in the control well (Figure 4N) since its decline was relatively slow and at 72 h did not exceed 23% of the initial value. In general, **1** demonstrated substantially higher stability than **2** under the experimental setup. Gradual formation of **5** in the control wells gives a clue that this dimer is formed from two molecules of arachidin-1, non-enzymatically. It is reasonable to suggest the same origin of arahypin-7 in the experiments with less aggressive strains (Figure 4I,K), when the original stilbenoid is not promptly involved in the interaction with the fungal strains.

In another set of experiments, the microorganisms were fed with **3** (Figure 5) that is suggested to be one of the end-products of compound **1** detoxification.[34] None of the species tested, with the exception of *Cladosporium* and *R. leguminosarum*, were able to degrade/consume **3** (Figure 5A,B). Therefore, it is reasonable to suggest the lack of substantial toxicity of **3** to *Aspergillus* and most of the bacterial species tested. The presence of *Cladosporium* dropped the initial concentration of **3** to almost zero in 5 days of incubation (Figure 5A). In preliminary experiments, the concentrations of all stilbenoids tested were chosen empirically so that fungal growth was not inhibited. Visually, stilbenoid **2** did not affect fungal growth in wells with *Aspergullus* spp. (Figure 6) at a concentration of 0.3 mM, which was the highest concentration available without stilbenoid precipitation. However, growth of *Cladosporium* was suppressed

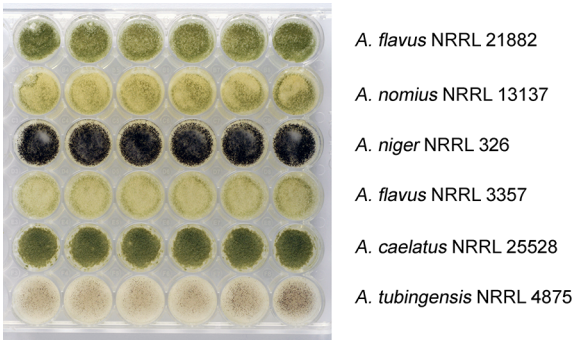

Figure 6. Experimental wells with the fungal species grown for 48 h on the PDA medium with arachidin-1 (**2**).

by 0.3 mM of **1** (Figure 7; *A. nomius* NRRL 26451 is shown for comparison). At present, the exact mechanism of degradation of **3** by *Cladosporium* and *R. leguminosarum* is unknown.

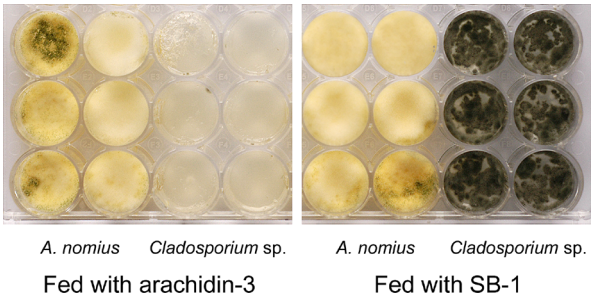

Figure 7. Experimental wells with *A. nomius* NRRL 26451 and a *Cladosporium* sp. grown for 72 h on PDA agar with arachidin-3 (**1**) and SB-1 (**3**).

Degradation of resveratrol by the fungal strains was rapid (Figure 8); in most experiments it was complete after 72−96 h. *A. niger* and *A. tubingensis* (Figure 8C) degraded 100% of resveratrol within 48 h. Products of resveratrol degradation were not monitored. The higher rate of resveratrol degradation compared to the arachidins tested was expected since resveratrol is not considered a peanut phytoalexin with antifungal properties.[24] *Aspergillus* from section *Flavi* (Figure 8A,B) were less potent than *Aspergillus* from sections *Nigri* and *Terrei* (Figure 8C). Two additional *A. tamarii* strains tested (Figure 8B) were the least potent. In contrast with the *Aspergillus* fungal

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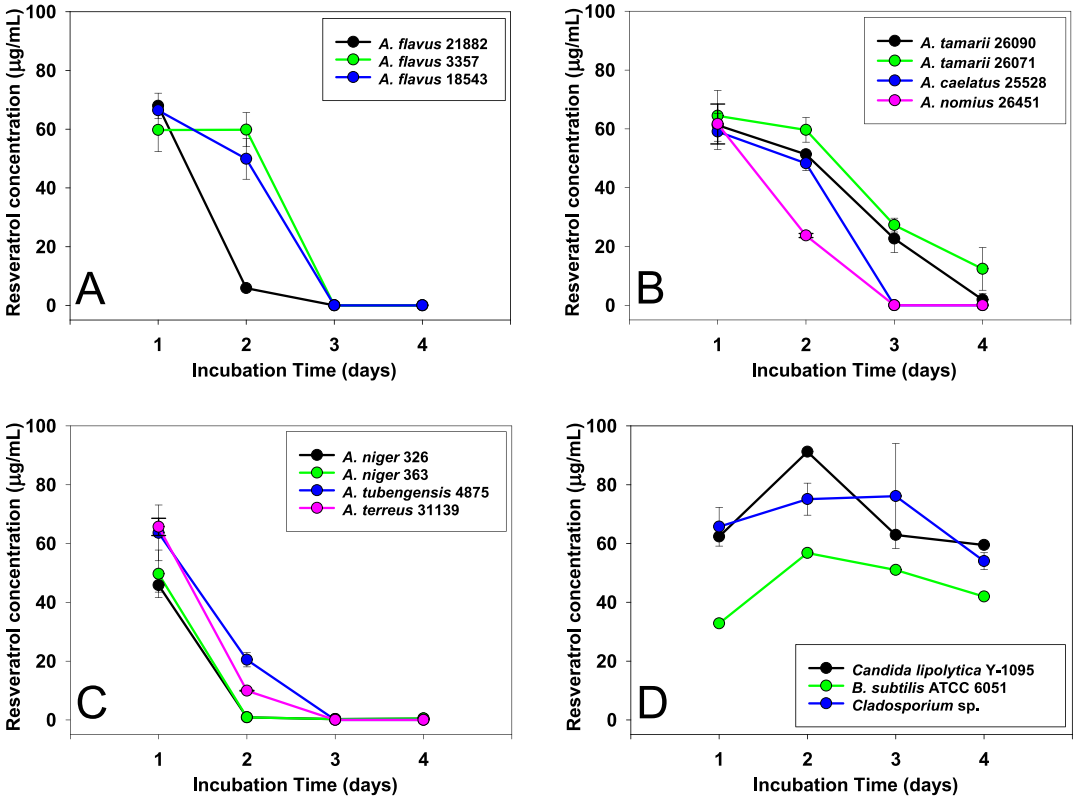

Figure 8. Dynamics of transformation of resveratrol (**4**) by selected microorganisms. The control graph is not shown since the concentrations of resveratrol in the course of incubation were not significantly different from the initial level. Numbers that follow the fungal species names are the Culture Collections numbers listed in the Materials and Methods section.

strains, the *Cladosporium* sp. and bacterial species tested (Figure 8D) did not show a substantial decline of resveratrol within the experimental time.

In summary, present research quantitatively revealed the concomitant degradation of arachidin-3 into its more polar hydroxylated homologs, arachidin-1 and/or SB-1, as well as similar degradation of arachidin-1 into SB-1. Statistically significant disappearance of these de-novo-formed compounds was not detected. Therefore, an educated suggestion that the observed stilbenoid transformations can be attributed to the stilbenoid detoxification is logical. Arachidin-1 was degraded by the same fungal species at higher rates compared to arachidin-3 with the formation of SB-1. *In vitro* experiments, *A. niger* from section *Nigri* as well as other fungal and bacterial species tested, were incapable of changing the structure of arachidin-3 in contrast to *Aspergillus* spp. that are known to invade preharvest peanut seeds. A better understanding of the detoxification mechanisms employed by fungi to compromise the plant's defense system could lead to new strategies for preventing plant invasion by the fungi that produce aflatoxins.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

HPLC, high-performance liquid chromatography; HPLC/MS, high-performance liquid chromatography-mass spectrometry; PDA, photodiode array (detector); APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; PDA agar, potato dextrose *Agar*

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