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MASTERARBEIT

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„Biotransformation and utilization of drimane sesquiterpenes by endophytic microorganisms“

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I GENERAL INTRODUCTION

1. Pepper bark tree (*Warburgia ugandensis*)

Warburgia ugandensis Sprague (= *Warburgia breyeri* Pott) is a highly aromatic evergreen tree within the family *Canellacae* with a characteristic bitter and peppery taste (1) and a wide range of medicinal properties. It is endemic to South East Africa and distributed in forests at altitudes between 1000 m to 2000 m, known under common names as the pepper-bark tree or greenheart (2).

Of a particular importance is its use in traditional medicine. As far as is known, all parts of the plant have medicinal properties and have been applied in the treatment of various diseases. Most frequently, bark and roots are used to treat diarrhea, coughing, colds, general muscular pains, and internal wounds, loss of appetite, malaria, syphilis, gonorrhea, stomachache, throat and chest infections, as well as skin diseases, among others. The plant material is sometimes only chewed, in other cases applied as powder, but usually boiled or soaked in water (3). Furthermore, stem bark and roots are the most often used plant parts to treat tuberculosis and diseases that compromise the immune system, especially AIDS. Leaves of *Warburgia ugandensis* possess similar albeit weaker properties compared to stem bark and roots. The main applications comprise baths for skin diseases and hot infusions against malaria (4), or other fevers (5). Apart from in traditional medicine it is also used in the household (2).

Pharmacological studies have confirmed antibacterial, antifungal (4, 6), antimycobacterial (1), cytotoxic (4, 7), antiplasmodial (5, 8), antitrypanosomal (7, 8) as well as *in vitro* (9) and *in vivo* (10) antileishmanial activities of *Warburgia ugandensis* extracts.

Phytochemical analyses identified drimane sesquiterpenes, which are characterized by α, β-unsaturated carbonyl functions in a *trans*-decalin ring system (1):

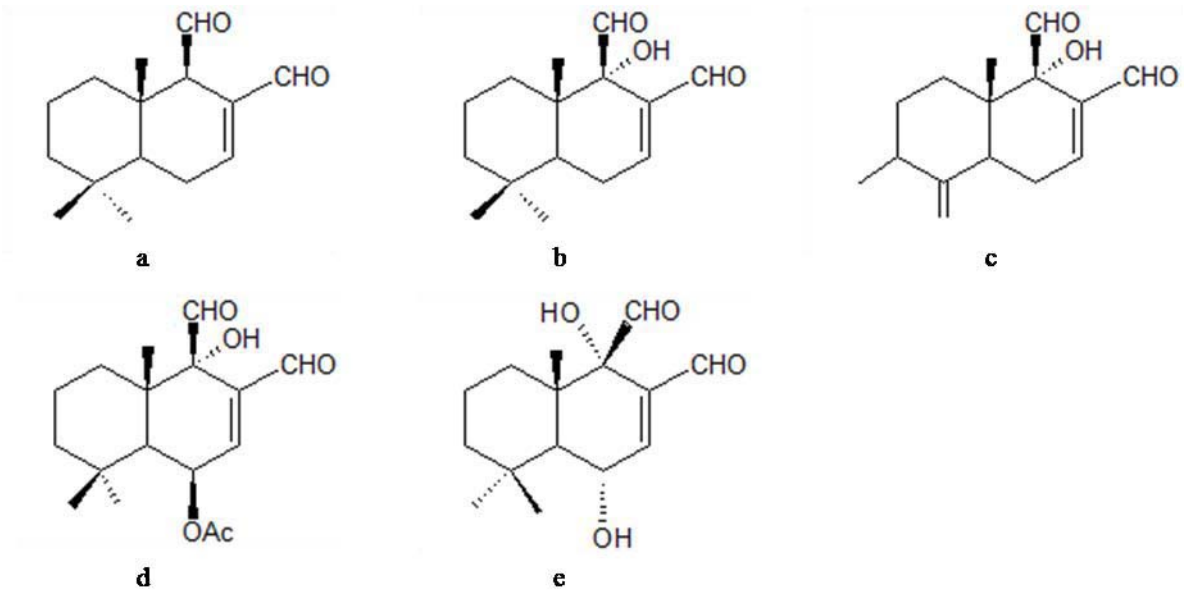


Figure 1: Drimane sesquiterpenes from *Warburgia ugandensis*: polygodial (a), warburganal (b), muzigadial (c), ugandensidial (d), and mukaadial (e).

Among the most active isolated derivates were muzigadial (7), mukaadial (8), polygodial and warburganal (11, 12). Muzigadial was isolated from the stem bark extract that showed antifungal activity against Ascomycota. Further, muzigadial inhibited trypanosomes and proved as highly cytotoxic to brine shrimps (7). The most active agent tested against the malaria-causing parasite *Plasmodium falciparum* was 11α-hydroxymuzigadiolide, and muzigadial, mukaadial, and ugandensidial were shown to possess antiplasmodial activities (8).

Other sesquiterpenes isolated from *Warburgia ugandensis* comprise: ugandenial A, dendocarbin A, dendocarbin L, dendocarbin M, 9-hydroxycinnamolide, cinnamolide-3-acetate, muzigadiolide, cinnamolide, ugandensolide, and ugandensidial (1, 13).

2. Drimane sesquiterpenes

A notable group of secondary metabolites, which are commonly believed to be produced as defense against predators (14), are drimane sesquiterpenes.

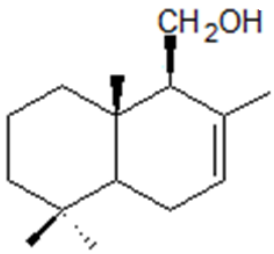


Figure 2: Structure of drimenol, the first drimane sesquiterpene isolated.

Drimanes are named after drimenol, a sesquiterpenoid alcohol isolated from *Drimys winterii* Frost. The currently proposed biosynthesis is cyclization of farnesyl pyrophosphate (FPP) (15). High contents of drimane sesquiterpenes are characteristic for members of the plant order *Canellales* comprising the New Zealand genus *Pseudowintera*, the African genus *Warburgia*, the Madagascan genus *Cinnamosma*, and the neotropic genus *Drymis* (1, 16, 17, 18, 19), all of them appreciated as medicinal plants. Drimanes were also detected in fungi (20, 21), ferns (15) and marine organisms, e.g. sponges, *Dysidea* sp. (22), molluscs, sea slugs and nudibranchs, e.g. *Doriopsilla sp.* and *Dendrodoris sp.* (23, 24).

Insecticidal, molluscicidal, and antimicrobial activities, cytotoxicity, plant growth regulation, were noted among drimane sesquiterpenes characteristics (15).

Polygodial is one of the most active drimane sesquiterpenes. First it was isolated from *Polygonum hydropiper* L (25). Warburganal and muzigadial were first isolated from *Warburgia*

ugandensis (11). Muzigadial was shown to be the most active drimane sesquiterpene against mycobacteria (1), but it also possessed antifeedant and cytotoxic properties. However, polygodial proved to be the most potent insect antifeedant of all three compounds, which also were characterized by notable antifungal activity (11, 25, 26). It was also the most active agent isolated from *Drimys brasiliensis* against dermatophytes (19). The aldehyde function on C-9 (11) and the lipophilicity (1) are thought to contribute to the observed activities. The orientation of the aldehyde group seems to be crucial for activity; polygodial possesses a β-aldehyde group at C-9, *epi*-polygodialan α-aldehyde group at C-9. The former is active, the latter not. Additionally, the presence of hydroxyl group at the position 9 enhances activity (8).

Polygodial`s mode of action is representative for all drimane sesquiterpenes. It was studied intensively using the yeast *Saccharomyces cerevisae* as model organism whose growth and respiration were inhibited by polygodial. Polygodial acts in the first place as toxin that after diffusing into the cytoplasm causes cell death (26, 27). However, depending on the chemical milieu, polygodial may lose or increase is activity. It is known that EDTA and phenylpropanoids, both transition metal chelators, may enhance polygodial's activity (28, 29).

Furthermore, polygodial enhance production of reactive oxygen species (ROS), which led into cell membrane disruption. Addition of antioxidants, however, reduced the extent of this effect (26, 27). For therapeutic use, combinations with other chemical compounds were recommended (29).

3. Farnesol

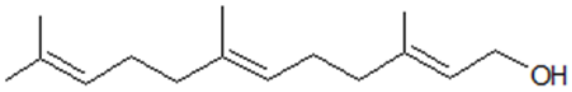
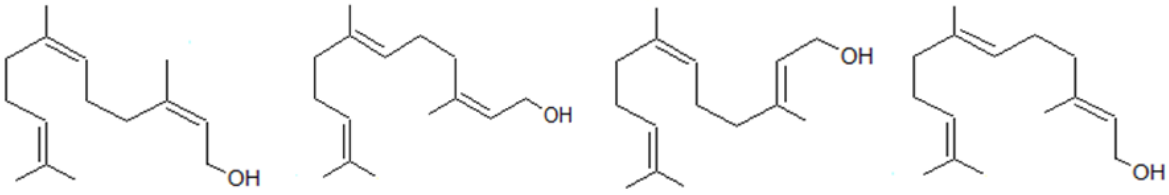


Figure 3: Structure of farnesol (3, 7, 11-trimethyl-2, 6, 10-dodecatrien-l-ol)

Farnesol is a 15-carbon isoprenoid alcohol, which is produced by many organisms, including plants, fungi (30) and mammals (31). It is a component of many plant essential oils, for example of two Myrtaceae *Eucalyptus* and *Leptospermum* (32, 33), citrus family and rose (34) and, together with its analogues, constitutes components in propolis (35). Besides, it may be utilized as a pheromone for insects and natural pesticide (34).

Farnesol occurs in four isomers, (*E*, *E*)-, (*E*, *Z*), (*Z*, *E*) -, and (*Z*, *Z*) (Figure 4) that differ in the *cis* and *trans* configurations of the double bonds (Figure 4, 36). The most active and the most common isomer in the nature is *E, E* (30).



(2Z, 6Z,)-Farnesol **(2Z, 6E,)-Farnesol** **(2E, 6Z,)-Farnesol** **(2E, 6E,)-Farnesol**

Figure 4: Farnesol isomers

It is thought to be formed by enzymatic dephosphorylation of farnesyl pyrophosphate (FPP), a major building unit in the mevalonate pathway (MVA) leading to terpenoid structures in eukaryotes (37, 38).

Farnesol may act as substrate regulator of the isoprenoid pathway by inhibiting one of its key enzymes, 3-hydroxy-3-methylglutaryl coenzyme A reductase (39).

Antibacterial activities are well documented for farnesol, e.g. cell wall disruption in *Staphylococcus aureus* (40), *Streptococcus mutans* and *S. sobrinus* (35), *Streptomyces tendae* (41) and *Pseudomonas aeruginosa* (42). Antifungal activities were demonstrated against pathogenic fungi *Paracoccidioides brasiliensis*. Lover farnesol concentrations (15µM) only affected hyphal morphology whereas higher (25µM) caused cytoplasm degradation. (43). This and other phenomena, such as cell wall damage and disruption of mitochondrial electron transport chains, are main characteristics of cell death, apoptosis, which is triggered by oxidative stress in living cells. Farnesol was definitely shown to cause apoptosis in *Sacharomyces cerevisae* (44) *Aspergillus nidulans* (45) *Fusarium graminearum* (46) and *Penicillium expansum* (47) by generating reactive oxygen species (ROS). Accordingly, farnesol promoted the efficacy of antibiotics (40, 48, 49) and is considered as anticancer agent (50).

Like of the most of the drimane sesquiterpenes, farnesol activity depends on environmental and metabolic conditions. For example, temperature and pH affect the activity (51, 52). The presence of other terpenes may increase (geraniol) or decrease (geranylgeraniol) farnesol efficacy (53).

4. Endophytes

Endophytes are *"microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects"* (54).

They occur in all plant tissues (55), at least one specific strain per host (56), while the real number is estimated to be much higher (57). Orchard grass (*Dactylis glomerata*) alone has shown to be host to over hundred fungal endophytes (58). They may be host specific or generalists (58, 59). It is suggested that endophytes evolved together with their hosts (55). Numerous abiotic and biotic factors may affect the actual relationship and determine if the microbe can establish itself or not, and if it remains an endophyte or turns into a pathogen (59–62).

Climatic conditions, season, type, tissue age, and host plant identity also are considered as non-negligible factors. Generally, plants species of warmer regions in the tropics not only host higher numbers but more diverse endophytes (59). Most common endophytes comprise fungi and bacteria, of which the most frequently isolated are ascomycete fungi (59, 60, 63, 64, 65).

The classification of endophytes is based on their transmission mode, colonization behavior, diversity, and benefit host plant. Carroll (1988) defined two types: (1) Type I comprises vertically transmitted through seeds, as it is the case for most grass endophytes; (2) type II endophytes are horizontally transmitted non-grass endophytes (66). Recently, Rodriguez et al. (2008) proposed an extended classification: (1) clavicipitaceous endophytes (class 1) colonize grasses, among of which the sexual genus *Epichloe* and the asexual genus *Neotyphodium* are most notable (67); (2) non-clavicipitaceous endophytes (class 2) colonize all plant species and include the ascomycete group *Pezizomycotina* and two basidiomycete groups, *Agaricomycotina*

and *Pucciniomycotina*; (3) class 3 endophytes are the most diverse consisting of all other fungi colonizing above ground plant organs; (4) class 4 contains fungi designated as “dark septate endophytes”, ascomycetous anamorphic fungi with melanized septa that are only found in plant roots (68).

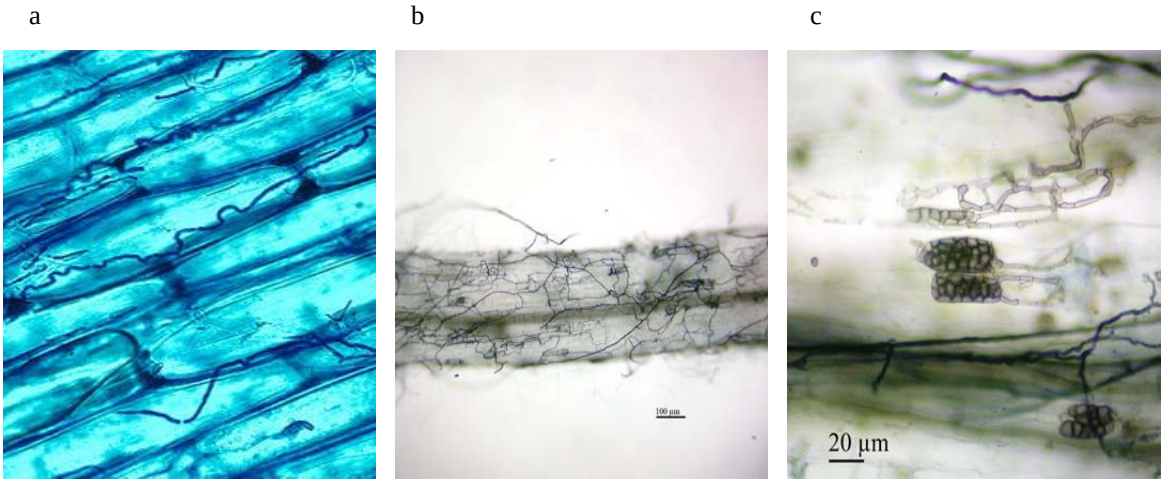


Figure 5: Endophytic fungi: a) *Neothypodium coenophialum* in tall fescue (*Festuca arundinacea* Schreb.) endophyte; (b, c) dark septate endophytes of *Eurybia divaricata*: fungal hyphae inside and outside of the roots (b) and hyphae ending in microsclerotia (c).

Plants provide endophytes with nutrients and habitat, endophytes may enhance host plant tolerance against biotic and abiotic stresses (67). Plant protection aspects have repeatedly been demonstrated for fescue and rye grass. Clavicipitaceous endophytes produce alkaloids, lolines, peramines, and lolitrems, indole alkaloids, which sometimes are also denoted as ergot alkaloids. They cause toxic effects on livestock, may protect against pathogens, insect herbivores, and plant pathogenic nematodes (67, 69, 70). Endophyte colonized *Theobroma Cacao* (Malvaceae) is more resistant against *Phytophthora* sp.; endophytes reduce leaf necrosis and mortality (71). In addition to biotic stresses, endophytes provide better resistance on environmental abiotic stresses like high temperatures (55), salt stress (72), and drought (61). *Dichanthelium lanuginosum* (panic grass) infected with *Curvularia* sp. (hyphomycete fungus) survives the high temperature in hot

springs; non-colonized plants perish (55). The ecology of endophytes definitely deserves more attention in the future.

5. Biotransformation

The biosynthesis of sesquiterpenes and other secondary metabolites by microorganisms, including endophytic fungi, is well known (20, 73, 74). They are also tools in efforts to synthesize drimane sesquiterpenes and their biosynthetic precursor farnesol (74, 75, 76, 77). Due to advance in availability and high production rate, microbial transformation of various substrates may be a good way to yield important bioactive natural products (78, 79, 80). It has been suggested that microbial biotransformation provide more stereoselective and -specific reactions (78, 79, 81, 82). Altering conditions may result in favor of production of specific compounds. Literature data suggest that biotransformation success may depend of microbial fitness (e.g., biomass production) as well as chemical properties of the substrate (e.g., solubility). Some additives (e.g. anionic polymers) may optimize these reactions (82).

The yeast *Saccharomyces cerevisiae* is used beside *E. coli* for production of farnesol (74) as stereospecific tool catalyzing reductive biotransformation of monoterpenoids and sesquiterpenoids (80). The ascomycete fungi *Aspergillus niger* may perform C-3 regioselective hydroxylation of drimenol and confertifolin converting them into 3β-hydroxyl derivates 3β-hydroxydrimane and 3β-hydroxyconfertifolin (81, 82). Furthermore, the biotransformation of drimane sesquiterpene muzigadial by the basidiomycete fungi *Cryptococcus neoformans* and Actinobacteria (*Streptomyces platensis* and *S. spectabilis*) into hemiacetal, hemiacetalepoxyde and lactone has been shown (Figure 6; 83). Several studies reported oxidative biotransformation of farnesol by ascomycetous and basidiomycetous fungi (Figure 7; 78, 84).

Alternative chemical syntheses for bioactive metabolites are difficult, expensive and time consuming (82, 83).

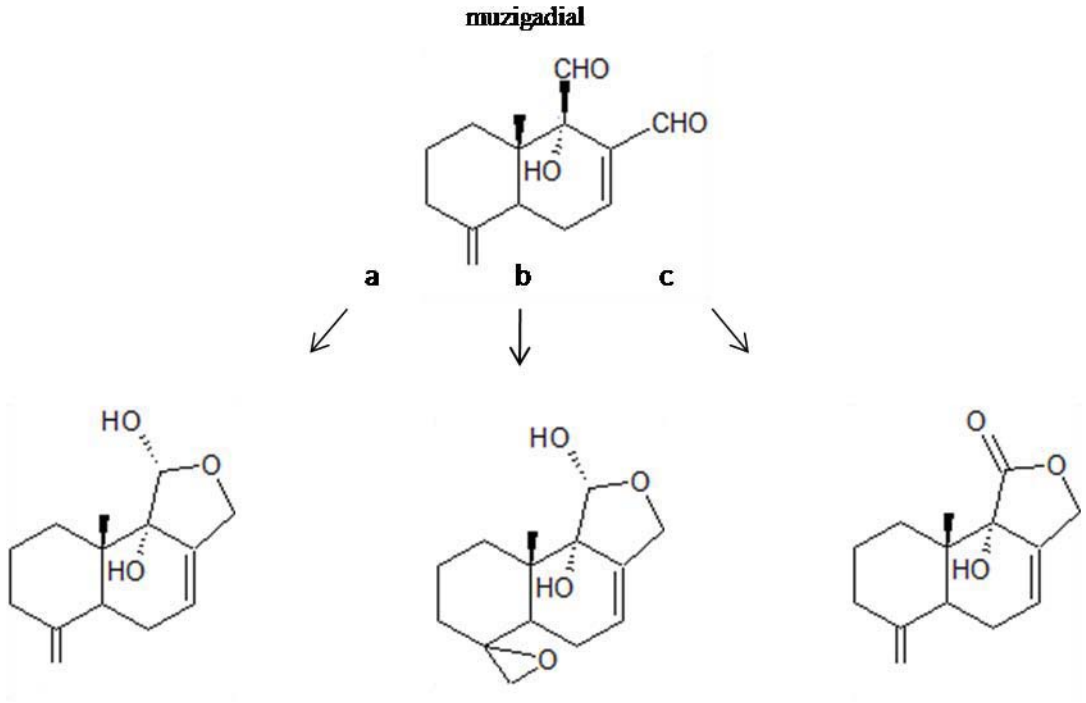


Figure 6: Biotransformation of muzigadial by *Cryptococcus neoformans* (a), *Streptomyces platensis* (b), and *S. spectabilis*.

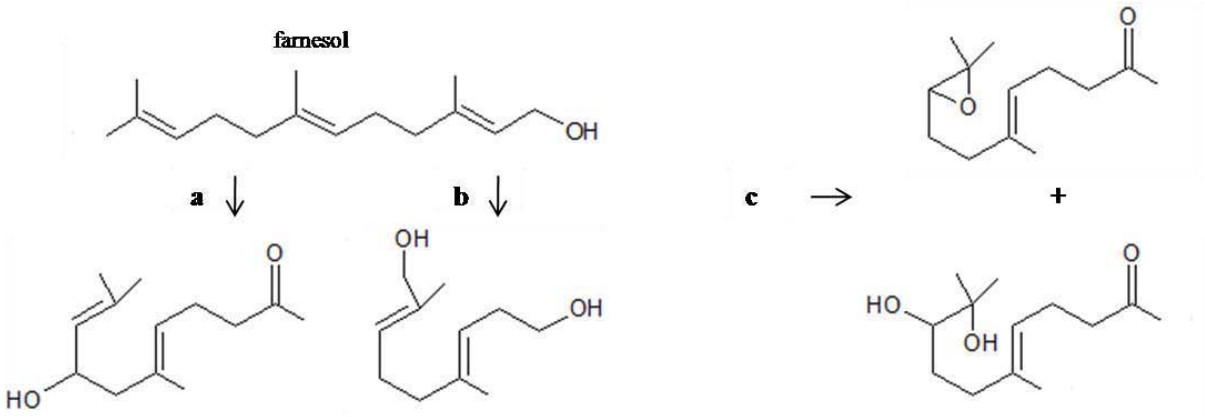


Figure 7: Biotransformation of farnesol by *Rhodotorula rubra*, *R. marina* (a), *Botrytis cinerea* (b), and *Fusarium culmorum* (c) into oxyderivates of geranilacetone.

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II BIOTRANSFORMATION AND UTILIZATION OF DRIMANE SESQUITERPENES BY ENDOPHYTIC MICROORGANISMS

1. Introduction

Warburgia *ugandensis* Sprague (= *Warburgia breyeri* Pott), the pepper bark tree, is a well-known medicinal tree endemic to South East Africa. Many studies confirmed its wide spectrum of biological activities and high content of drimane sesquiterpenes in all plant parts. It is generally assumed that secondary metabolites, such as drimane sesquiterpenes, are produced for protection (1, 2).

Microorganisms colonizing plant tissues without apparent negative effect on the host are denoted as endophytes (3). They colonize all plants, lower and higher, and are thought to represent, though unexplored, a rich source for bioactive compounds (2, 4, 5).

Warburgia ugandensis is host to diverse endophytes as many other trees. However, little is known about interactions between endophytes and their host plants (4). This study explores if drimane sesquiterpenes actually affect the growth of endophytic fungi of *Warburgia* (**hypothesis 1**), either positively or negatively, and compares the endophytic isolates with isolates from other host plants and an airborne fungus in terms of this aspect. Besides the crude extract, enriched drimane fractions and their hydrolysis products (to remove activity-masking esters and sugars) were tested. The drimane sesquiterpene fraction of the *Warburgia ugandensis* extracts was obtained by chromatography over Amberlite XAD 1180, which separated drimane sesquiterpenes from the predominating sugar alcohol (mannitol) in the crude extract.

Microbes are considered as successful agents in synthesis and biotransformation of drimane sesquiterpenes. They may be involved in oxidation and hydroxylation of drimanes (6, 7, 8, 9). In this context, the capabilities of endophytic fungal isolates from *W. ugandensis* to use drimane sesquiterpenes as substrate or their biotransformation were of interest (**hypothesis 2**).

However, due to the fact that all preliminary experiments with drimane sesquiterpene-supplemented media to study hypothesis 2 failed, their biosynthetic precursor farnesol was used instead. Farnesol is a natural sesquiterpene alcohol and occurs widely in plants and is also known to be biosynthesized by microbes (6, 10). It is derived from the mevalonate isoprenoid pathway by enzymatic dephosphorylation of farnesyl pyrophosphate (FPP). Oxidative biotransformation of farnesol similar to that of sesquiterpenes has been shown previously (11, 12). Consequently, it represented a possible and practicable substitute to the drimane sesquiterpenes.

2. Materials and methods

2.1. General

Farnesol (95% mixture of four isomers), methanol, MS medium, agar, D-mannitol, and cholestane, were purchased from Sigma Aldrich (Schnelldorf, Germany); sucrose, NaCl, Luria Bertani (LB) and malt extract broth from Roth (Karlsruhe, Germany); Amberlite XAD1180, from Fluka (Buchs, Switzerland); absolute ethanol and peptone from Merck (Darmstadt, Germany); *N*-methyl-*N*-TMS-trifluoroacetamide (MSTFA) from Pierce (Thermo Fisher Scientific Inc., Rockford, IL, USA). Distilled water was of the Milli-Q quality (Millipore, Bedford, MA). All the chemicals used were of p.a. quality.

2.2. Plant material

Plant material was collected by Sigrid Drage and Franz Hadacek (Department for Chemical Ecology and Ecosystem Research, University of Vienna) and Birgit Mitter and Angela Sessitsch (Department for Health and Environment, AIT) in Kenya in fall 2007. Leaves, bark, roots and fruits of ten randomly selected *Warburgia ugandensis* trees were collected at two locations, one near the town Rumuruti (0°19´N/36°30´E) and the second near the town Kitale (01°00´N/35°01´ E), both of which are located adjacent to the Rift Valley in Kenya. Plant material intended for isolation of fungal endophytes was cut in 1 cm^2 pieces, sterilized with 70 % aqueous ethanol (Sessitsch et al., 2002, Reiter et al., 2002) and incorporated into agar supplemented with MS mineral salts [1.5% (w/v)]. Plant material intended for chemical analysis was air-dried in an incubator at 40°C.

2.3. Plant material and hydrolysis

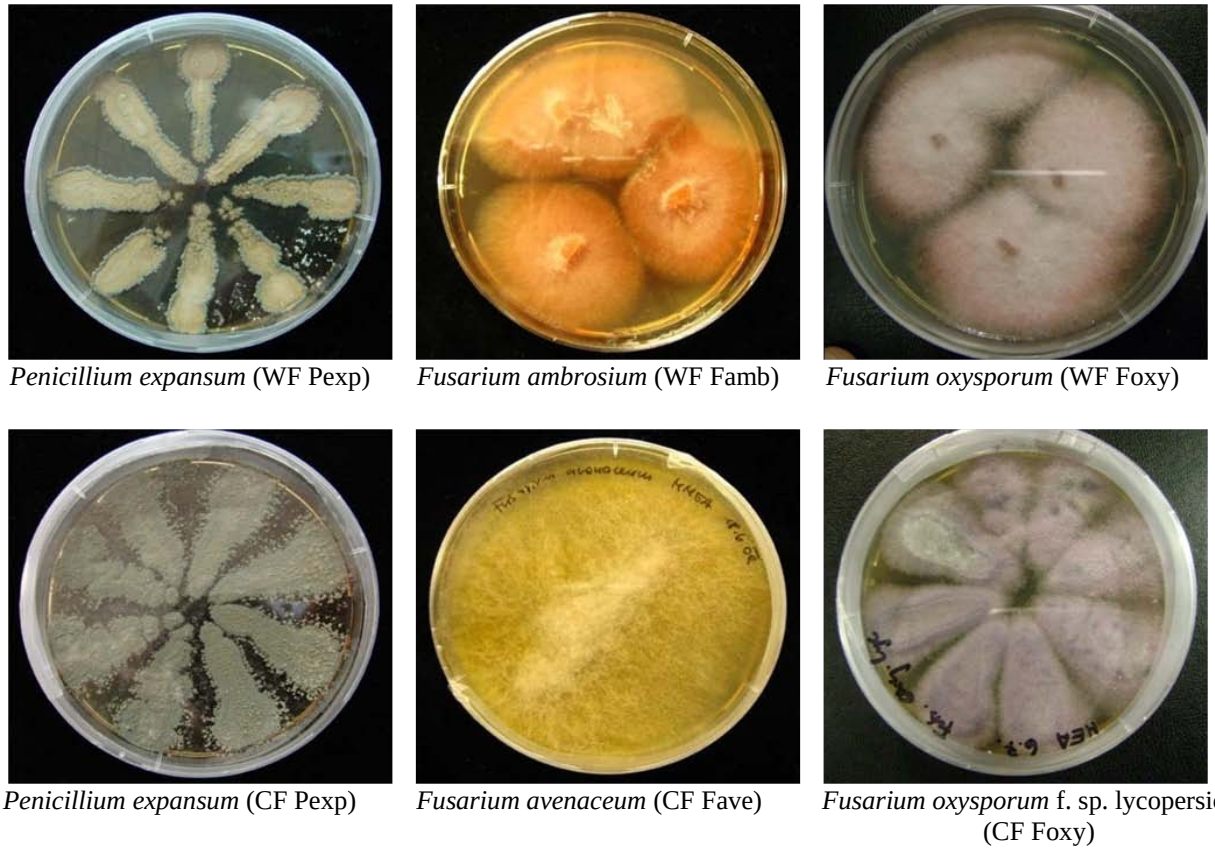
Three g plant material was extracted with methanol (80 ml, p.a. quality) for 24 hours. The extracts were filtrated (MN 615 1/4, Ø 240mm; Macherey-Nagel, Düren, Germany), evaporated under vacuum and dissolved in 10 ml methanol. Two hundred mg of the extract were subjected to fractionation over Amberlite XAD-1180 (following manufacturer's guidelines) in order to separate sugars from drimane sesquiterpenes as good as possible. The ethanolic fractions containing the drimane sesquiterpenes were evaporated, dried under vacuum and dissolved in methanol. For hydrolysis, the ethanol fraction of the extract was dissolved in butanol (2mg/ml); 1.7 ml of this solution was transferred into 2ml ampoules. Concentration was adjusted to 2N by addition of 0.3 ml concentrated (32%) HCl. Ampoules were closed under argon atmosphere and incubated at 80 °C for three hours, afterwards cooled to room temperature and neutralized with 5ml aqueous Na_2CO_3(1M). The hydrolyzed extract was phase-separated with butanol two-times.

2.4. Fungal isolates

Three endophytes isolated from *Warburgia ugandensis* and three fungal isolates of close taxonomic identity, two endophytes of other plant origin and one airborne, were chosen for the study (Table 1, Figure 1). *Warburgia ugandensis* endophytes included "*Fusarium ambrosium"*, isolated from leaves, "*Fusarium oxysporum"* and "*Penicillim expansum"* from roots. The endophytic strain of *Fusarium oxysporum f. sp. lycopersici* was isolated from *Solanum lycopersicum* roots; *Fusarium avenaceum* was recovered from *Cicuta virosa* rhizome and *Penicillium expansum* was an airborne isolate.

Table 1: Microorganisms used in the study; BLASTn analysis-retrieved closest relative to ITS region of *Warburgia ugandensis* isolates

strain	code	origin	identification (Blastn)		
			closest identified relative ITS	accession no./identity (%)	phylogenetic group
131	WF Pexp	*W. ugandensis*	*Penicillim expansum*	AB298711/ 99	Eurotiomycetes
108	WF Famb	*W. ugandensis*	*Fusarium ambrosium*	AF178397/ 95	Sordariomycetes
154	WF Foxy	*W. ugandensis*	*Fusarium oxysporum*	EU364854/ 99	Sordariomycetes
			identified species		
VIAM MA 2811	CF Pexp	Institute of Applied Microbiology, Agricultural University of Vienna (VIAM)	*Penicillim expansum*		Eurotiomycetes
VIAM MA1512	CF Fave	Institute of Applied Microbiology, Agricultural University of Vienna (VIAM)	*Fusarium avenaceum*		Sordariomycetes
Fol 007 (race 2)	CF Foxy	B.J. Cornelissen, Institute for Molecular Cell Biology, Amsterdam	*Fusarium oxysporum f. sp. lycopersici*		Sordariomycetes



Penicillium expansum (WF Pexp) *Fusarium ambrosium* (WF Famb) *Fusarium oxysporum* (WF Foxy)

Penicillium expansum (CF Pexp) *Fusarium avenaceum* (CF Fave) *Fusarium oxysporum* f. sp. lycopersici (CF Foxy)

Figure 1: Microorganisms used in the study, cultured on malt extract agar.

2.5. Isolation and identification of *Warburgia ugandensis* fungal endophytes

Agar-embedded plant material was incubated two weeks at room temperature. Based on morphology (examined with the naked eye and with an Olympus SZH10 research stereo microscope (x140)), agar plugs with emerging fungal hyphae were transferred to malt extract agar, MEA (30g malt extract, 3g peptone, 15g agar per litter distilled water) and further incubated at room temperature in the dark. This step was repeated until pure cultures were obtained. Agar plugs of actively growing mycelia with and without conidia were transferred in 2 ml sterile vials containing a 14% sucrose solution with 1% peptone, and stored at –20° C until further use. Identification was performed on basis of ITS ands partial LSU sequence analysis (Birgit Mitter, AIT, pers. comm.).

2.6. Preparation of fungal conidia suspensions

Fungal isolates were grown on a modified malt extract agar, MMEA (10g malt extract, 20g mannitol, 3g peptone, 15g agar per litter distilled water). Conidia were harvested with sterile 0.9% aqueous NaCl (w/v) supplemented with of 5% DMSO (v/v), following an established procedure (13). Conidia suspensions were stored in 2 ml sterile vials in sucrose (14%) solution with 1 % peptone added (v/v). Colony forming units (CFU) were determined by counting germinated spores formed from each of eight dilution series (1:10) on agar plates.

2.7. Gas chromatography mass spectrometry(GC–MS)

Samples were analyzed by gas chromatography–mass spectrometry (GC–MS). The instrument was an AutoSystem XL gas chromatograph linked to a TurboMass quadrupol mass spectrometer (Perkin Elmer, Waltham, MS, USA). Samples (100µg) were dissolved in 100µl *N*-

methyl-*N*-TMS-trifluoroacetamide (MSTFA) for silylation. One µl was injected in the splitless mode. The column was a JW 5ms (18 x 0.18 mm, 0.18 µm film thicknesses, Agilent Technologies Inc., Santa Barbara, CA). The column oven program started at a temperature of 70°C that was held for 3 minutes, rising to 300°C at a rate of 3°C/min. The carrier gas was helium with 0.8ml/min as flow rate. The transfer line temperature was set to 280°C, the ion source to 200°C, the filament to 70eV. The mass spectrometer was run in the TIC mode from 40 to 620 amu. The output chromatograms were integrated with Turbomas 4.1.1 software (Perkin Elmer, Waltham, MS, USA) and the peak areas converted to relative amounts (%) of the total peak area of every chromatogram. Mass spectra were tentatively identified by comparison with Wiley MS database, 6^{th} ed.

2.8. Assays

2.8.1. Minimum inhibitory concentration (MIC)

MIC concentrations were determined for six fungal strains (Table 1) against *Warburgia ugandensis* extract fractions and farnesol following a broth microdilution procedure (14, 15). Fungal strains were grown in 96-well U-shaped microplates with cover lids (Greiner BioOne, Kremsmünster, Austria) in modified MMEA (see 2.6.). Conidia stock solutions were prepared in the same medium.

The concentration of the conidia stock solutions was adjusted to 10^6CFU/ml. The extract and farnesol was first dissolved in methanol and diluted to reduce the methanol concentration to 5 % in the assay medium. One hundred µl of a 4000µg/ml extract/farnesol stock solution was added into the first well and diluted serially (1:1). Each well then was inoculated with 50µl of the

conidia stock solution. To control wells 50µl pure culture medium was added instead. Each sample was done in triplicate. Blanks (extract/farnesol in culture medium) were also included. The plates were incubated at room temperature in dark for at least two days (depending on the growth rate of the particular strain tested) and shaken (120 rpm). The lowest concentration that totally inhibited fungal growth was considered as MIC (NCCLS M27-A, 1997). The extract/farnesol concentrations, which inhibited fungal growth, were observed under an Olympus SZH10 research stereo microscope (x 140, Figure 2)

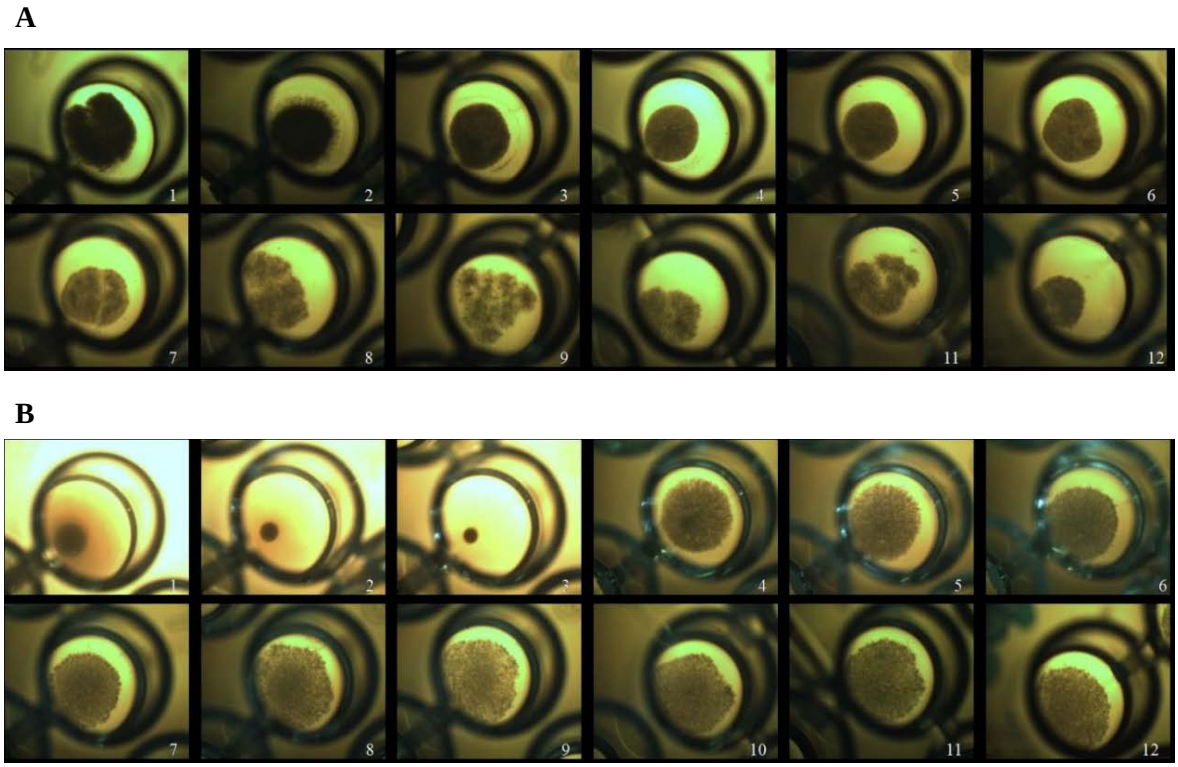


Figure 2: MIC determination; twelve *Warburgia ugandensis* extract concentration tested against two fungal strains (A and B). The lowest inhibiting concentration was determined as MIC; (A) no MIC; (B) MIC in third well.

2.8.2. Substrate utilization

Substrate utilization was evaluated by broth microdilution method (14, 15) and performed in sterile 96-well U-shaped microplates with lids (Greiner BioOne, Kremsmünster, Austria). Fungal strains (Table1) were grown in sterile distilled water spiked with *Warburgia* extracts and their respective fractions. The extract stock solutions were prepared in methanol and added to the medium, the control and the inoculums to a final concentration of 5 % methanol (v/v). The extract concentration in the stock solution was the sub inhibitory concentration previously determined in the MIC assay.

Fifty µl conidia stock solution, adjusted to 10^6CFU, and 50 µl control solution were added to the wells. To that, 100µl of extract stock solution/farnesol stock solution was added and diluted three-times (1:10). All samples were done in triplicate. Examination plates were closed with lids and sealed with Parafilm "M" (Pechiney Plastic Packaging, Chicago, IL, USA), and incubated on a horizontal shaker (120 rpm) in the dark at room temperature for four days. Fungal growth was evaluated by turbidity measurement with a microplate reader (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland) at 600 nm. Absorbance for each well was measured immediately after preparation (time point zero) and after four days (end time point when growth was visible).

2.8.3. Biotransformation

Two *Warburgia ugandensis* endophytes, *P. expansum* and *F. ambrosium*, *Cicuta virosa* endophyte *F. avenaceum*, and airborne *P. expansum* were grown in a modified LB culture broth. For the assay setup, 45.7 ml sterilized culture medium was poured into sterile 100 ml Erlenmeyer flasks. Conidia stock solution was added to adjust CFU to 10^6 in the medium. Erlenmeyer flasks were incubated on horizontal shaker (80 rpm) at room temperature and in dark until fungal

growth was visible (in average, two days). Farnesol was added as methanolic solution to a concentration of 500µg/ml. All samples were prepared in triplicates for zero (0 h) and end (48 h) time points. Zero time point media were extracted immediately, end time points two days later. The re-extraction was performed with 50 ml *tert*-butyl-methyl ether (TBME), twice with phase separation. The medium was filtrated (Whatman No.4 filter papers; Whatman International Ltd, Maidstone, UK) before extraction. Cholestane was added as internal standard. The MTBE extract was evaporated, dried under vacuum and dissolved in methanol for storage at –20 °C.

3. Results

3.1. GC-MS analyses of drimane extracts

Two root, two leaf and one fruit extract from *Warburgia ugandensis* were chosen to determine their effects on growth and development of several fungal strains, some endophytes from *W. ugandensis*, some endophytes of other plants and one airborne.

The original crude extracts predominately consisted of mannitol (Figure 3) and thus were fractionated to obtain a sugar fraction and an enriched drimane fraction. The drimane fraction was hydrolyzed with conc. HCl on basis of the assumption that some drimanes may occur as glycosides or esters in which the important functional groups were masked by sugars.

The GC–MS chromatograms of the drimane enriched extract fractions are shown in Figure 4. Even though the extract was fractionated on Amberlite XAD 1080, still sugars could be identified in the chromatogram on basis of the MS spectra. The leaves contained much lower amounts of drimanes (all unmarked peaks in the chromatogram) than the fruits and roots. The sugars comprised the sugar alcohols mannitol, xylitol and quercitol, the disaccharides sucrose and trehalose, and the trisaccharide raffinose. Furthermore, all extracts contained palmitic acid.

The GC–MS analyses revealed that roots and fruits represent organs that accumulate comparatively large amounts of drimanes. By contrast, drimane diversity as well as quantity was lower in the leaves (Figure 4). Surprisingly, the hydrolyzed drimane fractions showed a completely different picture. Both carbohydrate and drimane patterns had changed substantially. Instead of mannitol, the most prominent sugar alcohol now was xylitol, in all samples and even those where it had not been detected before. Also, both sucrose, the most prominent disaccharide

in leaves, and raffinose, the characteristic trisaccharide in roots, were not detectable any more (Figure 5). Moreover, the number and intensity of drimane peaks was considerably reduced compared to the non-hydrolized extract. Conversely, alkanes were visible in the hydrolyzed root drimane fractions that were not detectable in the non-hydrolized drimane fraction. Similarly, fatty acids were more prominent, even stearic acid that could not be detected in the non-hydrolized extract fraction.

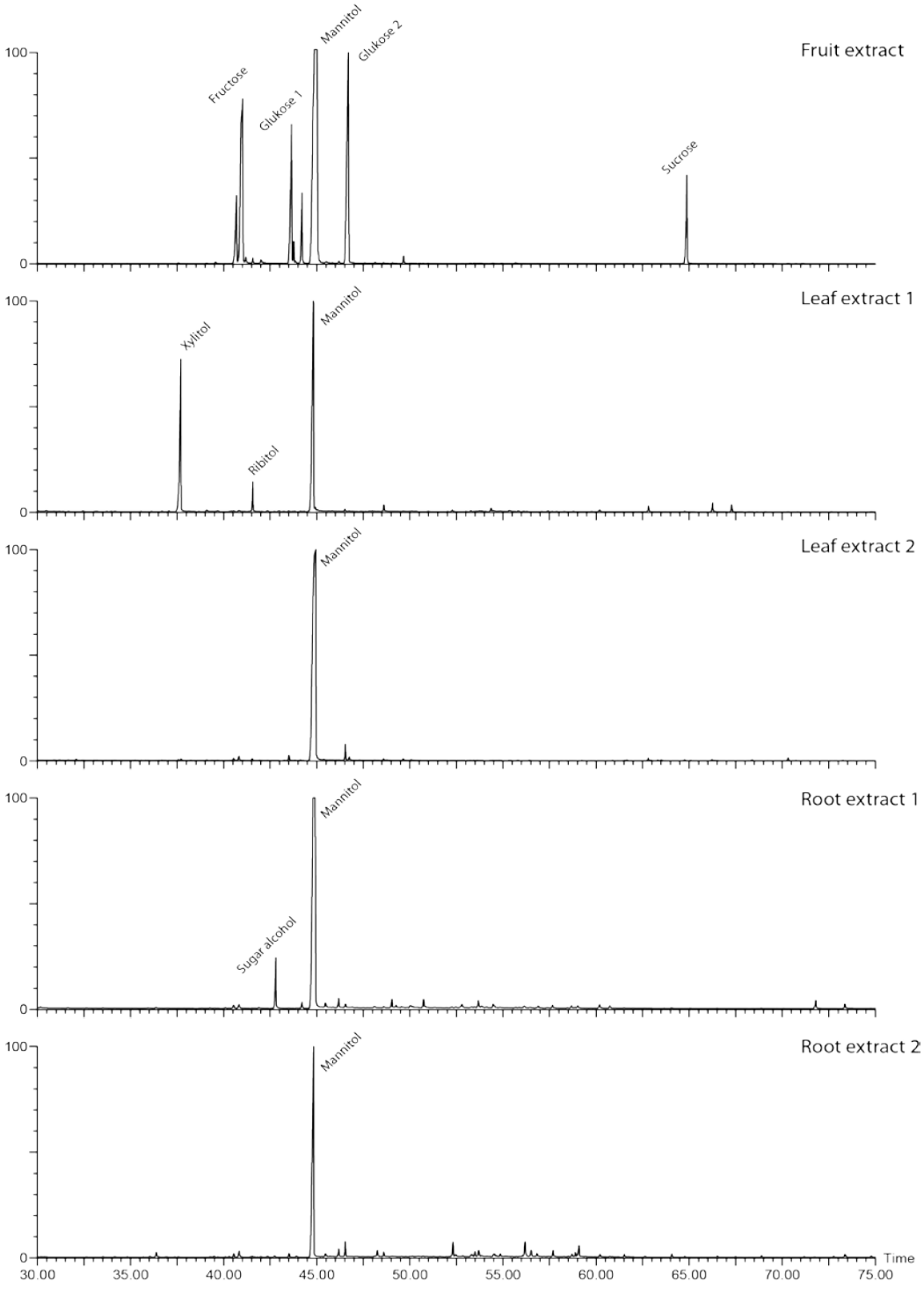


Figure 3: GC-MS chromatograms of crude *Warburgia ugandensis* extracts. The assigned peaks are carbohydrates and were identified on basis of their MS spectra.

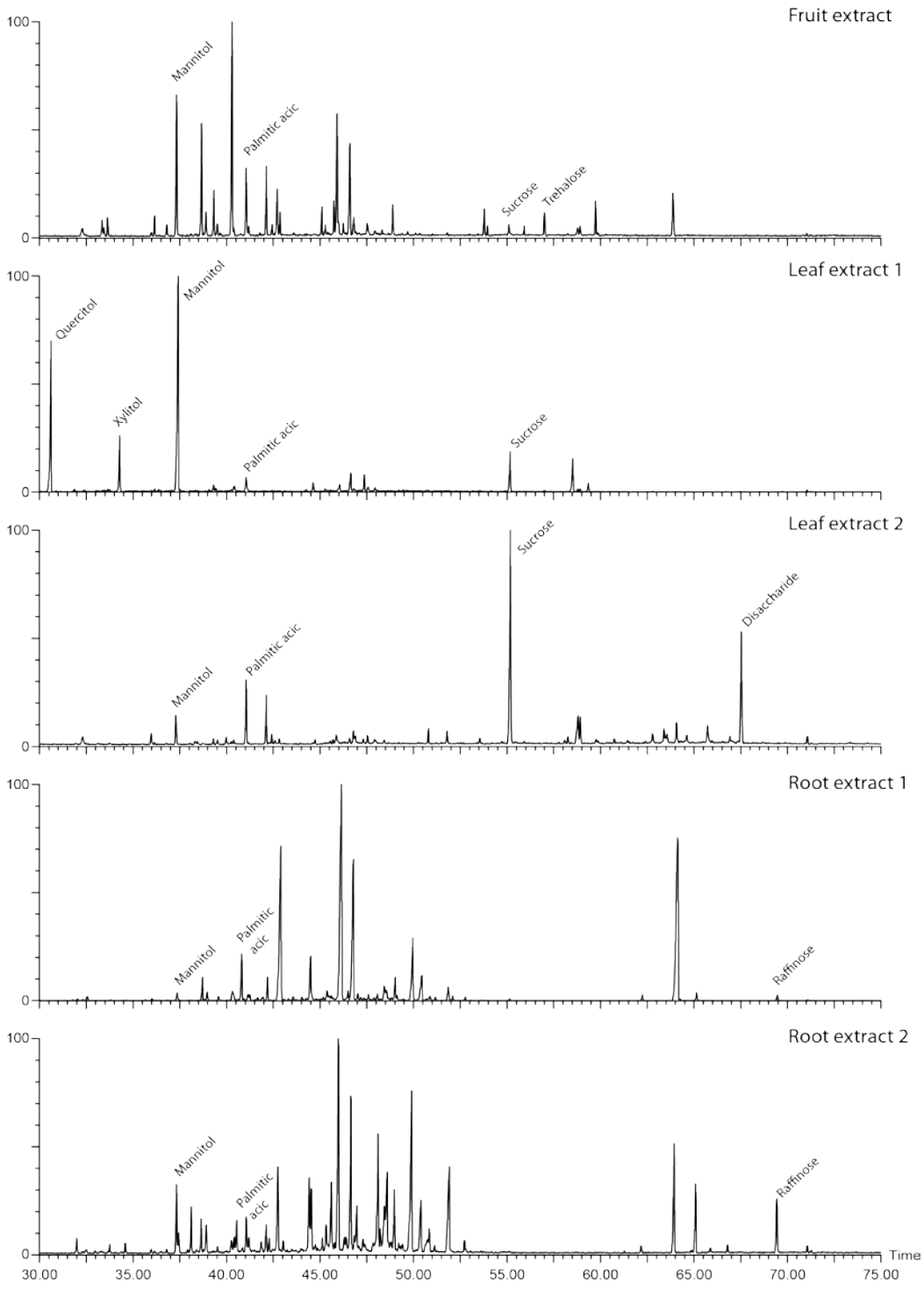


Figure 4: GC-MS chromatograms of drimane fractions of *Warburgia ugandensis* extracts. The assigned peaks are carbohydrates and were identified on basis of their MS spectra.

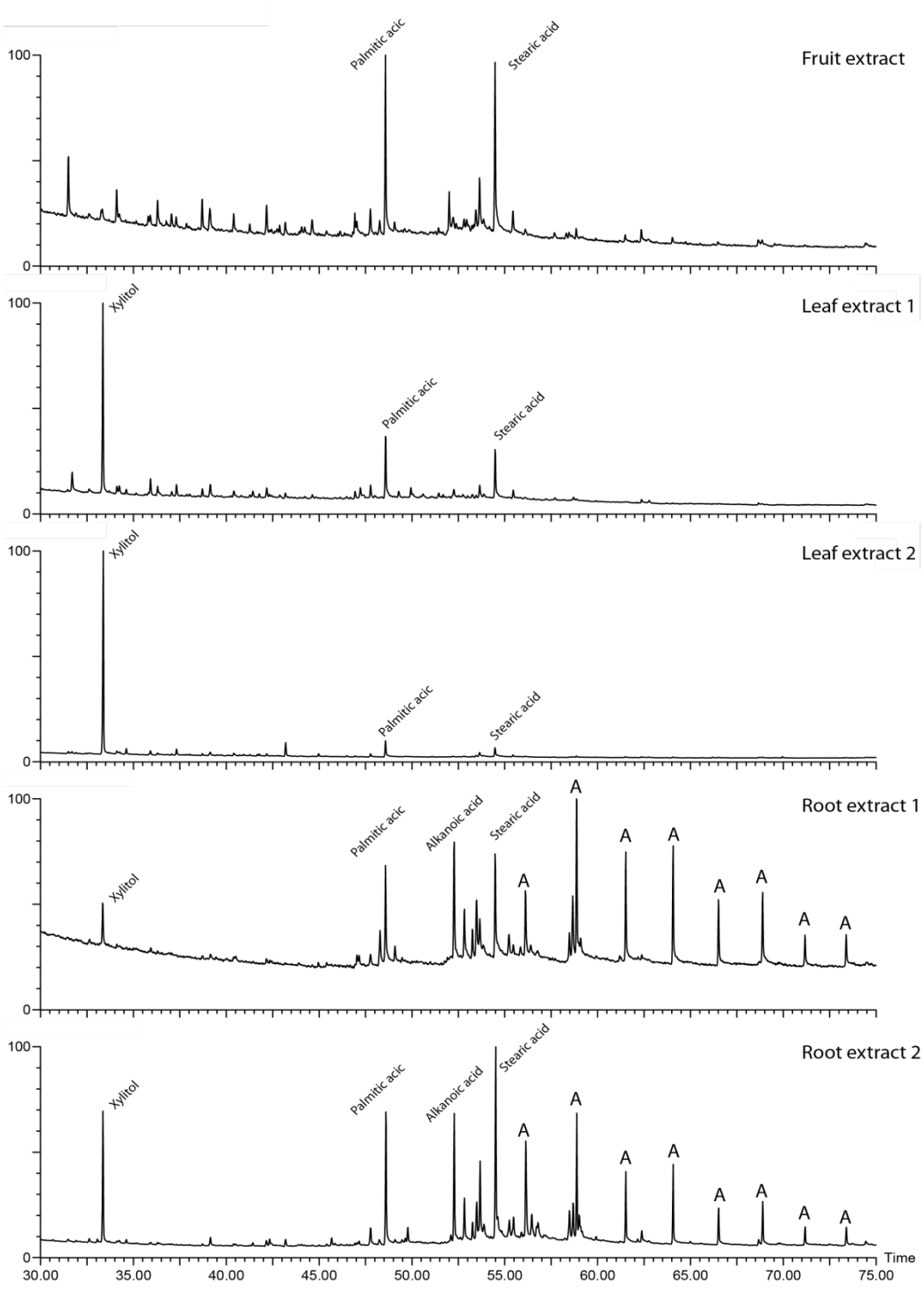


Figure 5: GC-MS chromatograms of the hydrolyzed drimane fractions of *Warburgia ugandensis* extracts. The assigned peaks are carbohydrates and were identified on basis of their MS spectra; A, alkanes.

3.2. Minimum inhibitory concentration (MIC) of farnesol, assayed extracts and extracts fractions

Minimum inhibitory concentrations of roots, leaf, and fruit extracts, extract fractions, and farnesol were determined for several *Warburgia ugandensis* endophytes (W) and other fungal isolated of similar taxonomic identity (Table 2).

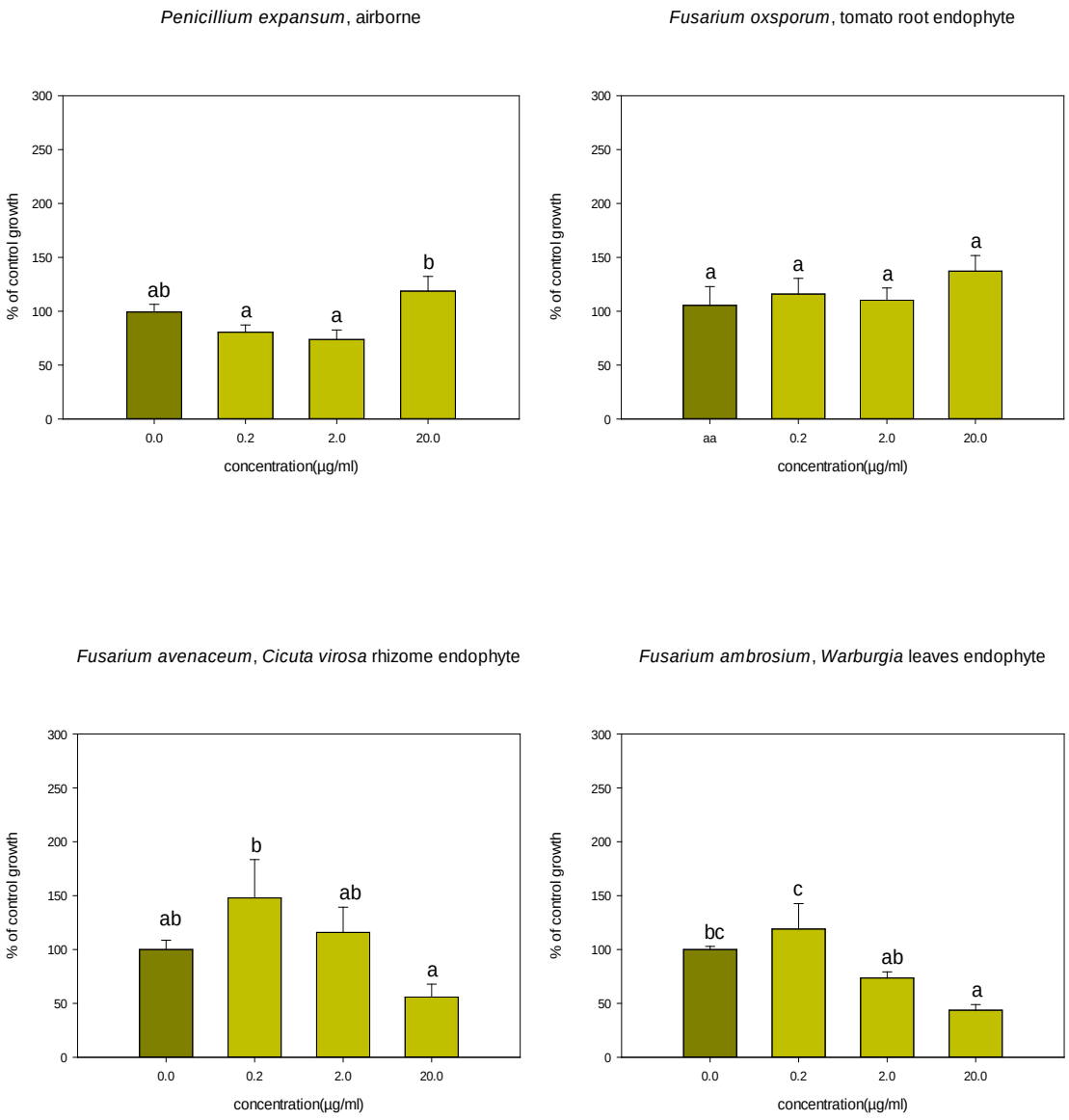
Table 2: Minimum inhibitory concentration (MIC, µg/ml) of farnesol and *Warburgia ugandensis* extracts/ extract fractions on fungal *Warburgia* endophytes (W) and other fungal isolates.

	W ***Fusarium ambrosium***	***Fusarium avenaceum***	**W** ***Fusarium oxysporum***	***Fusarium oxysporum f.sp. lycopersici***	**W** ***Penicillium expansum***	***Penicillium expansum***
farnesol	>4000	>4000	>4000	>4000	>4000	>4000
root 1, crude extract	30	30	10	15	1000	60
root 1, drimane fraction	15	15	15	30	>1000	60
root 1, hydrolized drimane fraction	>1000	>1000	>1000	>1000	>1000	>1000
root 2, crude extract	250	125	125	60	>1000	125
root 2, drimane fraction	250	125	60	125	>1000	125
root 2, hydrolized drimane fraction	>1000	>1000	>1000	>1000	>1000	>1000
leaves 1, crude extract	>1000	>1000	>1000	>1000	>1000	>1000
leaves 1, drimane fraction	>1000	>1000	>1000	>1000	>1000	>1000
leaves 1, hydrolized drimane fraction	>1000	>1000	>1000	>1000	>1000	>1000
leaves 2, crude extract	>1000	>1000	>1000	>1000	>1000	>1000
leaves 2, drimane fraction	>1000	>1000	>1000	>1000	>1000	>1000
leaves 2, hydrolized drimane fraction	>1000	>1000	>1000	>1000	1000	>1000
fruit, crude extract	250	60	60	125	250	250
fruit, drimane fraction	125	250	60	125	>1000	250
fruit, hydrolized drimane fraction	>1000	>1000	>1000	>1000	>1000	>1000

Only the fruit and both root extracts inhibited growth and development of most tested fungal isolates such that a MIC < 1000 µg/ml could be determined. The majority of the fungi were inhibited more by the root than the fruit extracts, the leaves showed no activity. All hydrolyzed drimane fractions were inactive or inhibited only at the highest concentration tested. Farnesol showed no activity at all. The *Warburgia* endophytes did not differ from the other tested fungi by specific susceptibility patterns. The most notable isolate was *P. expansum Warburgia* endophyte; only the fruit crude extract yielded a MIC value < 1000 µg/ml.

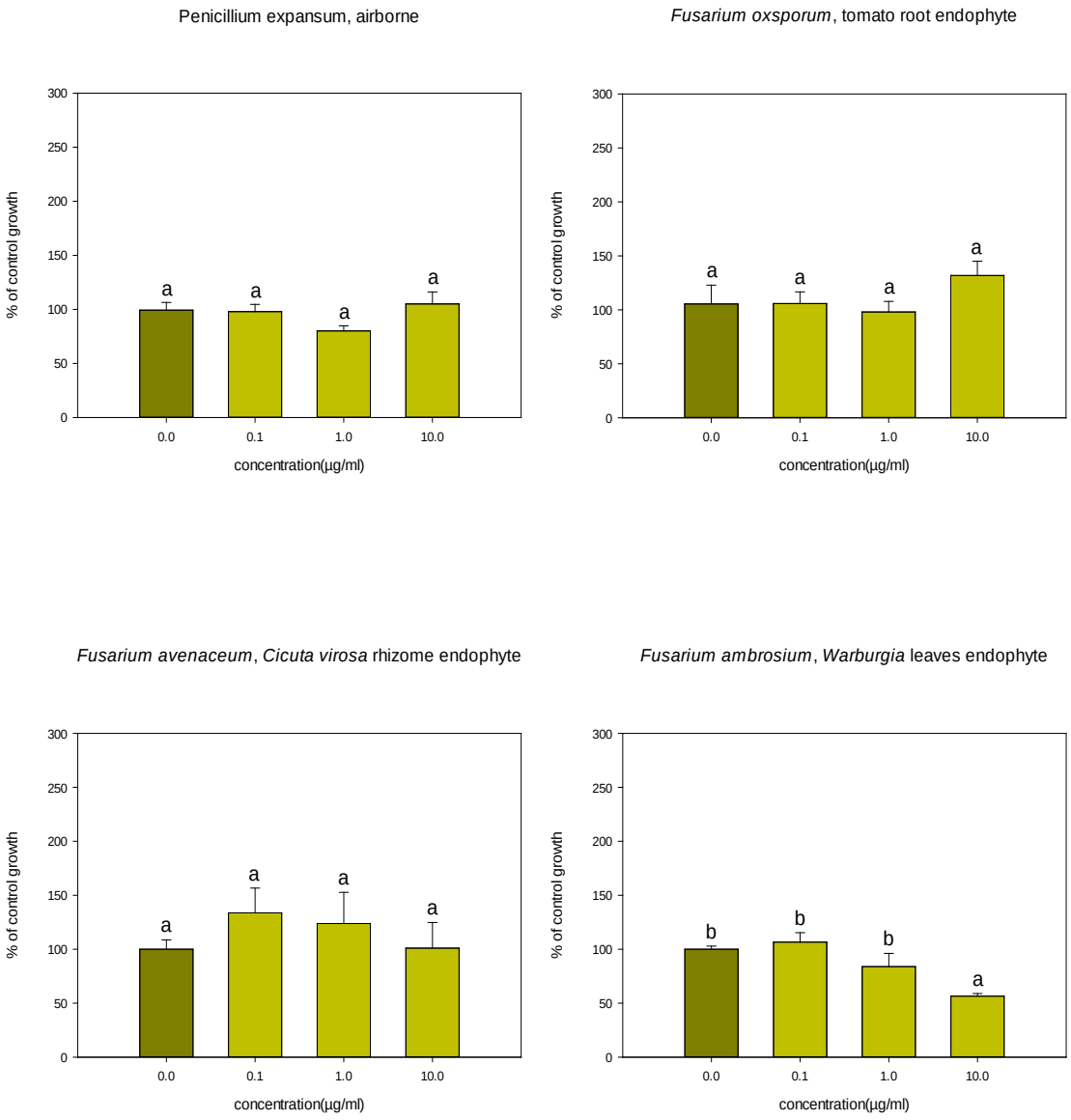
3.3. Substrate utilization of drimane crude extracts and extracts fractions

Root 1 crude extract



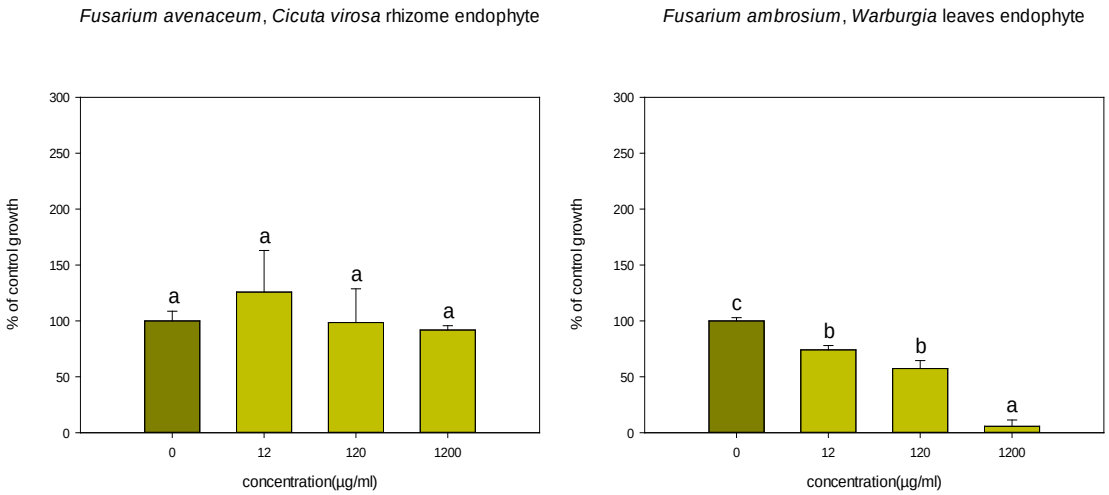
The root 1 crude extract caused variable effects on the tested fungal strains. *Fusarium ambrosium* and *F. avenaceum* were inhibited with increasing concentrations. *Fusarium oxysporum* remained unaffected and *P. expansum*, by contrast, was stimulated by higher concentrations.

Root 1 drimane fraction



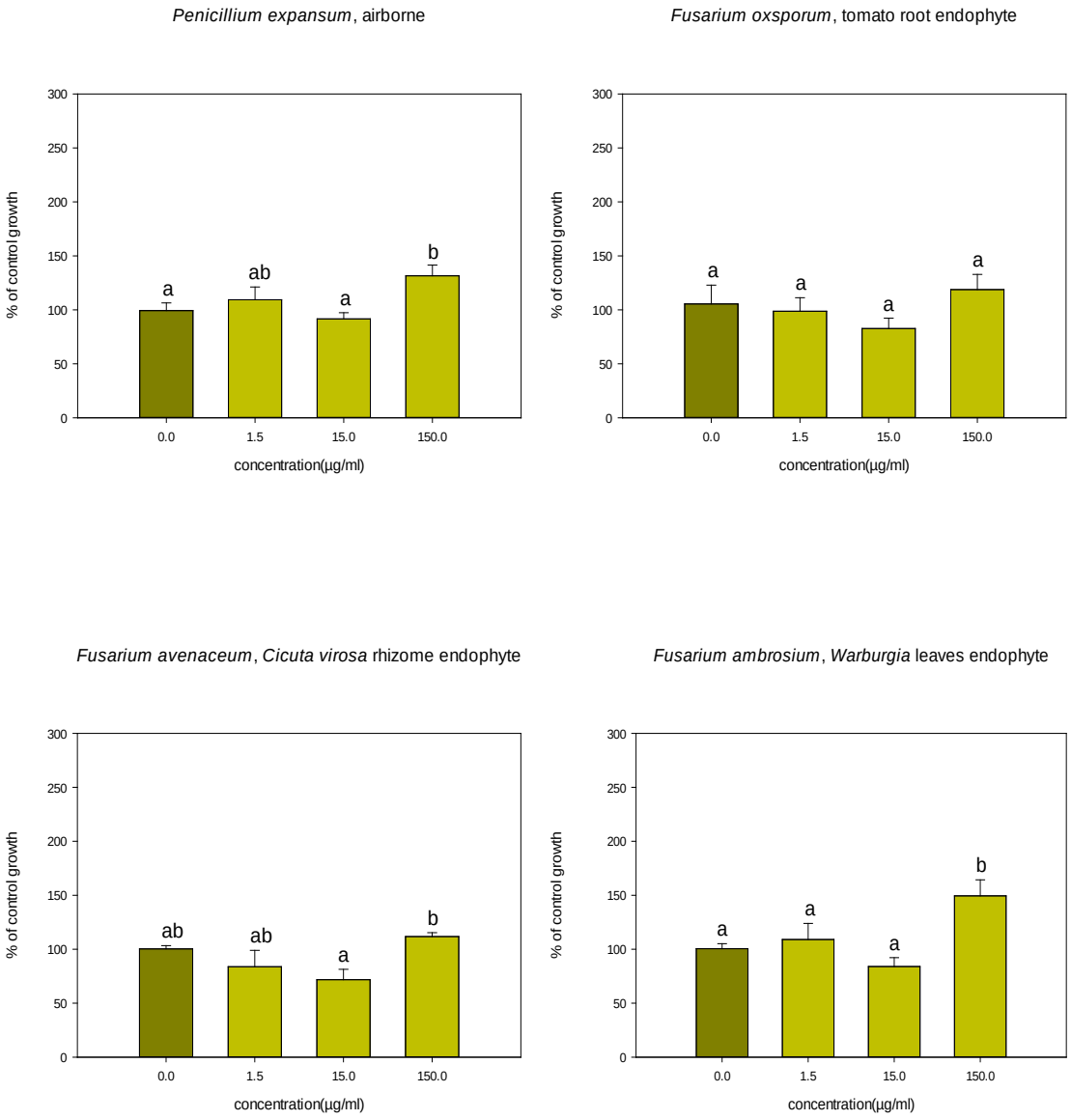
The root 1 drimane fraction only inhibited *F. ambrosium* with rising concentrations. All other fungi remained unaffected.

Root 1 hydrolyzed drimane fraction



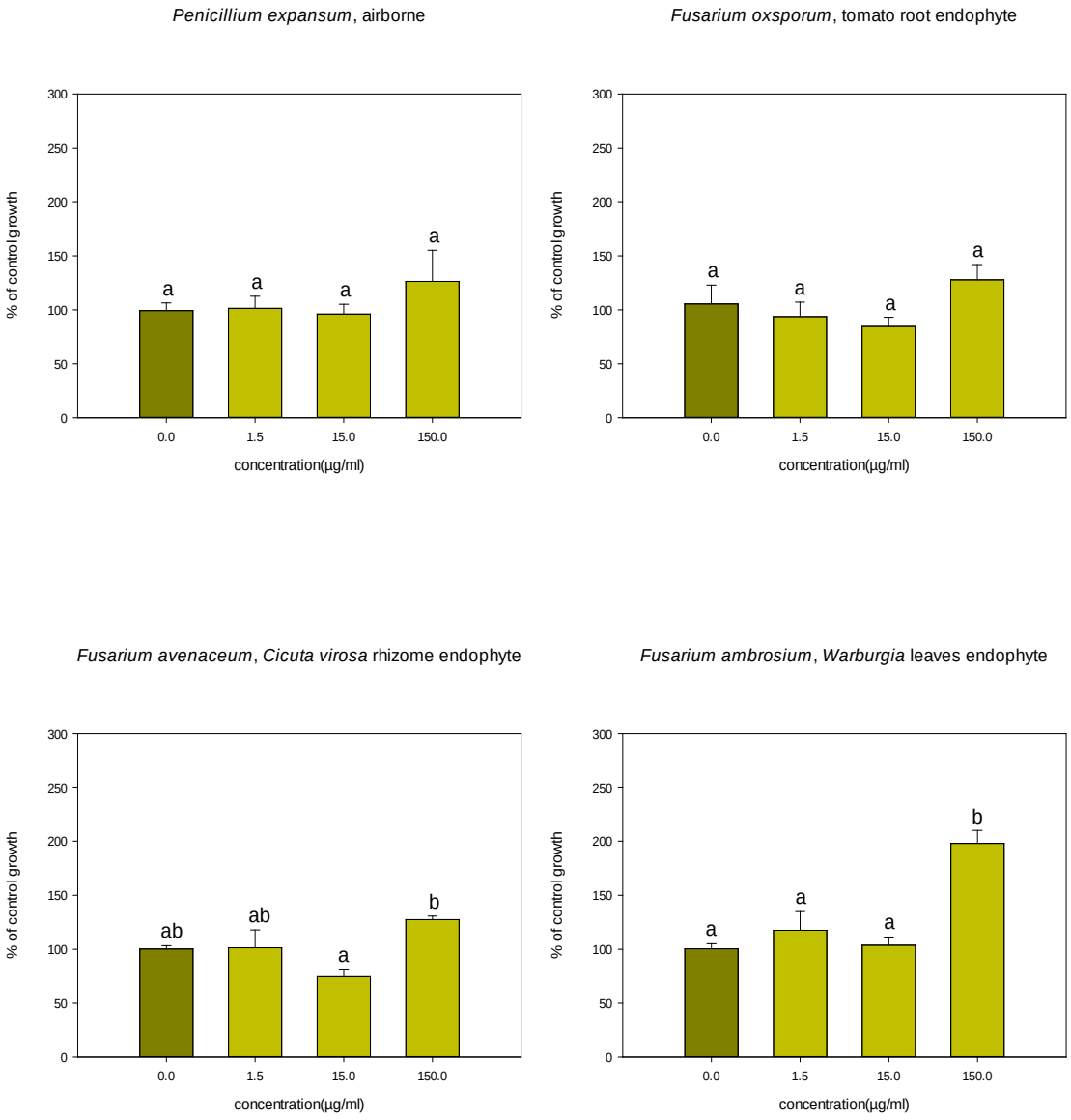
The root 1 hydrolyzed drimane fraction inhibited the growth of *F. ambrosium* with rising concentrations. *Fusarium avenaceum*, by contrast, remained unaffected. The high amounts of substrate required for this assay precluded assays with *F. oxysporum* and *P. expansum.*

Root 2 crude extract



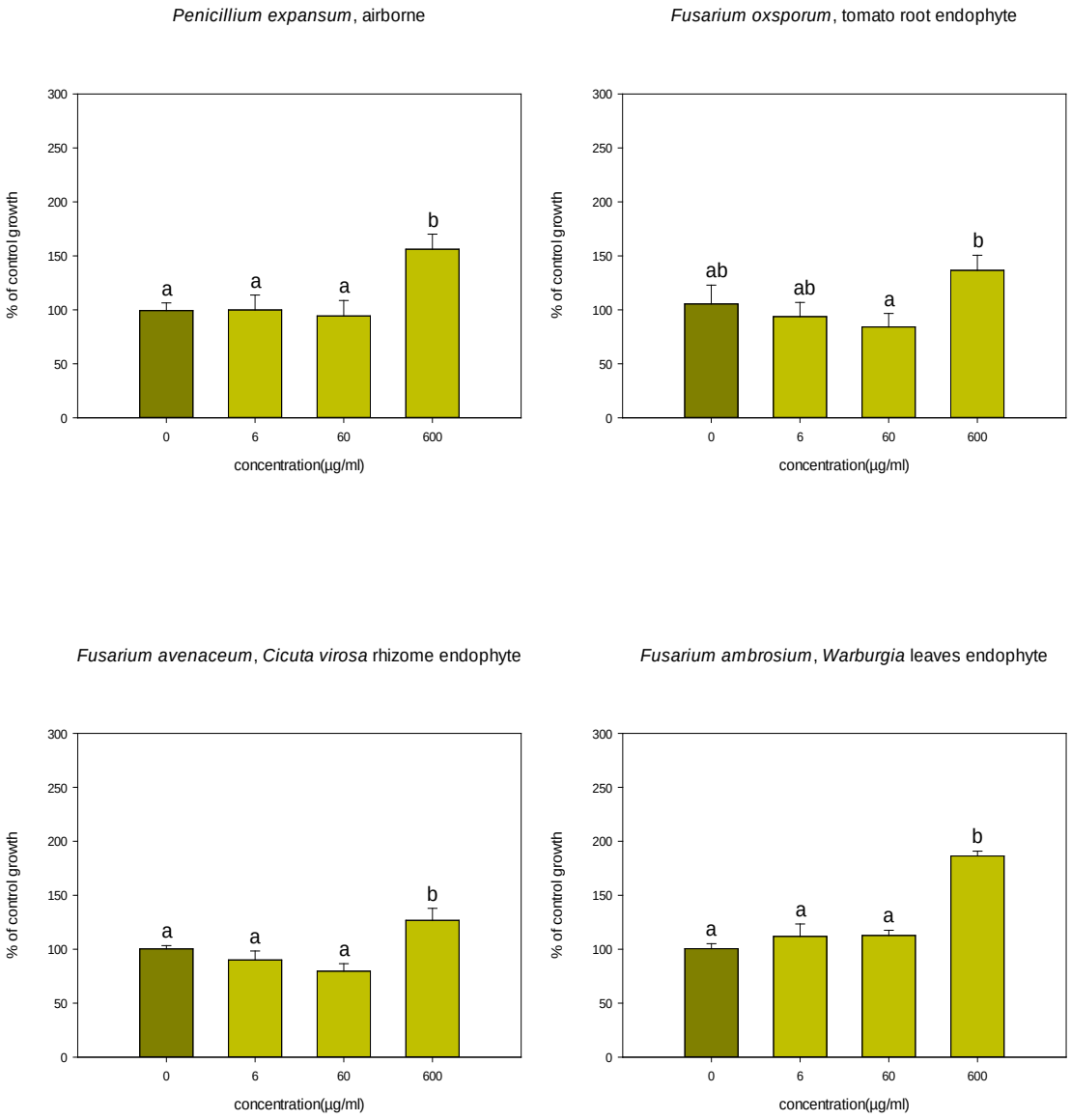
The root 2 crude extract inhibit none of the assayed but stimulated all fungi except *F. oxysporum* at the highest concentration tested.

Root 2 drimane fraction



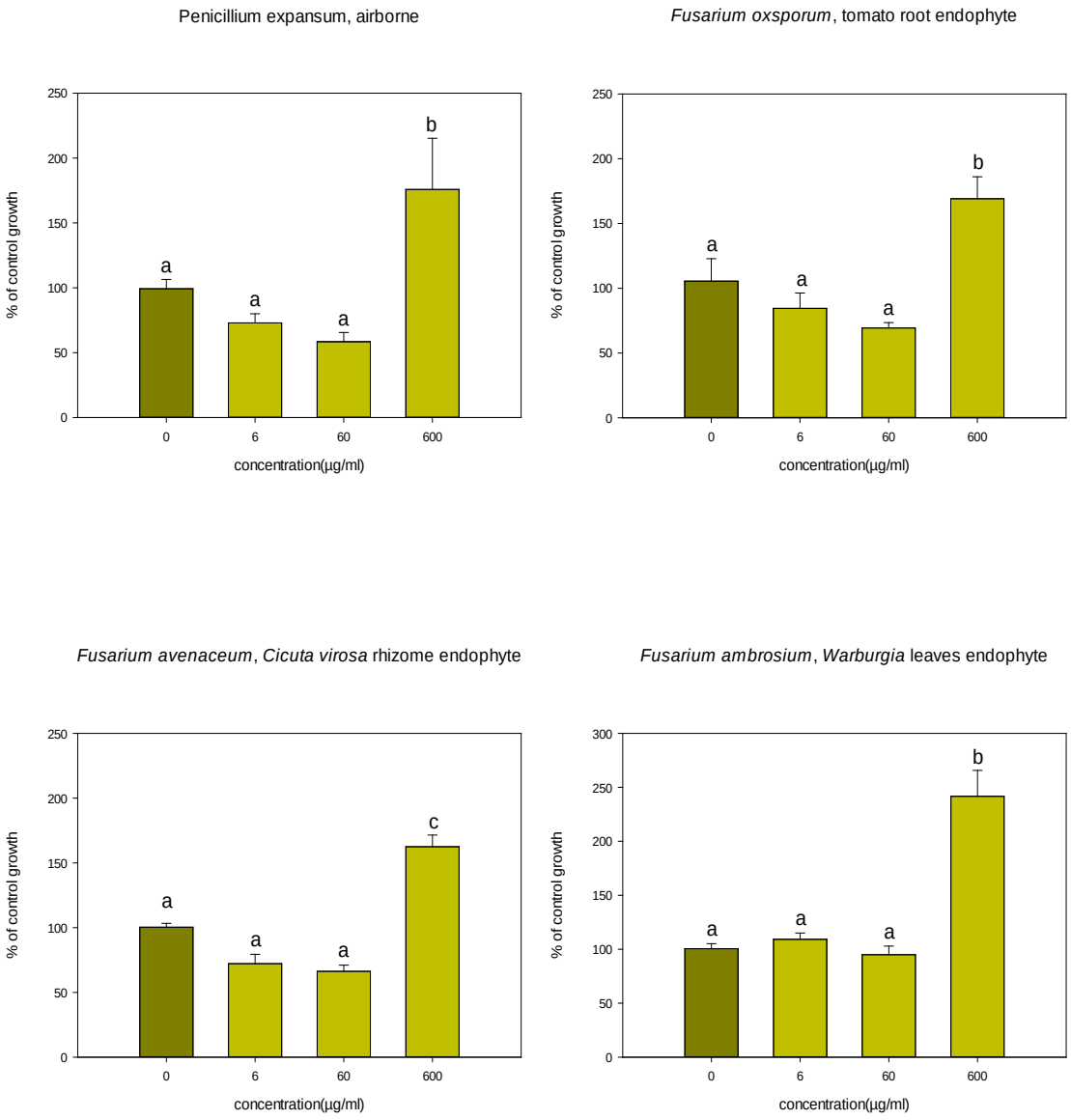
The root 2 drimane fraction stimulated growth of *F. ambrosium* and top a lesser extent *F. avenaceum* at the highest concentration. The other two fungi remained unaffected.

Root 2 hydrolyzed drimane fraction



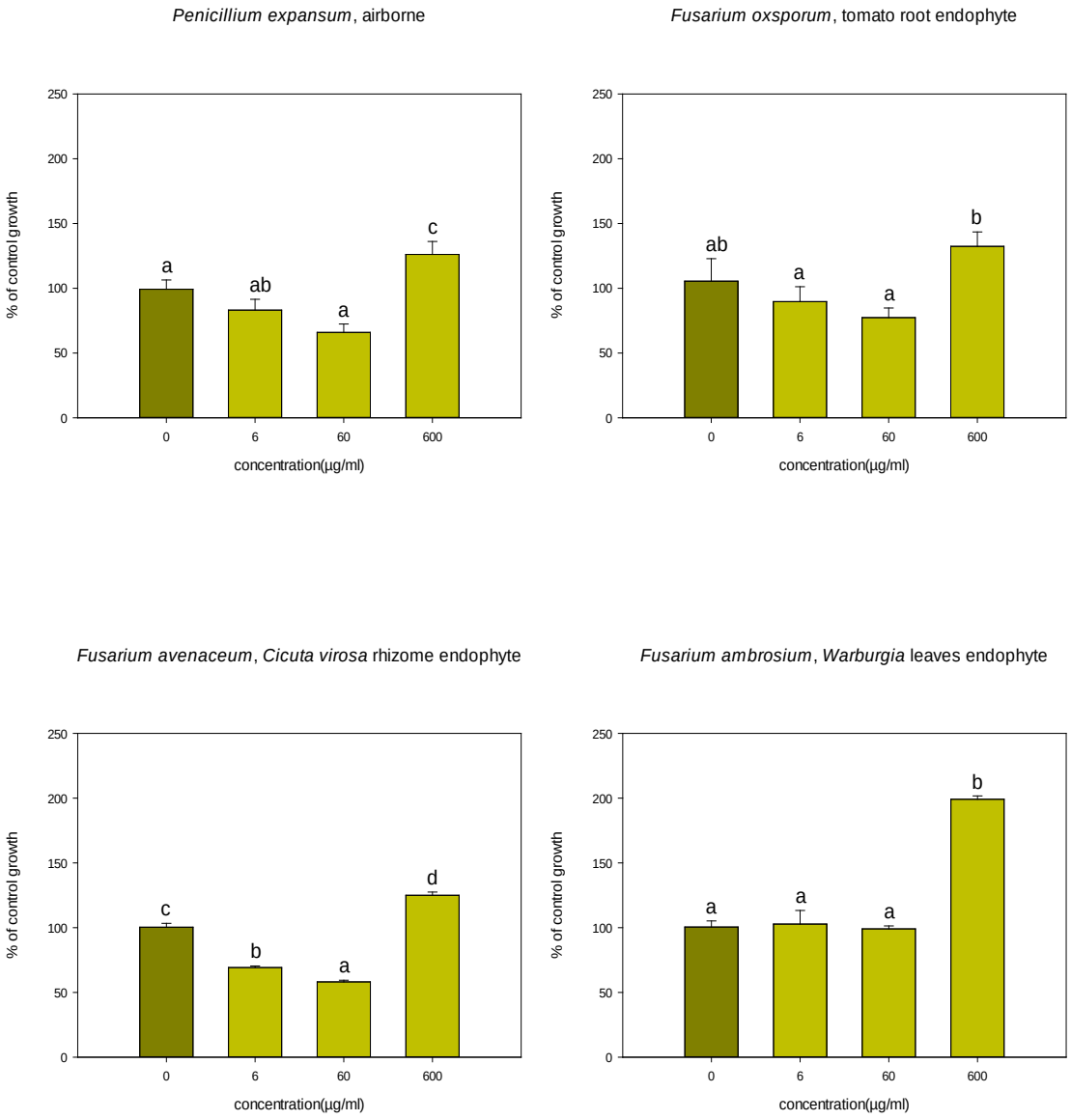
The root 2 hydrolyzed drimane fraction stimulated all the fungi at the highest concentration tested.

Leaf 1 drimane fraction



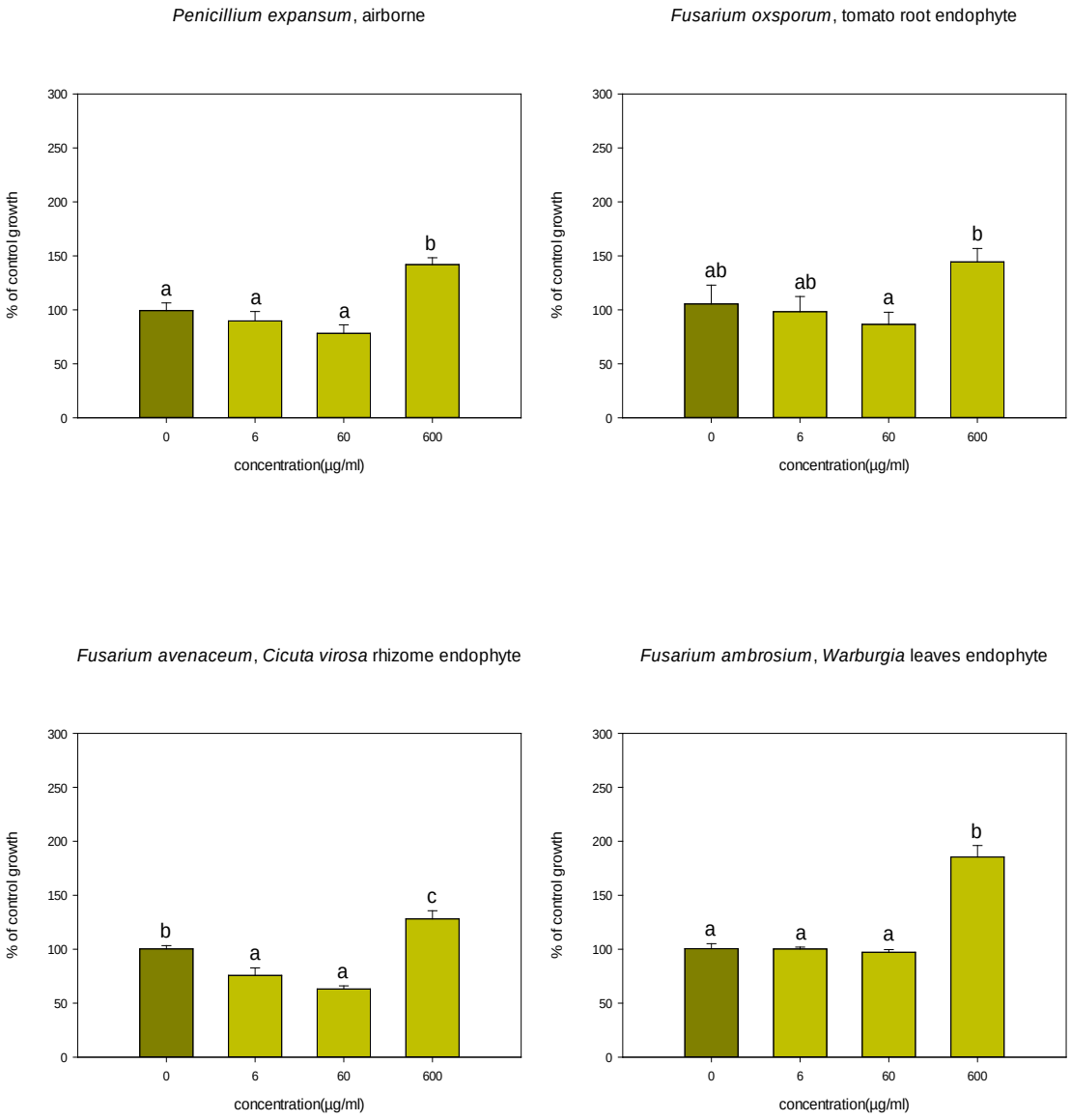
The leaf 1 drimane fraction simulated significantly all fungi at the highest concentration.

Leaf 1 hydrolyzed drimane fraction



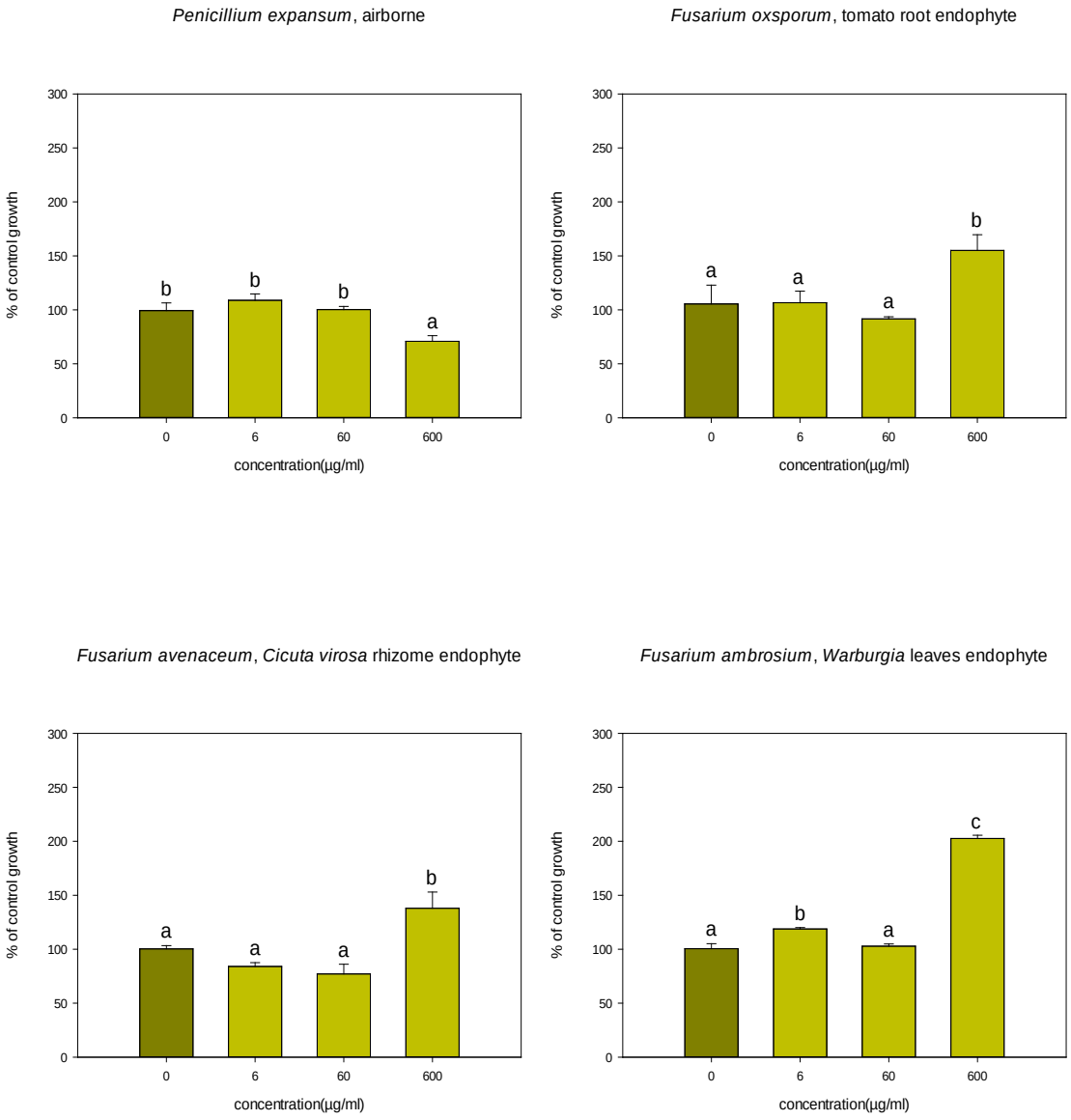
The leaf 1 hydrolyzed drimane fraction stimulated all the fungi with the strongest activity on *F. ambrosium*.

Leaf 2 drimane fraction



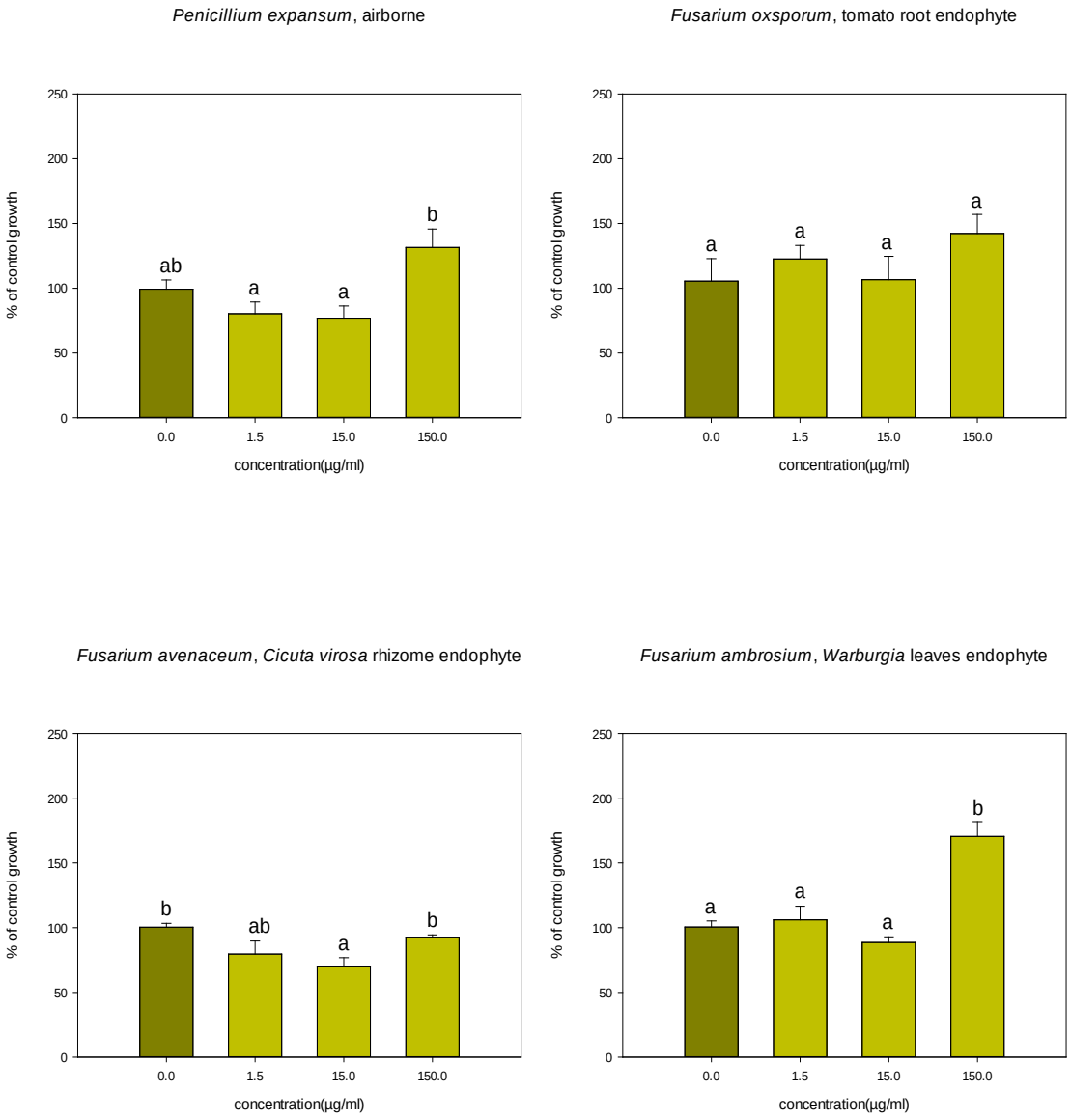
The leaf 2 drimane fraction stimulated all tested fungi at highest concentration all the fungi.

Leaf 2 hydrolyzed drimane fraction



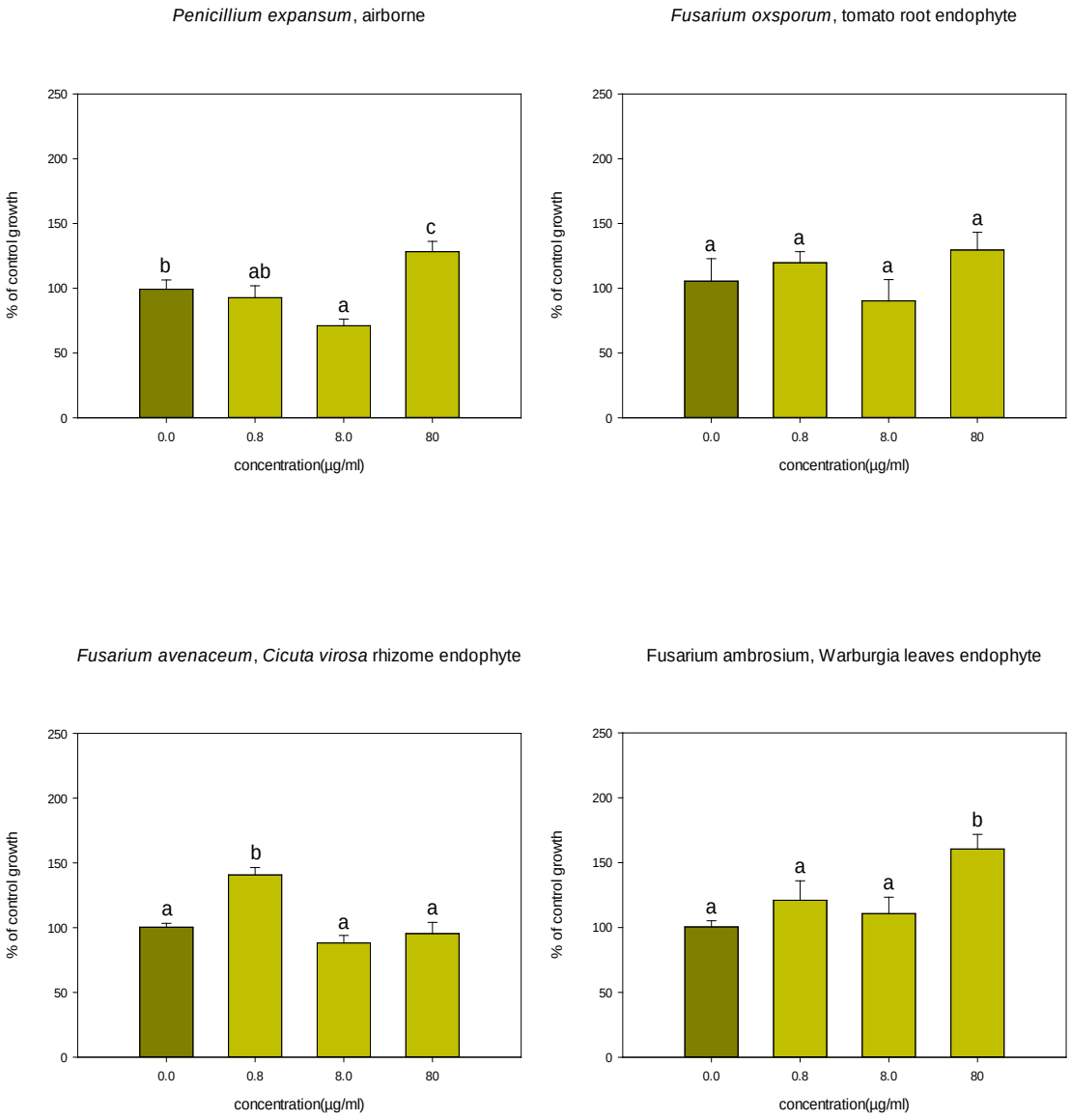
The leaf 2 hydrolyzed drimane fraction inhibited the airborne isolate P. expansum at the highest concentrations while all the endophytic isolates were all stimulated.

Fruit crude extract



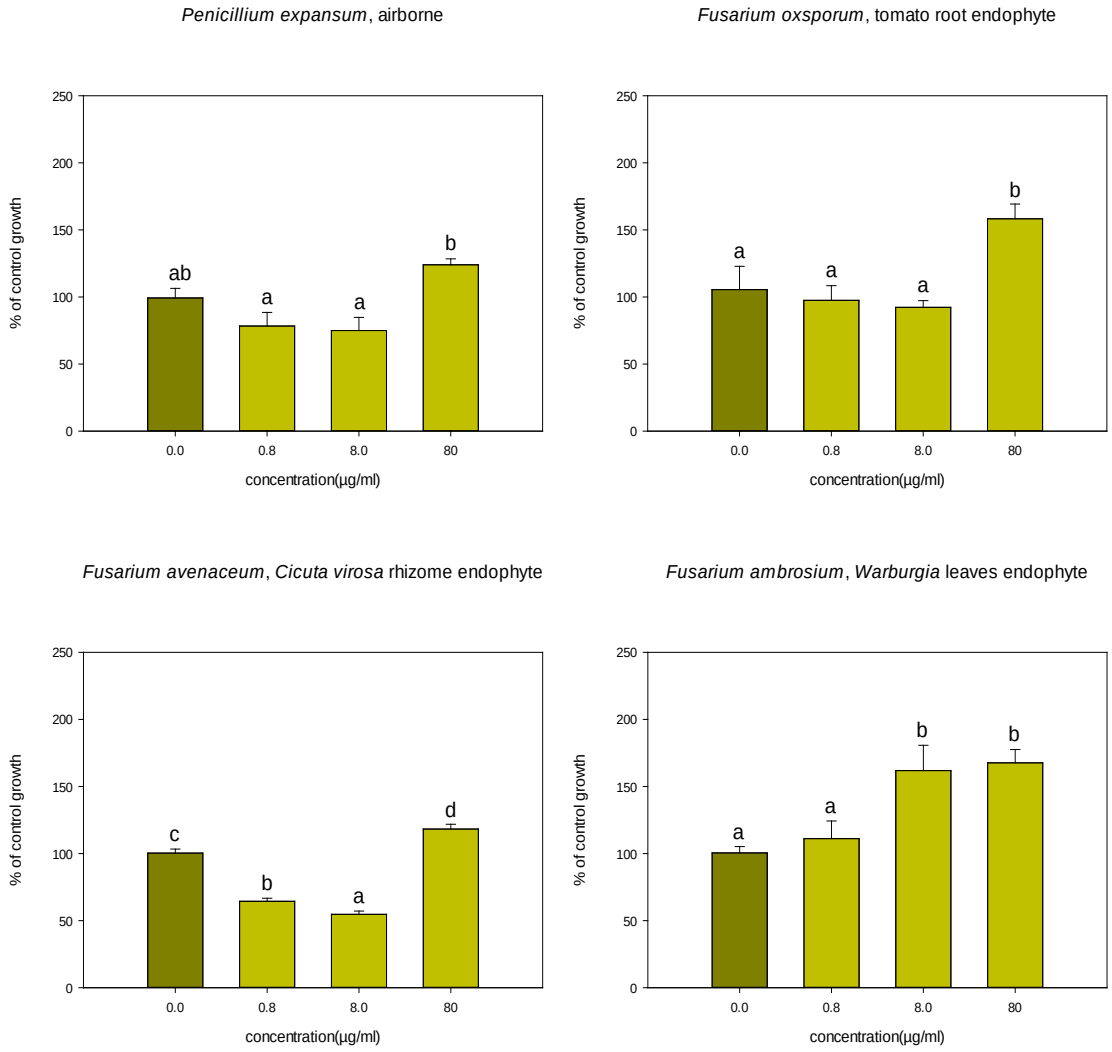
The fruit crude extract stimulated *F. ambrosium* growth at the highest concentration. *Fusarium avenaceum* was inhibited by the second lowest concentration.

Fruit drimane fraction



The fruit drimane fraction inhibited *P. expansum* growth at 8 µg/ml but stimulated at 80 µg/ml.*Fusarium avenaceum* was stimulated at 0.8 µg/ml, the lowest concentration tested, and *F. ambrosium* at 80 µg/ml, the highest concentration tested.

Fruit hydrolyzed drimane fraction



The fruit hydrolyzed drimane fraction stimulated *F. ambrosium* with increasing concentrations. All other fungal isolates at least were stimulated by the highest concentration tested. *Fusarium avenaceum* was inhibited by lower concentrations.

The error bars presented in figures, each extract and extract fraction separately, indicate the turbidity (mean, se of the three replicates) calculated as percentage of the control (0.0). The letters represent statistical significance, ANOVA with a 95% Duncan multiple range test.

3.4. Farnesol biotransformation

Here only results are presented from experiments where fungal growth was detectable in the controls. These included one pair of isolates of *P. expansum*, a *Warburgia* endophyte and an airborne isolate (Figure 6 and 7).

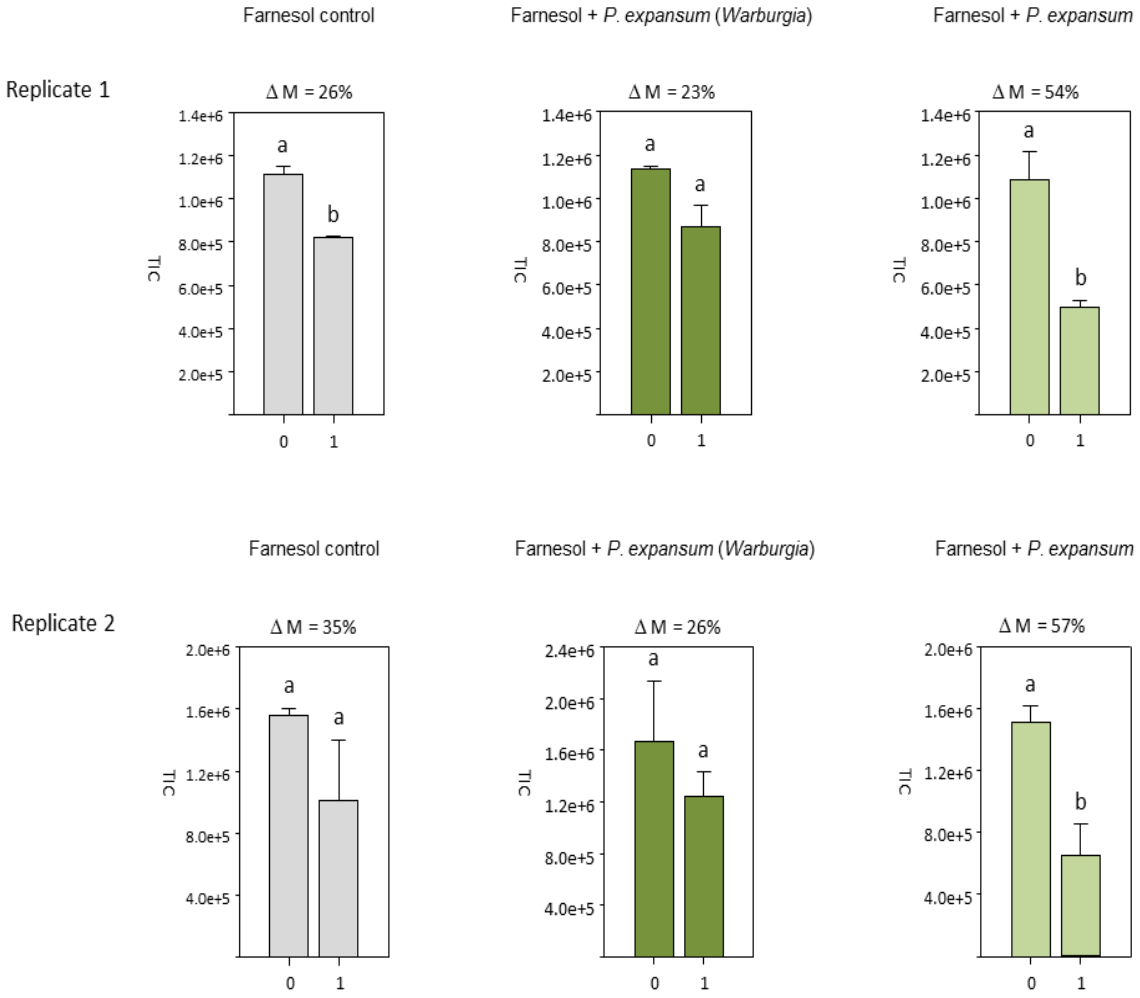


Figure 6: Effects of the *Warburgia* endophytic isolate (dark green) and an airborne isolate (light green) of *Penicillium expansum* on GC–MS detectable farnesol during 43 h of growth; control farnesol without fungal inoculation (grey); bars represent total ion concentration (TIC) of the farnesol isomer peaks (mean, standard error) at zero (**0**) and end time point (**1**); ΔM, mean difference of time points in %; ANOVA with a 95% Duncan multiple range test; $n = 3$.

The airborne isolate decreased the detectable farnesol nearly twice as much as the endophytic isolate. The effect was visible in both replicates. In replicate 1, however, the farnesol concentration decreased significantly at time point 1 whereas that was not the case in replicate 2 despite a more pronounced difference in the means.

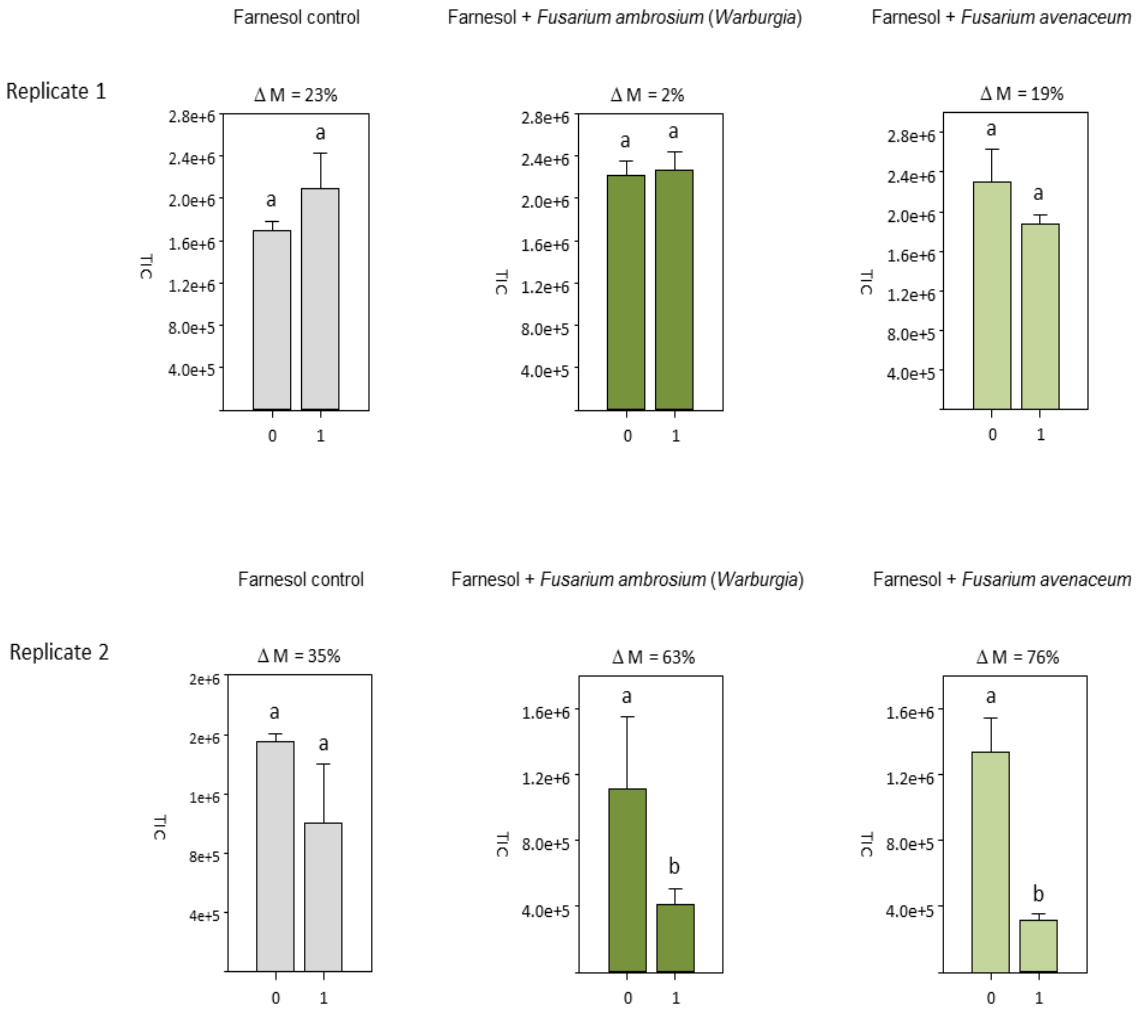


Figure 7: Effects of the *Warburgia* endophytic isolate *F. ambrosium* (dark green) and an endophyte from a different host plant, *F. avenaceum* (light green) on GC–MS detectable farnesol during 43 h of growth; control farnesol without fungal inoculation (grey);bars represent total ion concentration (TIC) of the farnesol isomer peaks (mean, standard error) at zero (**0**) and end time point (**1**); ΔM, mean difference of time points in %; ANOVA with a 95% Duncan multiple range test; $n = 3$.

No corresponding isolate to the endophyte *F. ambrosium* was available. Thus, an endophytic isolate of *F. avenaceum*, an endophyte of water hemlock *Cicuta virosa* L. from another host plant family (Apiaceae), was compared to *F. ambrosium*. In both replicates, *F. avenaceum* reduced the farnesol concentration in the medium more (% mean difference). However, the two replicates were not as comparable as in the experiment with *P. expansum*. Replicate 1 farnesol control showed a slightly higher though not significant increase at the time point t1; in replicate 2, by contrast, the farnesol concentration decreased at time point 1 as in all other experiments.

Attempts to use cholestane as standard failed because of irreproducible re-extraction from the medium.

4. Discussion

According to expectations, the drimane rich root and fruit extracts and their respective specific extract fractions inhibited the growth of all tested fungal isolates. Interestingly, the corresponding hydrolized drimane fractions did not reflect this effect at all. The GC–MS analysis showed no drimanes peaks, contrary to expectations, despite the fact that similar amounts had been used to prepare the samples. The only assumption in support of this scenario would be that either before or during silylation, the standard derivatisation of GC–MS samples, some form of polymerization occurred, which caused the substantial peak absence in the chromatogram of the hydrolized extract fractions. The dramatic decrease of inhibitory activity further suggests that polymerization most probably occurred immediately after the hydrolization procedure. This fact puts the initially asked questions into a completely different perspective and points to the polymerization phenomenon as a non-negligible aspect in terms of obtaining some understanding of the obtained results.

Drimane sesquiterpenes affect the growth and development of fungi and may constitute substrates when present in higher concentrations and no too pronounced toxic effects are exerted. There exist, however, differences between the fungal strains. One of them, *P. expansum*, isolated as an endophyte from *Warburgia* roots proved as rather insensitive to the antimicrobial drimanes in the MIC test. Unfortunately, the substrate utilization assays with this interesting isolate failed—the fungus did not grow despite positive preliminary tests and replication was impossible because of limited substrate amounts. The other two *Warburgia* endophytes, however, showed similar susceptibility to drimanes as fungal isolates from other sources. This suggests that reduced susceptibility against drimane sesquiterpenes may facilitate the endophytic life style in a

plant accumulating drimanes, but does not constitute an indispensable prerequisite. Hypothesis 1 thus neither can be rejected nor confirmed.

The tested concentrations in the substrate utilization of drimanes were chosen depending on the MIC of the offered extract or extract fraction to facilitate a more realistic comparison. Generally, all tested extracts from roots, fruits and leaves served as substrates at the highest concentration offered compared to the control that had to grow in water. Only one extract was different: root 1 was more antimicrobial and thus could not be offered in high enough concentrations to serve as substrate.

Chromatography suggested remarkable difference between the three extract preparations. In this context, however, it has to be pointed out that any polymerization products, the majority of the analytes in the extract, did not show as distinctive peaks in the chromatogram. Obviously, the quality of the polymers was somehow different. This assumption is supported by the assays. Perhaps, the extent of de- and repolimerization differed. In the GC, slightly less diversity in the prominent drimane peaks of similar intensity shows in root 1 compared to root 2 and the fruit extract. To what extent this phenomenon may be linked to polymer quality remains, however, elusive. The polymers are not only comprised of drimane sesquiterpenes, but also of sugar alcohols, especially mannitol because this analyte also is missing in the hydrolized drimane fraction, most possibly also due to increased polymerization. The similarity of the effects of the three extract preparations can be only explained by specific effects of the polymers; the detectable analytes differ considerably. The polymers, by contrast, constitute the only analytes that are unequivocally present in all three preparations. For definite conclusions, however, the scope of this study had to remain too limited, but in spite of this, one conclusion is supported: notonly a specific metabolite class may affect growth and development of endophytic fungi in

their host plants, but their polymerization chemistry with other metabolites present in the same solution.

Antimicrobial properties of polymers were first realized in peptides and antimicrobial peptides, which occur widely in multicellular organisms (16). Moreover, randomly formed polymers can mimic antimicrobial peptides (17). Today, various antimicrobial polymers are recognized and their activity was attributed to nitrogen functions that may occur as cations and was shown to depend on pH (18, 19). By contrast, drimanes contain no amine groups, but both nitrogen and oxygen functions can participate in redox chemical reaction, which may create, always depending on the present reactants and reaction milieu, specific “random” polymer structures (20).

The biotransformation of farnesol—that of the drimanes eluded the experimental attempts—did not yield any oxidized (10, 11) or other derivatives apart from the four known isomers (21). Instead, the GC–MS analyses revealed that the concentration of all isomers decreased. This affected all four isomers without exception (data not shown). The experiment was started in a well-defined chemical milieu and, depending on which fungus was present, the polymerization of farnesol proceeded with different speed. In this assay, the pair of the *P. expansum* isolates could be tested and the endophyte reduced the farnesol concentration less efficiently than the airborne isolate. *Fusarium ambrosium* and *F. avenaceum* are both endophytes, but only the former occurred in *W. ugandensis.* The first experiment produced some idiosyncratic results, which may be explained by an initially incomplete solution process of farnesol, but the second experiment suggested that *F. avenaceum*, the endophyte from another host plant, was slightly more efficient in reducing the farnesol concentration. The first experiment, though being idiosyncratic, also shows the same trend.

The decrease rate in farnesol may be related with the fungus's ability to create an oxidative milieu, which is required for polymerization. The strength of an oxidative milieu is determined by reactive oxygen species concentrations, which also have been shown to determine fungal development and its colonization of plant tissues (22). ROS are characteristic products of redox chemistry of oxygen and affect both polymerization and depolymerization of drimanes as well as that of farnesol. It is somehow justified to hypothesize that endophytes and non-endopyhtes may differ in this characteristic.

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Abstract

Endophytes comprise all microbes colonizing plant tissues which cause no disease symptoms. The pepper bark tree, *Warburgia ugandensis*, occurs in tropical South East Africa and is well known for its secondary metabolites, drimane sesquiterpenes, which are renowned for various biological activities and use in traditional medicine. Drimane sesquiterpenes are especially known for their antimicrobial activity. In context with endophytes, this tree represents an interesting model system to explore how endophytic microbes survive in host plants with efficient antimicrobial secondary metabolites and to what extent endophytes affect the patterns of secondary metabolites in their host plants. Accordingly, several experiments were carried out.

Several endophytic fungi and taxonomically closely related strains that were recovered from other sources were included in the study. The susceptibility of all fungal strains was determined against various root, leaf, and fruit crude extract, the respective enriched drimane fraction and their hydrolysis products (in attempts to remove masking sugars and esters). The same crude extracts and extract preparations were offered as substrates to the same fungi and their ability to utilize them was assessed by comparing them to water-grown cultures. Biotransformation assays with the crude extracts and extract preparations failed. Thus, the commercially available sesquiterpene farnesol was used instead for this experiment.

Drimane richness generally caused inhibition of the tested fungi, one of the endophyte isolates was less susceptible but other endophytic isolates were similarly susceptible than non-endophytic strains. In all successful experiments, the highest concentration tested (depending on the susceptibility) served as substrate for all tested fungi. No biotransformation products of farnesol could be detected and all tested fungal isolated decreased the detectable farnesol

concentrations. The enigmatic character of the results is discussed in terms of depolymerization and repolimerization dynamics of the drimane sesquiterpenes, which is caused by their high redox activity. Redox chemistry seems also to be a factor that contributes to adaptation of a fungal strain to an endophytic life style.

Keywords: *Warburgia ugandensis*, endophytes, secondary plant metabolites, drimane sesquiterpenes, farnesol

Zusammenfassung

Endophyten umfassen alle Mikroorganismen, die Pflanzengewebe besiedeln ohne Krankheitssymptome hervor zu rufen. Der Pfefferrindenbaum, *Warburgia ugandensis*, kommt im tropischen Südostafrika vor und ist bekannt hinsichtlich seiner Sekundärmetaboliten, Drimansesquiterpene, die sich durch eine Reihe von verschiedenen biologischen Aktivitäten und dem Einsatz in der Volksmedizin auszeichnen, insbesondere für ihre antimikrobielle Wirkung. In Zusammenhang mit Endophyten stellt dieser Baum ein interessantes Modellsystem dar, in dem erforscht werden kann, wie Endophyten in einer Wirtspflanze mit effizienten antimikrobiellen Sekundärmetaboliten überleben können beziehungsweise in welchem Ausmaß Endophyten Sekundärstoffwechselmuster der Wirtspflanze beeinflussen können. In diesem Zusammenhang wurden verschiedene Experimente ausgeführt.

Mehrere endophytische Pilze und taxonomisch verwandte Isolate von anderen Quellen wurden in die Studie aufgenommen. Deren Empfindlichkeit gegenüber verschiedenen Wurzel, Blatt- und Fruchtextrakten, deren jeweilige angereicherte und hydrolisierte Drimanfraktion (letztere als Versuch, maskierende Zucker- und Estergruppen abzutrennen) wurde für alle Pilze überprüft. Dieselben Extrakte und Extrakpräperationen wurden als Substrat den Pilzisolaten angeboten und die Verwertung mittels Vergleich mit Wasserkulturen überprüft. Biotransformationsversuche mit denselben Extraktfraktionen schlugen fehl, daher wurde der kommerziell erhältliche Sesquiterpenealkohol Farnesol als Ersatz verwendet.

Hoher Drimangehalt führte zu einer Hemmung der getesteten Pilze. Einer der Endophyten war weniger empfindlich, doch andere Endophyten wurden ähnlich gehemmt wie Nichtendophyten. In allen erfolgreichen Experimenten führten höhere Substratkontentrationen

(sofern es die antimikrobielle Aktivität zuließ) zu einer Entwicklungsförderung. Keine Biotransformationsprodukte von Farnesol konnten detektiert werden und alle getesteten Isolate verringerten die Farnesolkonzentration. Der schwer zu interpretierende Charakter der Ergebnisse wird im Lichte von Depolymerisations und Repolymerisationsdynamik der Drimane diskutiert, welche durch ihre Redoxaktivität hervorgerufen wird. Redoxchemie ist offenbar ein Faktor, der zur Anpassung eines Pilzes an einen endophytischen Lebensstil beizutragen scheint.

Curriculum Vitae

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