Bull. Chem. Soc. Ethiop. **2003**, 17(2), 185-190. ISSN 1011-3924
Printed in Ethiopia

BIOACTIVE METABOLITES FROM *TRICHODERMA HARZIANUM* AND *TRICHODERMA LONGIBRACHIATUM*

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(Received April 8, 2002; revised March 26, 2003)

ABSTRACT. The tea plant, *Camellia sinensis* (L.) O. Kuntze is an important crop in the agriculturally based economy of Kenya. Many diseases affect the tea plant but the most prevalent is armillaria root rot caused by the fungus *Armillaria mellea*. Compounds from the fermentation of *Trichoderma* species in different media were bioassayed against some selected gram-positive and gram-negative bacteria, fungi including *Armillaria mellea*, a yeast and a Mucor. Compounds obtained from *T. harzianum*, and *T. longibrachiatum* when cultured in various media were investigated individually for *in-vitro* antifungal and antibacterial activities by agar diffusion technique. Some of the compounds produced definite antifungal and antibacterial activities. 2-Phenylethanol (**1**) and tyrosol (**2**) obtained from *T. harzianum* are reported for the first time from *Trichoderma* species. The most active metabolite isolated from these strains was 6-n-pentyl-•-pyrone (**3**), which showed the highest antifungal and antibacterial activity and completely inhibited the growth of *Armillaria mellea* fungus at a concentration of 200 ppm. Compound **4** (sorbicillin) exhibited moderate activity against the fungal test organisms.

KEY WORDS: *Trichoderma harzianum*, *Trichoderma longibrachiatum*, 2-Phenylethanol, Tyrosol, 6-n-Pentyl-•-pyrone, *Armillaria Mellea*

INTRODUCTION

Tea is an important cash crop in Kenya and is the leading foreign exchange earner in the country and provides a livelihood for more than a million people [1]. The most prevalent tea disease in Kenya especially in the Nyayo Tea Zone plantations is armillaria root rot caused by the fungus *Armillaria mellea* Vahl [2]. In these plantations, forestlands are cleared and immediately tea is planted before the fields are adequately prepared to limit the presence of the inoculum of the pathogenic *A. mellea* fungus [2].

Armillaria root rot is currently controlled through the use of synthetic soil fumigants such as methyl bromide and carbon disulphide. These compounds have adverse environmental effects and their use is being discouraged internationally. *Trichoderma* species have been used as biocontrol agents against plant pathogens and could be a possible source of fungicides against *A. mellea* fungus. It is therefore essential to identify and bioassay the secondary metabolites from *Trichoderma* isolates against *A. mellea*.

Some *Trichoderma* species are antagonistic to many soil borne phytopathogenic fungi and significantly decreased infection and disease through antibiosis and mycoparasitism [3]. In many cases, suppression of soil borne plant pathogens has been attributed to *Trichoderma* species [4-6]. The culture broth of *T. harzianum* has been shown to be bioactive against *A. mellea* [7].

Investigations have shown that 6-pentyl-α-pyrone and other α-pyrone analogues exhibit antibiotic activity against the growth of the fungus *Gaeumanmyces graminis* Vartriticis [8]. 6-Pentyl-α-pyrone has been reported to inhibit growth *in vitro* of a number of fungi and that it reduced the rate of damping-off in lettuce by inhibiting the growth of *Rhizoctonia solani* [9].

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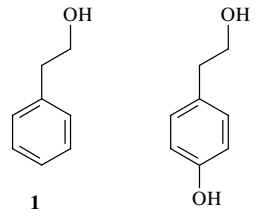
In the present investigation, five metabolites were isolated from the culture broth of *T. harzianum* and the mycelium of *T. longibrachiatum*. 2-Phenylethanol (**1**), and tyrosol (**2**), are reported for the first time from *Trichoderma* species. 6-*n*-Pentyl-α-pyrone (**3**), sorbicillin (**4**) and ergosterol (**5**) had earlier been reported from *T. koningii, T. longibrachiatum* and *T. hamatum*, respectively [10-12]. The metabolites were bioassayed against standard bacterial and fungal test organisms and *A. mellea*.



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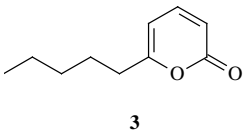

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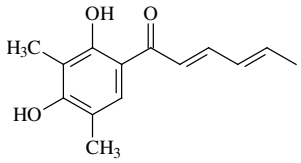

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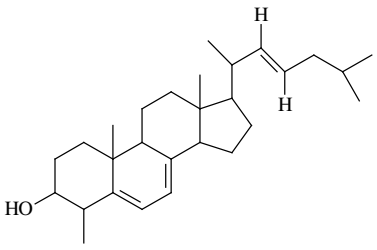
EXPERIMENTAL

General experimental procedures. IR spectra: Perkin-Elmer 598 FTIR series spectrometer in KBr pellet. UV: Perkin–Elmer lambda 16 UV/VIS spectrometer in MeOH. NMR: Varian VXR 200 in $CDCl_3$ at 200 MHz for ^{1}H NMR and 50 MHz for ^{13}C NMR. The purification of the compounds was carried out using column chromatography followed by prep. HPLC as described by Ouma [13].



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In the present investigation the *Trichoderma* species studied were *T. harzianum* (IMI 339496) and *T. longibrachiatum* (IMI 339495). These species of *Trichoderma* were isolated at Tea Research Foundation, Kericho from soils obtained from tea growing areas in Eastern and Western Kenya and identified at the International Mycological Institute (IMI), Kew, UK [7].

The *Trichoderma* species were cultured in various media as reported by Schneider [14] and Omolo [15]. The fungal test organisms were grown in yeast glucose malt (modified) agar medium while the bacterial test organisms were cultured in nutrient broth (Difco, USA) [14].

Bioassay tests. The bioassay tests of the isolated compounds was against the test organisms which included gram-positive bacterium; *Bacillus brevis* (ATCC 6633), *B. Subtilis* (ATCC 9999) and *Sarcina lutea* (*Micrococcus leuteus*) (ATCC 381) and a gram-negative bacterium, *Enterobacter dissolvens* (LMG 2683); fungal test organism *Paecilomyces variotii* (ETH 114646), *Penicillium notatum* (isolated in the Department of Biotechnology, University of

Kaiserslautern, Germany); mucor, *Mucor miehei* (TÜ 284 and a yeast, *Nematospora corylii* (ATCC 10647). The bioassay was carried out using agar diffusion technique [14, 16].

Assessment of *Trichoderma* metabolites for antibiosis against *A. mellea* was carried out as described by Onsando [7]. The data was analyzed using the ANOVA, One Factor Randomized Complete Block Design.

All strains of the *Trichoderma* species were separately fermented in various media and the parameters that were monitored during the fermentation included pH, glucose content, growth (mycelial dry weight), and the antibiotic activity against *Nematospora corylli* [13, 14, 17].

Extraction and isolation of Trichoderma metabolites. The culture broth of *T. harzianum* was extracted with the resin DIA-ION HP 21 (Mitsubishi, Düsseldorf, Germany) while the mycelium of *T. longibrachiatum* was separated from the culture broth and extracted as outlined by Schneider [14] and Tarus [17]. Isolation of bioactive metabolites from the extracts of *T. harzianum* and *T. longibrachiatum* was carried out using column chromatography (silica gel and sephadex), prep. HPLC and recrystallizations to obtain compounds **1-3** from *T. harzianum* and **4-5** from *T. longibrachiatum* as described by Tarus [17].

RESULTS AND DISCUSSION

Fermentation monitoring

Most of the fermentation profiles of *T. harzianum* (IMI 339496) and *T. longibrachiatum* (IMI 339495) isolates followed the expected trends of fungal growth with a few exceptions. Some fermentation profiles of *Trichoderma* species obtained in this study are illustrated in Figures 1 and 2.

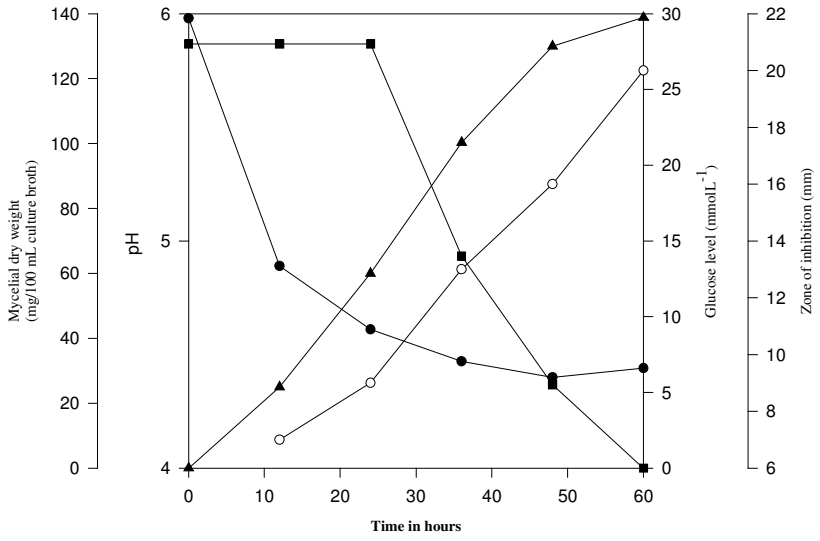

Figure 1. Fermentation of *Trichoderma harzianum* (T4, IMI 339496) in 20 litres double malt medium (• pH, □ glucose, ▲ Mycelial dry weight, ○ Zone of inhibition).

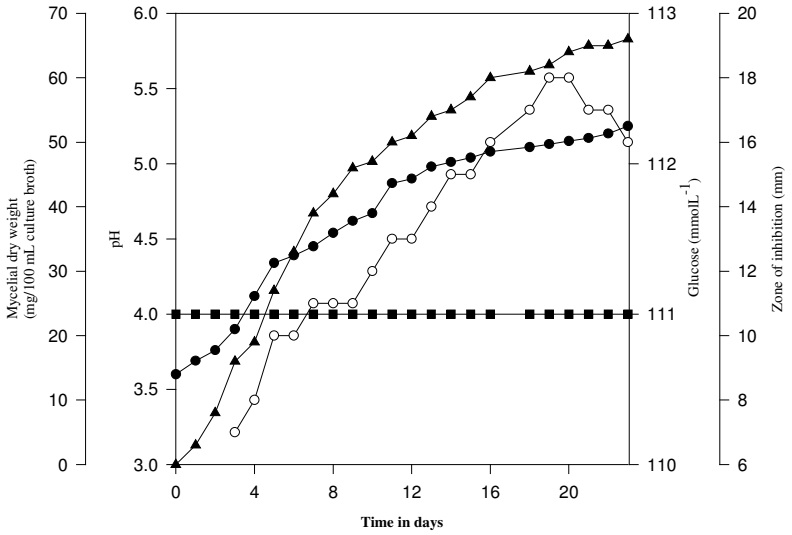

Figure 2. Fermentation of *Trichoderma longibrachiatum* (T3, IMI 339495) in potato-glucose medium (• pH, □ glucose, ▲ Mycelial dry weight, ○ Zone of inhibition).

Isolated compounds

From the comparison of the UV, IR and NMR spectral data of compounds **1-5** and with literature data, the structures of these compounds were determined to be **1** (2-phenylethanol); **2** (2-(hydroxyphenyl) ethanol) [18]; **3** (6-n-pentyl-α-pyrone) [10, 19]; **4** (sorbicillin) [8, 20] and **5** (ergosterol) [12, 21].

Bioassay against test organisms

The isolated compounds were bioassayed against the test organisms and the results are presented in Table 1. The antifungal activity of **3** was highest against *P. variotii* (30 mm), *P. notatum* (27 mm) and the yeast *N. coryli* (35 mm) when tested at 100 µg per 6 mm filter paper disk. The antibacterial activity of **3** was much lower with inhibition zones of between 11 mm and 13 mm for the gram-positive bacteria, *B. brevis, B. subtilis* and *S. lutea* and an inhibition zone of 9 mm against the gram-negative bacteria, *E. dissolvens*. Compound **4** exhibited antifungal properties against the fungi *P. variotii* (11 mm) and *P. notatum* (12 mm) and exhibited no activity against *N. corylii*, *M. miehei*, *B. brevis*, *B. subtilis*, *S. lutea* and *E. dissolvens*. Compounds **1**, **2** and **5** did not exhibit any activity against the fungal and bacterial test organisms used in the present study.

Bioassay against A. Mellea fungus

The results of the bioassays of isolated compounds against *A. mellea* isolate N are shown in Table 2. Compound **3**, significantly (P = 0.05) inhibited the mycelial and rhizormorphal growth of *A. mellea* isolate N at a concentration of 100 ppm as compared to the control up to 21 days. The fungus did not grow when 200 ppm of **3** had been incorporated in the medium. The compounds **1**, **2** and **5** did not significantly (P = 0.05) inhibit mycelial growth at concentrations of 200 ppm while compound **4** did not significantly (P = 0.05) inhibit rhizormorphal growth at concentration of 100 ppm.

Table 1. Zones of inhibition in millimetres of isolated compounds tested at 100 μg per disk.

Isolated pure compounds	**1**	**2**	**3**	**4**	**5**
Paecilomyces variotii	-	-	30	11	-
Penicillium notatum	-	-	27	12	-
Nematospora corylii	-	-	35	-	-
Mucor miehei	-	-	18	-	-
Bacillus brevis	-	-	13	-	-
Bacillus subtilis	-	-	12	-	-
Sarcina lutea	-	-	11	-	-
Enterobacter dissolvens	-	-	9	-	-

- no inhibition.

Table 2. Bioassay results of the isolated compounds against *Armillaria mellea* isolate N.

Compounds/days Concentration (ppm)	14 days	18 days	21 days
1 (100)	7.5a	11.8a	15.3a
2 (100)	7.5a	11.7a	15.7a
3 (200)	0.0d	0.0d	0.0c
3 (100)	0.5c	1.6c	4.5b
4 (100)	7.3a	11.0a	14.8a
5 (100)	7.3a	10.8a	15.0a
Control	6.3a	9.7a	13.3a

Mean values followed by the same letter are not significantly different from each other (P = 0.05) according to Duncan's Multiple Range Test.

CONCLUSION

Compounds **1** and **2** were the major metabolites obtained in most of the fermentation of *T. harzianum* in the various media. It is the first time that these compounds are isolated from the *Trichoderma* species. Compound **3** showed the highest activity against the test organisms and completely inhibited the growth of *A. melea* at 200 ppm. Compound **3** was therefore a promising prospect for field trial in a bid to control *A. mellea* in tea.

ACKNOWLEDGEMENTS

The authors are grateful to German Academic Exchange Services (DAAD) for financial support both in Kenya and Germany. Special thanks to Prof. Dr. H. Anke of the Institute of Biotechnology and Drug Research, University of Kaiserslautern for hosting PKT in her laboratory. Finally we wish to express our gratitude to Mr Washington Otieno and Joel Lang'at of the Tea Research Foundation, Kericho, Kenya for helping with the *A. mellea* assays.

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