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TOLYPOCLADIUM TERRICOLA SP. N., A NEW MOSQUITO-KILLING SPECIES OF THE GENUS TOLYPOCLADIUM GAMS (HYPHOMYCETES)

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Abstract. A new entomopathogenic species of the genus *Tolypocladium, T. terricola* is described from a soil sample from Finland. From other known *Tolypocladium* species, *T. terricola* differs in morphology, production of secondary metabolites and possession of relatively strong mosquitocidal activity. The fungus is characterized by broad oval conidia (2.5 × 2 μm) with one pointed end which are produced in grape-like clusters, and are not firmly adherent. When treated with *T. terricola*, mosquito larvae show typical features of intoxication characterized by the concentration of larvae in the centre of cup, hanging by their siphons on the surface.

Fungi of the genus *Tolypocladium* Gams, 1971, were in many cases isolated from field specimens of dead invertebrates, including insects and rotifers. *T. cylindrosporum* Gams was involved in natural infections of mosquito larvae (Weiser and Pillai 1981). Experimental infectivity for mosquito larvae was demonstrated also with *T. inflatum* Gams (= *niveum* (Rostrup) Bisset), *T. geodes* Gams, and *T. cylindrosporum* (Weiser 1986, 1987).

Among different natural isolates, which were evaluated for direct mosquito-killing activity, a strain isolated from soil collected near Tampere, Finland, was found to induce a rapid mosquito larval mortality. This isolate differs in morphology from described members of the genus *Tolypocladium*. The aim of the presented paper is to describe this isolate and compare its mosquito-killing activity with *T. cylindrosporum*, CCEF 1679 as a standard mosquito-killing pathogen.

MATERIALS AND METHODS

The fungus was isolated in Martin's agar with bengal red from one of the nonfertile soil samples collected near Tampere, Finland, in a search for mosquitocidal sporeformers. The isolate was cultivated on Sabouraud's nutrient agar and Bacto Mycological broth (with 1 % Soytone and 4 % Dextrose). On both media the growth was rather slow at 20 °C. Conidiation was studied on wet mounts and Giemsa stained preparations under the light microscope. Fragments of the mycelium were fixed with 2 % glutaraldehyde and inspected in the scanning electron microscope. For transmission electron microscopy studies, mycelium was fixed in 4 % glutaraldehyde in cacodylate buffer, embeded in Vestopal resin and cut in ultrathin sections for examination by Philips EM 420 electron microscope at 80 kV.

The same conditions as described by Weiser (1987) were used for the evaluation of mosquitocidal activity of the new strain. Briefly, laboratory standard strain 1979 of *T. cylindrosporum* and *T. terricola* were cultivated on Sabouraud agar slants. After 20 days cultivation at 25 °C suspension of

conidia was prepared in sterile water. L_4 *Culex pipiens autogenicus* larvae were placed in plastic cups with 100 ml of conidia suspension ranging from $5 \cdot 10^5$ to $5 \cdot 10^7$ conidia · ml^{-1}. Dead larvae were examined under the dissecting microscope for any movement of the heart or for growth of the fungus. Mortalities were corrected according to Abott's formula. In experiments extending over 24 hrs food was added in the form of powdered rat food pellets.

The mycelial mass was extracted with methyl alcohol and screened for the presence of secondary metabolites by HPLC. The Varian Vista HPLC apparatus, equipped with a DS 604 data station, a UV 200 variable wavelength detector and Hypersil ODS, 5 µm (250 × 4.6 mm, I.D.) column from Keystone Scientific, Inc. (Bellefonte, PA, U.S.A.) was used for analysis.

RESULTS

The new species differs from other known *Tolypocladium* species and is defined as follows:

Tolypocladium terricola sp. n.

Coloniae 15–20 mm in diametro post 10 dies (medio Sabouraud, 26 °C), candidae et floccosae, cremescentes, reverso albo, sine odore distincto. Hyphae hyalinae, leves, 1.5–3 µm latae. Sudor in guttis hyalinis tempore sporulationis. Conidiophora hyalina, 20–30 × 3–6 µm. Phialides verticillatae vel solitariae, laterales vel terminales conidiophoris insident, parte basali 2.8–3.5 × 2–3 µm inflata usque globosa, colle angusto saepe deflexo 3–4 × 0.5–1 µm. Conidia singularia sive pauce congregata, subglobosa, hyalina, uniformes 2.5–3 × 2–2.5 µm, uno extremo rotundo, altero in papillam minutam contracto seu acuminato.

Habitat: In terra frigida, non arata.
Typus: Vicinitas civitatis Tampere, Finlandia. Cultura desiccata et cultura vivens deposita in Collectione CCEF sub. No. 2201, Instituto Entomologici Budejovicensi.

Colonies are slowly growing on Sabouraud agar (at 26 °C in 10 day 15–20 mm in diameter), without distinct odor, white, when sporulating floccose and creamy. Reverse white, watery clear droplets of exudate are present on the surface of colonies. Hyphae are hyaline, smooth-walled, 1.5–3 µm wide. Conidiophores are hyaline, smooth, cylindrical, 20–30 × 3–6 µm (Fig. 1a; Pl. I, Figs. 2, 3). Phialides arising single or in verticils on the end of conidiophores, consisting of swollen broad ellipsoidal base, 2.8–3.5 × 2–3 µm and a thin neck 3–4 × 0.5–1 µm, curved laterally (Fig. 1b). Conidia are rather solitary, in minor heads (Fig. 2A), not firmly adherent in globose heads, uniform in size and shape, 2.5–3 × 2–2.5 µm, broad oval, smooth-walled (Pl. I, Fig. 1). Distant pole broadly rounded, other end with a minute round papilla (Fig. 1c), forming in low magnifications a pointed end (Pl. II, Fig. 2). In ultrathin section the conidia have no surface coating of any kind (Pl. I, Fig. 3), which can explain the grapelike deposition, without formation of slimy heads (compare Figs. 2A, B). In fresh conidia the papilla and the pointed end are prominent (Pl. II, Fig. 3), in old conidia pointed end is less prominent. In fixed

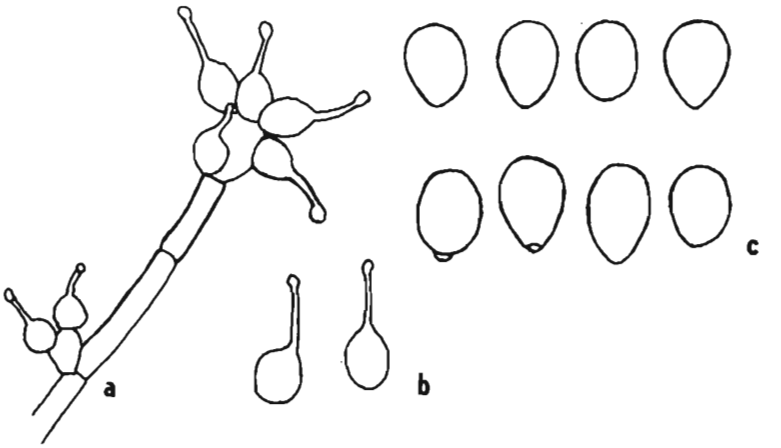

Fig. 1. *Tolypocladium terricola*: a) mycelium with conidiophores and phialides; b) two phialides; c) conidia.

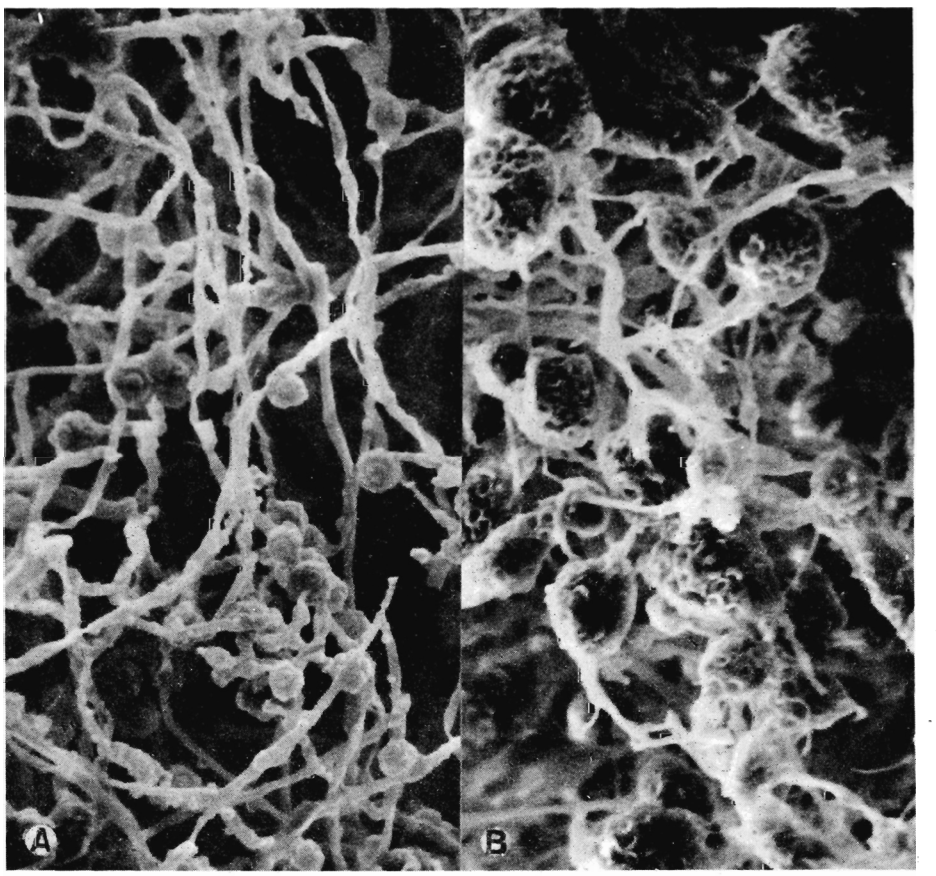

Fig. 2. Scanning EM of the mycelium and conidial heads of *Tolypocladium terricola* (A) and *T. niveum* (B) at magn. 2000×.

material for scanning EM conidia are more elongated than in dry smears or water mounts (compare Pl. II, Figs. 2, 3).

Holotype originates from a sample of soil collected between blocks of granit rocks, outskirts of Tampere, Finland. Culture is deposited at the CCEF Collection, Institute of Entomology, Czechoslovak Academy of Sciences, České Budějovice, No. 2201.

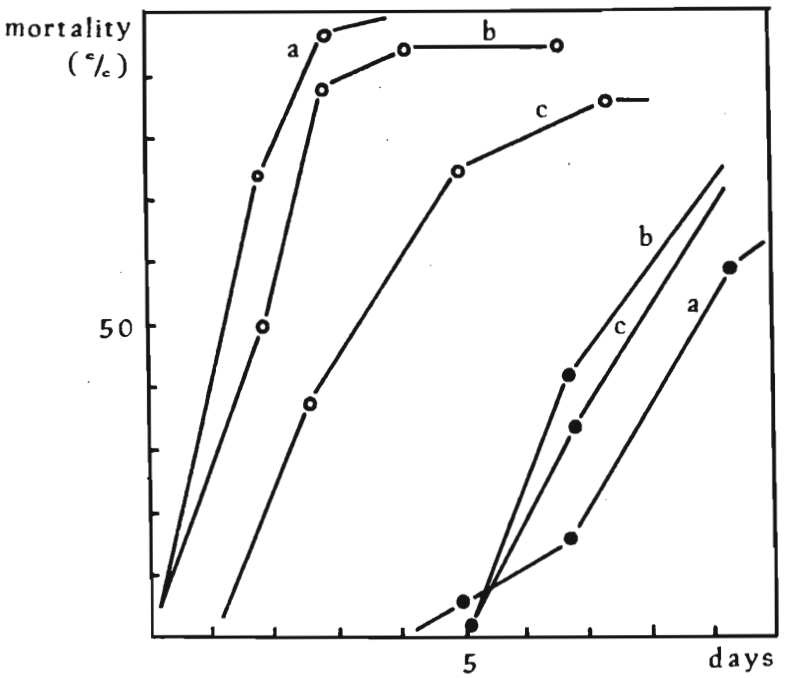

Fig. 3. Mortality of L_4 larvae of *Culex pipiens autogenicus* in water with conidia of *T. cylindrosporum* 1679 (•) and *T. terricola* (o). Concentration of conidia a) $5 \cdot 10^7$ ml^{-1}; b) $5 \cdot 10^6$ ml^{-1}; c) $5 \cdot 10^5$ ml^{-1}.

There was a striking difference in the mosquitocidal activity of conidia of the two compared fungi. The standard strain of *Tolypocladium cylindrosporum* 1679 caused mortality starting on the 5th day with the maximum of activity between 7–9 days after treatment. Dead larvae had in their body cavity hyphae of the fungus and more than 50 % produced hyphal bodies on their surface. In the case of *T. terricola* practically the same mosquitocidal activity of conidia was detected (Fig. 3) but it was much faster and was not accompanied by the growth of fungus in or on the cadavers of mosquito larvae. Mosquitocidal activity of *T. terricola* reflects the effect of intoxication rather than the development of a real mycosis. Depending on the concentration of conidia, the effect of intoxication was apparent 3 or more hours after the treatment. The affected larvae were concentrated in the centre of the cup, hanging by their siphons at the surface.

Table 1. Comparison of morphological characteristics of *T. niveum* and *T. terricola*

Fungus	Conidiophores	Phialides	Conidia
T. niveum	hyaline, smooth walled, cylindrical up to 50 µm long 1.4–2.5 µm wide irregularly branched	solitary or in verticils of two to five 4.8–8.8 × 2.1–3.4 µm	one celled, hyaline smoth walled, broadly ellipsoidal to subglobose 1.9–3.1 × 1.3–2.4 µm average 2.3 × 1.7 µm
T. terricola	hyaline, smooth walled, cylindrical 20–30 µm long 3–6 µm wide irregularly branched	solitary or in verticils of two to five 2.8–3.5 × 2–3 µm	one celled, hyaline smooth walled broadly oval with a minute round papilla on the pole uniform in size and shape 2.5–3 × 2–2.5 µm

Differential diagnosis based on the revision published by Bissett (1983) indicates that the new species is close to *T. niveum*. Morphological characteristics of both species are summarized in Table 1. As it is evident from this table, *Tolypocladium terricola* differs distinctly by the morphology of conidiophores, size homogenicity and morphology of conidia. Moreover in *T. terricola* conidia are solitary, not organized in large slimy heads (compare Figs. 2A, B).

The pattern of secondary metabolites analyzed by HPLC has shown valuable supporting information. Generally, *T. inflatum* (= *niveum*) and *T. geodes* exhibited some quite different secondary metabolites, e.g. pigments, than *T. terricola, T. nubicola* Bisset and *T. cylindrosporum*, which seem to be more closely related (Jegorov, unpubl. data). On the other hand, *T. terricola* differs markedly from strains *T. niveum, T. nubicola* and *T. cylindrosporum* by its ability to produce cyclic peptides cyclosporins (Weiser and Maťha 1988) (Fig. 4) as well as some additional differences found under various chromatographic conditions in the pattern of other, yet unidentified, secondary metabolites.

DISCUSSION

The new species studied is more distinct in morphology, with a very uniform type of conidia with a typical pointed end and with specific differences in the formation of minute clusters of conidia compared with strains reviewed by Bissett (1983). It was shown in the recent studies of *T. extinguens*, Soares et Samson and *T. cylindrosporum* that in case of minor morphological diversity the identification of new species can be supported with data on differences in physiological activity (Samson and Soares 1984, Soares et al. 1985). In the case of *T. extingues*, where the indistinct conidial morphology (subglobose to ellipsoidal, sometimes kidney-shaped conidia 3 × 2 or 1.5 × 5 µm) may not be the deciding morphological distinction, hence, the different infectivity and isoenzyme patterns were used simi-

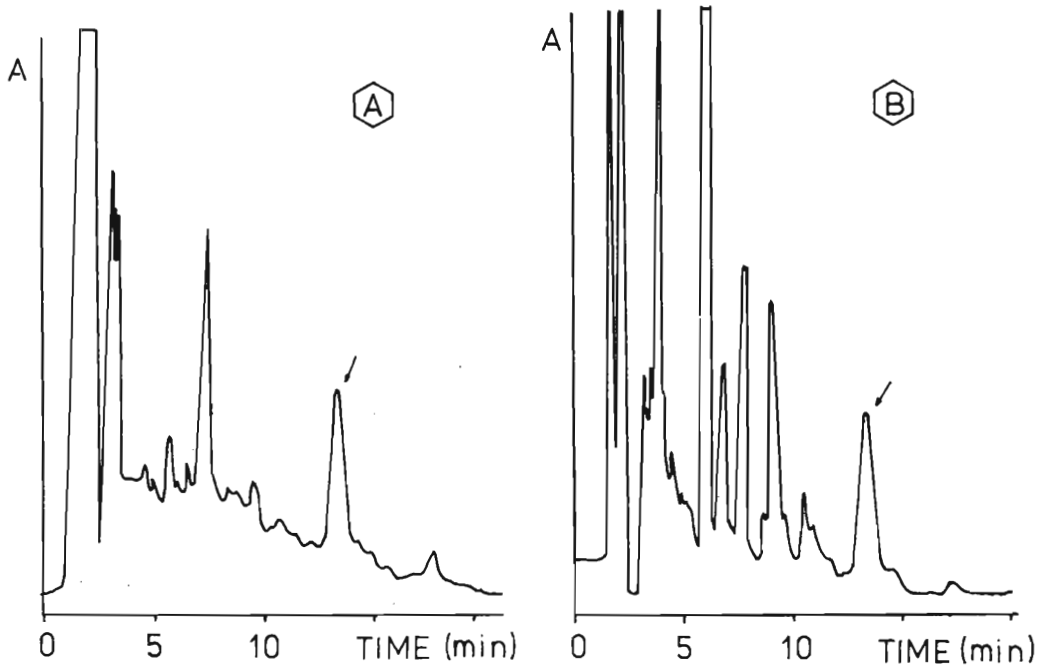

Fig. 4. Comparison of HPLC chromatograms of methanolic extracts of isolated spores: A) *T. terricola*, B) *T. inflatum* (= *niveum*). Conditions: Hypersil ODS, 5 µm, 250 × 4.6 mm column, isocratic elution with acetonitrile/water (68 : 32, v/v), 70 °C, 1 ml/min, det. 214 nm. (arrow indicates cyclosporin A).

larly to support the descriptions of authors. Since teleomorphs are unknown in *Tolypocladium*, this may present difficulties in determinations of conspecific strains which in fact may represent in their perfect form different species, or perhaps even genera.

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H. P. Saluz and J. P. Jost: A laboratory guide for *in vivo* studies of DNA methylation and protein/DNA interactions. *In: Biomethods (A. Azzi, J. M. Polak and H. P. Saluz, Eds.), Vol. 3, Birkhauser Verlag, Basel, 1990, 286 pp., Price 108 DM.*

This book represents the third volume of the Biomethods series of specialized laboratory guides.

Recombinant DNA technology enables the isolation and cloning of many genes of higher eukaryotes, permitting analysis of the structure of individual genes at the nucleotide level of resolution. While this methodology provides a means for analysing structure in great detail, it is not amenable for the study of gene changes during the transition from inactive to active state.

The first method available for studying the methylation state of cytosines used methylation-sensitive restriction enzymes and their isoschizomers. This method has limitations because it detects methylation sites within restriction recognition sites only and, moreover, it is not DNA strand-specific.

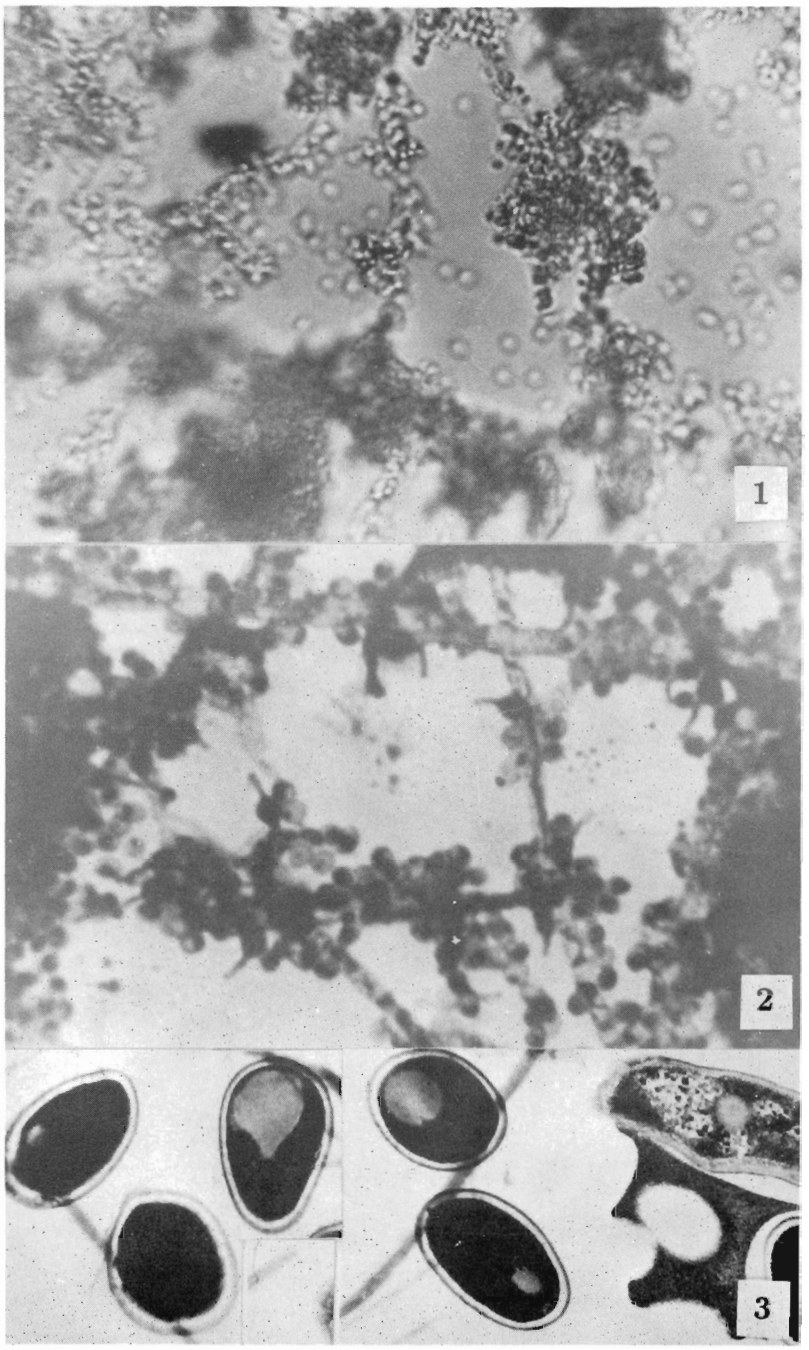
An alternative approach that overcomes the limitations of the former method was brought by the development of genomic sequencing by Church and Gilbert. This procedure provided an excellent tool not only for studying DNA methylation but also protein-DNA interactions, mutations etc. Unfortunately this method is rather complex and time consuming that prevents it from being used widespread. The novel procedure published by Saluz and Jost (1989), using Taq polymerase, radically simplifies previous methods and makes sequencing of genomic DNA and *in vivo* footprinting more accessible to a much larger number of investigators.

The book is divided into six sections and several appendices (sections could be arranged in the different order). The major part of this volume deals with a detailed description and discussion of genomic sequencing techniques.

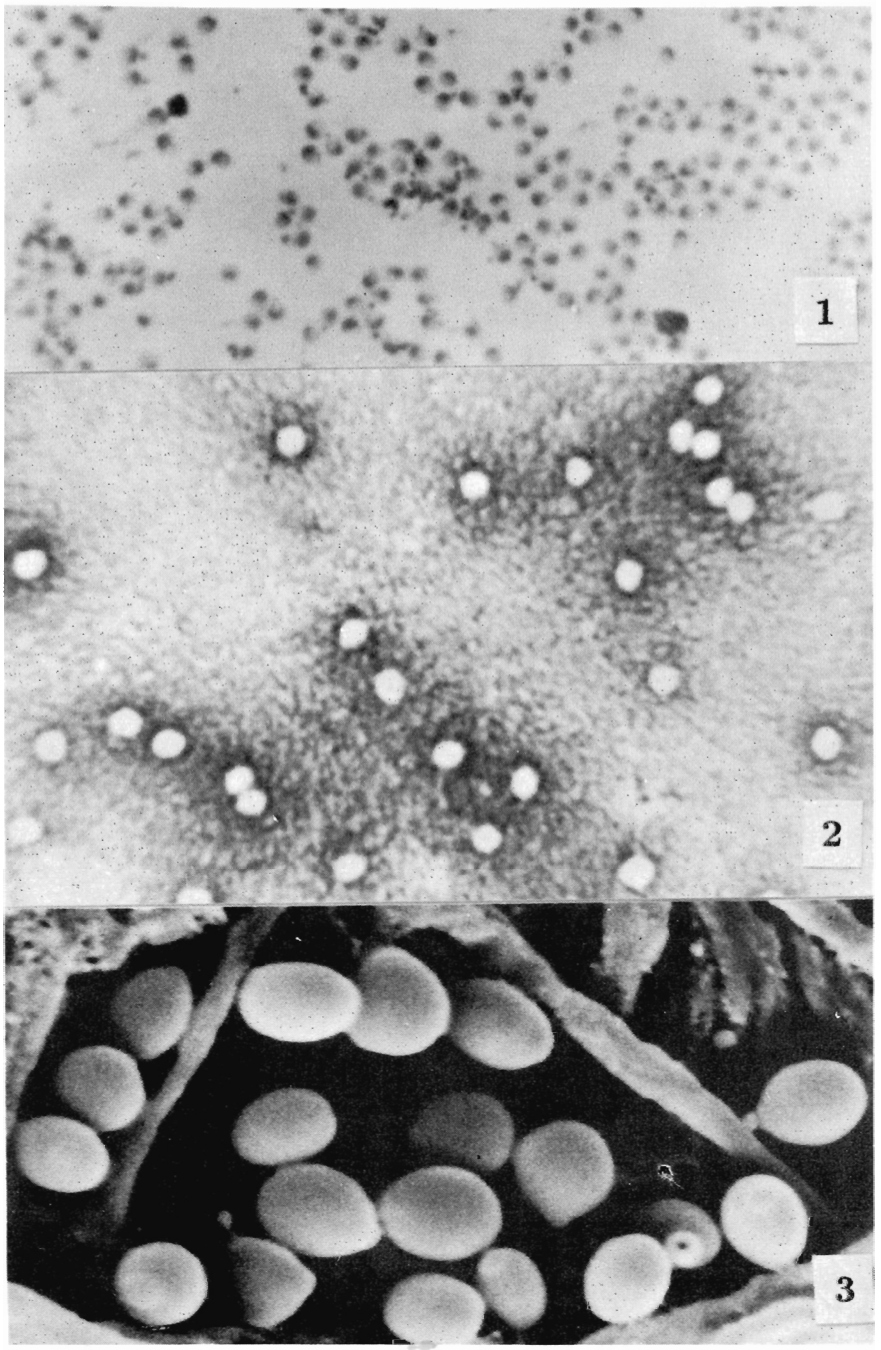
The "classical" genomic sequencing (section VI) combines the chemical sequencing procedure of Maxam and Gilbert with the detection of DNA sequences by electroblotting and indirect end-labelling by hybridization. According to this procedure the total genomic DNA is digested with a restriction endonuclease, after that the digests are subjected to a cytosine specific hydrazine reaction and chemical cleavage. Resulting DNA fragments are then separated on a sequencing gel, electrotransferred and UV crosslinked to nylon membranes, followed by hybridization of membranes with cloned single stranded probes with high specific radioactivity. The modified base 5-methylcytosine can be distinguished from cytosine because it does not react with hydrasine which results in the absence of a band within the cytosine-specific sequencing lane.

The novel method of genomic sequencing developed by the authors (section IV) uses direct labelling and linear amplification of genomic DNA fragments originated in chemical sequencing reactions. In this procedure the hydrazine reaction is followed by selective, linear amplification using a primer labelled to a very high specific radioactivity and thermostable DNA polymerase (Taq polymerase). The reaction products are directly separated on a sequencing gel and used for autoradiography.

In addition, section II is particularly stimulating – it covers the detailed description of procedures used in the isolation of intact genomic DNA suitable for genomic sequencing.



Figs. 1–3. *Tolypocladium terricola*. **Fig. 1.** Grape-like clusters of conidia. **Fig. 2.** Conidiophores, phialides and conidia. **Fig. 3.** Conidia in ultrathin sections with smooth surface and fat droplet in their anterior pole. On right mycelium with surface deposit of metabolites.



Figs. 1–3. *Tolypocladium terricola.* **Fig. 1.** Conidia in Giemsa stained smear, empty ends are fat droplets. **Fig. 2.** Conidia in smear with Indian ink, pointed end visible in some, note uniformity of size. **Fig. 3.** Conidia in scanning EM, constricted end visible (the fixation of a conidium on the mycelium on right is an accidental).