The Isolation of a Phytotoxin from Cultures of *Didymella applanata* (Niessl) *sacc.*

The first communication on the fungus nowadays called *Didymella applanata* (Niessl) *sacc.*, appeared in 1875 from the hand of VON NIESSL[1]. This parasite was reported to occur on raspberries. Since then many publications have dealt with it, and with the disease it causes. A review of the literature is given by LABRUYERE and ENGELS[2].

Ascospores as well as pycnospores may infect leaves of young canes. The fungus makes its way through the tissues of the leaves and petioles to the cortex of the stems, where it causes dark discolorations. When the canes enter their second year, in the following spring, perithecia appear and later on also pycnidia with spores able to provoke infections. The fungus probably inhibits development of the buds that produce flowers and fruit. It appears that *D. applanata*, when grown in a synthetic nutrient solution, produces material that is toxic to young sprouts placed in the culture filtrate. Typical interveinal small necrotic spots appear on the leaves, and the sprouts wilt within 18–36 h. The toxicity of fractions derived from the culture filtrate was tested with sprouts which regularly developed throughout the year from roots of the variety 'Rode Radboud', planted in peat soil in a greenhouse. In autumn and winter, artificial light was supplied from 06.00 until 22.00. 4 rows of 40 W TLS lamps were used at a height of 60 cm, illuminating about 0.70 m^2 of the bench on which the plants stood. The temperature was 20 °C, the relative humidity about 70%.

Each isolated fraction was tested by placing the base of a sprout into an aqueous solution of the fraction which had been brought to a concentration of the same order as that of the original filtrate, into fresh nutrient medium, and into water as control. All sprouts were kept under the same environmental conditions for 18–36 h.

The fungus was grown in shake culture for 10 days at 20 °C in a Czapek Dox medium, which was supplemented with 10 g casein hydrolysate and 3 g yeast extract per litre and adjusted to pH 6.5. This culture medium proved to be phytotoxic itself, due to the casein hydrolysate, which, however stimulated mycelium growth and toxin production. It could not be replaced by another non-phytotoxic factor with the same effect.

In order to eliminate the toxic effect, 'replacement cultures' were grown: After shaking for 10 days, the solucion was decanted and the mycelium was washed twice with sterilized water. Then it was added to a fresh medium of similar composition, to which, however, no casein hydrolysate or yeast extract were added. A sample of this solution was tested on phytotoxicity after shaking the mycelium for 1 h at 20 °C. It proved to be negative.

After shaking the culture for another 10 days, the solution induced pronounced phytotoxic effects on the sprouts. When mycelium, shaken for 10 days in the medium containing casein hydrolysate and yeast extract, was transferred to sterilized water and shaken for 10 days, no phytotoxic effect of the filtrate could be detected. This is an indication that an extracellular factor is formed by the fungus during the second period of shaking in the replacement culture and has not been extracted from the mycelium.

Non-incubated 'yeast extract' culture medium was treated as the incubated cultures were. No phytotoxic material could be isolated. This excluded the presence of any toxic factor in the medium.

The phytotoxic material was isolated by passing 30 l culture filtrate at a rate of flow of 1 l/h over charcoal (Norite RII) packed in a column (80 · 3 cm), which was heated to 50 °C. The phytotoxic factor was adsorbed on the charcoal and was eluted by passing 5 l of pyridine-water (1:1 v/v) through the column at a rate of 1 l/h. Through repeated concentration in vacuo and dilution with water, a pyridine-free residue could be obtained. This material was submitted to gel filtration in a Sephadex G25 coarse column (80 · 5 cm) with water as eluent. Each hour a fraction of 36 ml was collected, the fractions 16–23 contained the toxin(s). These active fractions were combined and the solution was concentrated and subjected to elution chromatography on charcoal (Norite RII, column 80 · 3 cm, 25 °C) with a water-ethanol gradient. The rate of flow was 60 ml/h. The toxic material was present in the 'hour' fractions 23–33 (starting volume of water: 1 l). Fractions 23–33 were combined and the solution was concentrated and subjected to partition chromatography on cellulose (columns 40 · 5 cm) with butanol-pyridine-water (6:4:3 v/v). The rate of flow was 15 ml/h. The active material was isolated from the 'hour' fractions 40–56 and proved to be chromatographically pure. This substance was found to be still active in solutions of 1 mg/l. 0.5–2.0 mg phytotoxin was obtained from 1 l culture liquid.

The isolated substance has a molecular weight lower than 2500 (gel filtration). The usual methods of molecular weight determination yielded no results. The behaviour of the compound on ion exchangers indicated neutral character. The IR-spectrum of the colourless solid is represented in the Figure.

Certain data of this spectrum indicate the presence of a carbohydrate. This fact was supported by positive periodate, anthrone and orcinol reactions. The naphthoresorcine, benzidine and ammoniacal silver reactions were negative. Nitrogen and sulphur could not be detected. After hydrolysis of the toxin, three compounds, which could be separated as trimethylsilyl derivatives by gas chromatography, were present. One of them was D-glucose.

Zusammenfassung. Die Isolierung eines phytotoxischen Stoffes aus der Kulturflüssigkeit von *Didymella applanata* (Niessl) *sacc.*, der eine Krankheit bei Himbeeren verursacht, wird beschrieben. Die Symptome dieser Krankheit und die Züchtungsbedingungen für den Mikroorganismus und die Testpflanzen werden angegeben. Das Toxin dürfte ein Oligosaccharid sein.

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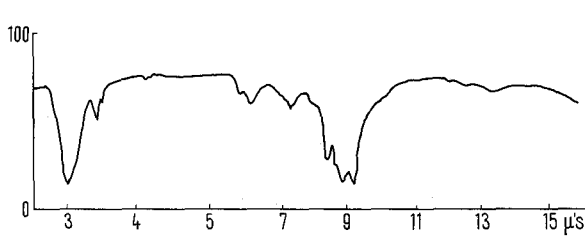

[1] G. VON NIESSL, Österr. Bot. Zeitschr. *25*, 129 (1875).
[2] R. E. LABRUYÈRE and GERDA M. M. ENGELS, Neth. J. Pl. Pathol. *69*, 235 (1963).