On Anti-Fungal Factors in Potato Leaves

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There are considerable varietal differences in the damage of potato (*Solanum tuberosum*) leaves caused by late blight (*Phytophthora infestans* de By). Anti-fungal factors have been demonstrated in all cultivated plants so far investigated in this laboratory¹ and new light has thus been thrown on the nature of the resistance of these plants to pathogenic fungi. Attention was also paid to corresponding factors against *Phytophthora infestans* in potato leaves. *Phytophthora infestans* and *Fusarium nivale* have been used as test organisms in these experiments. The latter fungus was used because its cultivation on an entirely synthetic medium was easy and results were soon obtained. *Phytophthora infestans* was cultivated on pea-agar, on which its growth was good.

The leaf material used in the experiments was taken at various stages of development from potato plants grown under natural conditions in the field. A highly active extract was obtained when the leaves were macerated in 96 % ethanol for 5 min. The leaf material was taken from Aquila, a variety highly resistant to late blight. Ethanol was evaporated before biological activity estimations. The ethanol residue of 0.5 g fresh leaf material in 1 ml synthetic medium totally inhibited the mycelial growth of *Fusarium nivale*. On pea-agar the inhibition limit was 0.7 g fresh wt/1 ml medium. The growth of *Phytophthora infestans* on pea-agar was inhibited when the concentration exceeded 1 g fresh wt/1 ml medium. An anti-fungal effect was also observed in an extremely late blight-susceptible variety, Sieglinde, but the inhibition limits were considerably higher.

For the investigation of the active factor, 3.8 kg of sound leaves of Aquila were taken on August 30, and homogenised by grinding. 96 % ethanol was added immediately, until there was 70 % alcohol in the whole homogenate. The homogenate was kept for 2 days at +5°C and then filtered under pressure through filter paper. No activity was observed in the compact residue. The filtrate was concentrated *in vacuo* at 30°C to 350 ml; the pH

of this press juice was 4.7. It was extracted with equal amounts of ether ten times. From each fraction both a biological activity determination and a one-dimensional paper chromatogram using acetic acid-water (15:85) as solvent was made. The highest activity was found in fractions 1 and 2. In fractions 3—10 an activity, though considerably weaker than in the first two fractions, was still observed.

Three fluorescent spots with the R_F -values ~ 0.02 , ~ 0.50 , and ~ 0.70 were found in fractions 1 and 2 in chromatograms exposed to ultraviolet radiation. In fractions 3—10 only the last spot was found. The blue fluorescence of the first two spots was unchanged in NH_3 vapour, the light blue fluorescence of the last changed into yellowgreen. The spots with $R_F \sim 0.50$ and 0.70 were eluted with 96 % ethanol and the ultraviolet absorption spectrum of the two solutions was determined. The spectra were much alike, the spot with $R_F \sim 0.70$ having a maximum at 245 and 330 $m\mu$ and a minimum at 266 $m\mu$, the spot with $R_F \sim 0.50$ a maximum at 237 and 325 $m\mu$ and a minimum at 264 $m\mu$ in ethanol. (Fig. 1.) These results suggested chlorogenic and caffeic acids. The spectra and the corresponding fluorescence of the pure preparations of these acids supported this view. Chromatograms of a mixture of caffeic acid and the spot with $R_F \sim 0.50$ gave only one spot when different solvents were used and this was also the case with a mixture of chlorogenic acid and the spot with $R_F \sim 0.70$. The following solvent-systems were used: methanol-water (1:2), isopropanol-ammonia (25 %)-water (8:1:1), butanol-acetic acid-water (6.3:2.7:1) and (5:4:1), butanol saturated with water, benzene, chloroform, dist. water.

A very strong blue fluorescence was still found in the press juice of Aquila after the ether extractions. The pH of the juice was lowered with phosphoric acid to 3 and immediately extracted with ether. The ether extract was active, the extract from 5 g fresh wt in 1 ml medium totally inhibiting the growth of *Fusarium nivale*. The spectrum of this fraction, measured in ethanol, was exactly the same as that of pure chlorogenic acid. The calculated content of chlorogenic acid ($E_{1\text{cm}}^{1\%} = 526$)² in this fraction was 480 μg fresh wt.

The pH of the press juice was further lowered to 1 and the juice extracted with ether. A weak activity was found in the ether extract, 10 g fresh wt/1 ml medium had a clearly inhibiting effect on the growth of *Fusarium nivale*. A light blue fluorescence was found in the chromato-

Fig. 1. The molar absorption spectra of chlorogenic (—) and caffeic (-----) acids.

gram from the starting point to the liquid front, the strongest fluorescence, however, in the region of chlorogenic acid (R_F 0.65–0.75). The calculated content of chlorogenic acid amounted to 60 $\mu\text{g/g}$ fresh wt. The press juice, which had now lost its fluorescence, was allowed to stand at -10°C for 7 days and was then extracted with ether. Even this extract had a weak activity and the chromatogram of the extract was about the same as above. It is probable that chlorogenic acid was slowly liberated through hydrolysis at pH 1. On stronger hydrolysis (2 h at 100°C in bomb in 1 % HCl) a very weak light blue fluorescence all over the chromatogram was obtained from the press juice. After hydrolysis the spectrum of the juice was very uncharacteristic, bearing no relation to those of the above-mentioned acids.

The author tried to isolate chlorogenic acid from the ether fraction, which was obtained after lowering the pH to 3. The ether was evaporated *in vacuo* and the residue carefully washed with chloroform and small amounts of cold dist. water. The residue was dissolved in 50 % ethanol and kept at room temperature until crystals began to form. Ethanol was added and the solution cooled to -10°C . The crystals were washed with 50 % ethanol and dried in a vacuum desiccator above P_2O_5

at room temperature. The m.p. of the crystals obtained was $207\text{--}209^\circ\text{C}$, which is in agreement with that given for chlorogenic acid². The mixed m.p. with pure chlorogenic acid was $206\text{--}209^\circ\text{C}$.

For biological activity tests with pure substances, a preparation of chlorogenic acid (m.p. $206\text{--}209^\circ\text{C}$) was obtained from the Laboratory of Biochemistry, University of Helsinki. A commercial product of caffeic acid (Light & Co.) was used. Because chlorogenic acid is composed of one caffeic and one quinic acid molecule³, the activity of quinic acid was also investigated. On a synthetic medium, chlorogenic acid totally inhibited the growth of *Fusarium nivale* in a concentration of ~ 1 mg/ml medium, on pea-agar in a concentration of ~ 1.5 mg/ml.

The anti-fungal effect of caffeic acid was considerably higher, the corresponding inhibition limits being ~ 0.5 mg/ml and ~ 0.8 mg/ml. The very weak solubility of caffeic acid in water made the determination of the inhibition limit more difficult. Quinic acid had no inhibiting effect on the growth of *Fusarium nivale* in corresponding concentrations. The growth of *Phytophthora infestans* was inhibited on pea-agar when the amount of caffeic acid exceeded 1 mg/ml and that of chlorogenic acid 2 mg/ml. Quinic acid had no effect.

The quantitative determinations of chlorogenic and caffeic acids in potato leaves show that the amount of free chlorogenic acid is often sufficient to inhibit the mycelial growth of various fungi, since determinations with various potato varieties indicated 0.5–4 mg free chlorogenic acid in one g fresh leaves. Because it is evident that considerable amounts of chlorogenic acid may be bound in complex form, the anti-fungal effect of leaves improves when the complex is disorganized. It is possible that such a defence mechanism begins to function chiefly after infection. The amount of free caffeic acid in potato leaves is so insignificant that only a minor part of the plant's resistance can be due to it.

Among the above mentioned fluorescent spots on the paper chromatogram, of which the spot with $R_F \sim 0.70$ contained chlorogenic acid, the spot with $R_F \sim 0.02$ was also found to have an anti-fungal effect. The substance has absorption maxima at 272 and 310 $m\mu$ in ethanol and it appears to be comparatively unstable. Its chemical nature is not established yet. The substance is found both in leaves, stems and tubers although in so small amounts that

its effect is of no importance. Chlorogenic and caffeic acids are thus not the only anti-fungal factors in the potato plant.

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The Assay and Specific Activity of Crystalline Alcohol Dehydrogenase of Horse Liver

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Crystalline alcohol dehydrogenase was first prepared from horse liver by Bonnichsen and Wassén¹. Modifications of the procedure were reported by Bonnichsen² and Bonnichsen and Brink³. Assay of the enzyme, based on spectrophotometric measurements of the reduction of diphosphopyridine nucleotide (DPN) coenzyme by excess ethanol substrate at alkaline pH, was described by Theorell and Bonnichsen^{4,5}. A modification of this assay method is described below.

Reagents. 0.1 M glycine-NaOH buffer, pH 10.0. DPN solution 0.83 mg/ml (1 mg/ml cozymase 90, Sigma Chemical Co.). Ethanol solution, 1 ml 96 % to 100 ml with water.

Procedure. The reagents and the cell compartment of the spectrophotometer are kept at 23.0°. 1.85 ml buffer, 1.0 ml DPN solution and 0.15 ml ethanol solution are mixed in a cuvette of 1 cm optical depth, and the extinction at 340 mμ (E_{340}) is measured. A small volume (1 to 10 μl) of enzyme solution is

Fig. 1.

introduced into the reaction mixture on a stirrer, and the time ($t_{0.2}$) required for an increase of 0.200 in E_{340} ($\lambda_{\max}$ DPNH) is measured.

Theoretically, the enzyme concentration should be more closely proportional to the reciprocal of $t_{0.2}$, the time required for the reduction of a definite fraction of the DPN, than to the amount of DPN reduced in a fixed time (E_{340} in 3 min) measured in the earlier method³, provided that the enzyme is stable in the test mixture⁵. This was found to be the case, as shown in Fig. 1 by a plot of measured values of $1/t_{0.2}$ against the weight of enzyme used in the test, obtained by adding different volumes (0.9 to 10 μl) of the same enzyme solution. The plot is a satisfactory straight line passing through the origin, i.e. the product, $t_{0.2} \times \text{amount of enzyme}$, is constant to within 2 %. The method has proved consistently reproducible to this extent.

The ethanol concentration (9 mM) is the optimum value⁶; the concentration of 300 mM used originally causes considerable inhibition. Neither the ethanol nor the DPN concentration (0.42 mM) is critical, variations of 50 % in either causing only 10 % variation in the results. Semicarbazide has no effect, as at pH 10.0 the reduction of DPN goes practically to completion with these reactant concentrations⁴.

Specific Activity. The enzyme used for the assays of Fig. 1 had been recrystallized with ethanol to constant activity/ E_{280} ratio. The enzyme was prepared as described by Bonnichsen and Brink³; after removal of the haemoglobin, the enzyme

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