THE DECAY OF CUTIN AND CUTICULAR COMPONENTS BY SOIL MICROORGANISMS IN THEIR NATURAL ENVIRONMENT

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SUMMARY

The cuticular components cellulose and cutin are decomposed in a period of 3–8 months (depending on the season) by soil microorganisms under natural conditions. The cellulose is attacked prior to the cutin layer. The latter is invaded by microorganisms which settle first in the cellulose layer. If purified cutin strips with the cellulose removed, are exposed to the action of soil microorganisms, the degradation also starts from the "inside" of the layer.

The progress of degradation depends on the soil used, active garden soil giving the best, and poor sand soil the slowest attack. The degradation of the cutin layers starts with a swelling of the former boundaries of the epidermal cells, then follows the removal of successive sub-layers and finally the detachment of entire subunits from the weakened structure, ultimately leading to a total decay of the structure. The detachment of the lamellae within the cutin layer could be shown in cross sections of the exposed material by electron microscopical examination.

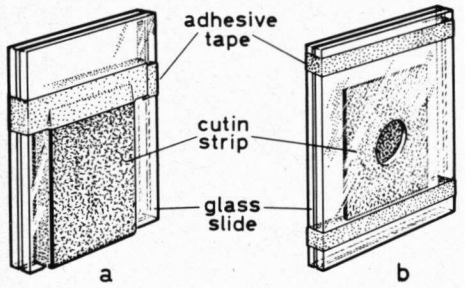
Halo-formation around the invading microorganisms, and the lipid accumulation as a result of fatty acid release from the cutin by far-distance decomposition, showed that cutin decay by exo-enzyme action is a widespread property of soil microorganisms.

1. INTRODUCTION

The degradation of cutin has so far mostly been studied under laboratory conditions (HEINEN 1960; 1962; LINSKENS, HEINEN & STOFFERS 1965; HEINEN & de VRIES 1966; RUINEN 1966). However, the main breakdown of this material – which is abundantly supplied each fall – should be expected to occur under the natural conditions of the normal environment, the soil. Microorganisms originating from the soil have already been shown to be well-fit for the degradation of cutin (HEINEN & de VRIES 1966). Since other polymer plant products like cellulose are also attacked quite rapidly in the soil by a variety of microorganisms (SIU 1951; WHITE & DOWNING 1953; TRIBE 1957) we tried to follow also the degradation of cuticular components under similar conditions.

2. MATERIALS AND METHODS

The material used in this study was derived from *Gasteria verrucosa* leaves. We mostly used the layers which are separated from the bulk of the leaf tissue after treatment with pectinase in acetate buffer according to ORGELL (1955). This material consists mainly of cutin if objects with a true cutin layer are used, but contains the rest-layers of cellulose and pectic substances, and the wax film at the outer surface. For some experiments we used cutin layers which were further purified according to the methods applied earlier (HEINEN & de VRIES 1966), which is referred to as "purified cutin" in the text.



Scheme 1. Mounting of cutin or cuticle strips between glass slides for exposure in the soil. See methods.

Small oblong strips (1 × 3 cm) of the layers were mounted between glass slides (2,5 × 3,5 cm) in such a way that half of the strip could be bent over and fastened on the outer side of one slide with adhesive tape, which also kept the two glasses fixed (scheme *1a*). In other experiments smaller strips (1 × 2 cm) were placed between two glass slides, one of which had a hole of about 0,8 cm in diameter cut out. Both slides were again held together with adhesive tape (*scheme 1 b*).

In both cases either the former "inside" or "outside" of the layers could be exposed to the action of the soil, and that part of the strips which were squeezed between the slides was under both conditions protected from a direct attack and could serve as reference material.

After exposure for several months in the rhizosphere in rich garden soil (3.1×10^8 bacteria, and 7.2×10^7 molds and yeasts per gram dry weight) about 5–7 cm deep, the material was recollected, removed from the glass slides, cleaned, and either studied directly by light microscopy, or embedded for the preparation of cross sections.

For electron microscopical examination the material was treated with a fixative (5% glutaraldehyde in phosphate buffer pH 7.2) for two hours, rinsed over night in the buffer, stained with 2% $KMnO_4$ and embedded in Epon (Epon A and B in even proportions). Cross sections were made on a Porter-Blum ultramicrotome and examined with an EM 100 PHILIPS electron microscope.

3. RESULTS

The decomposition of the cutin strips which were exposed to the action of soil microorganisms was found to proceed in a slow but steady path. Some macroscopical observations already revealed the alterations of the material: After exposure for about 2–4 months, the material showed a tendency to stick to the glass surface, and after prolonged incubation the strips had been altered to a gummy material, forming extensible threads upon removal. After one year no material at all was detectable on the outer glass surface, while remnants of the strips were still visible between the two slides.

Again we could confirm our earlier observation (HEINEN 1962) that the degradation always starts at that part of the layer which originally represented the

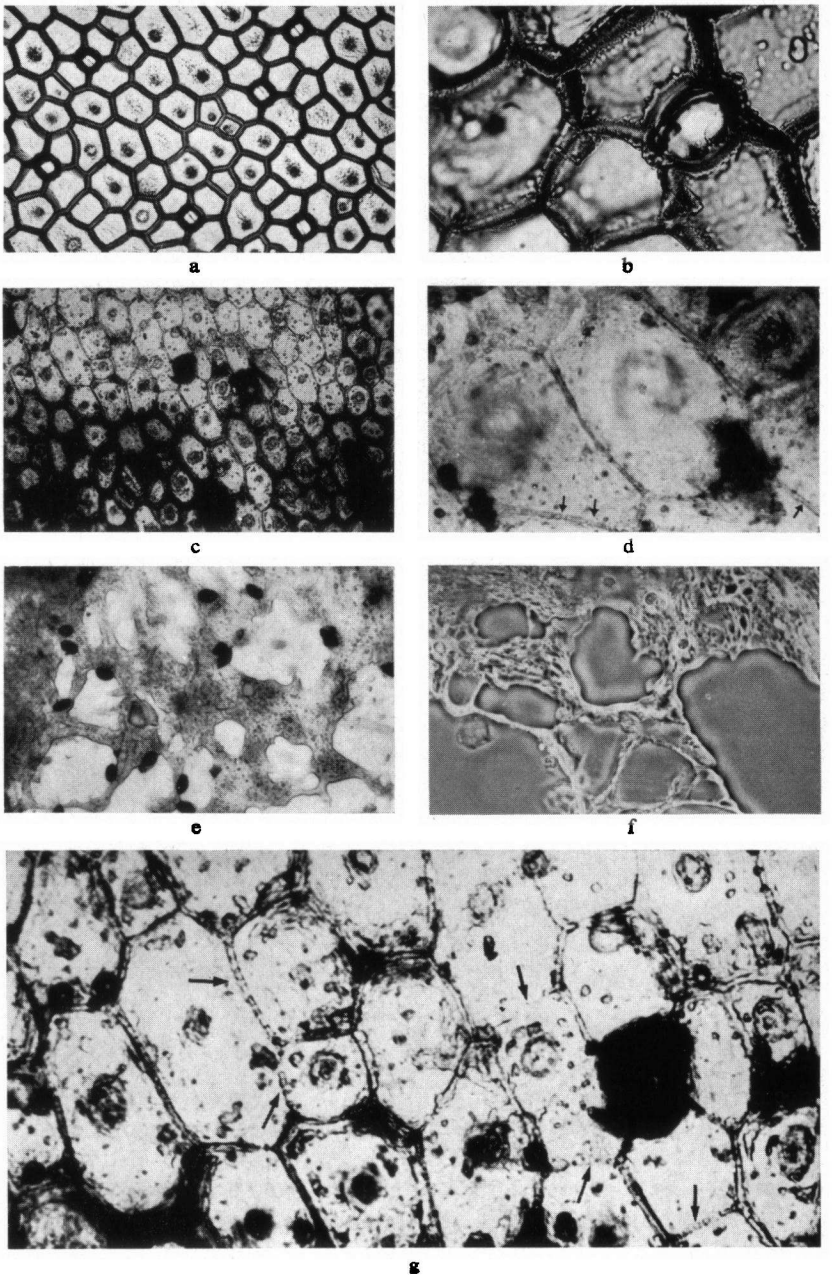


Plate 1.

"inside", facing the pectin and cellulose layers within the intact plant. If the former "outside" was exposed, decomposition proceeded in a much slower path. The adhesive tape used for mounting the strips on the slides was also found to be decomposed after prolonged exposure, which in turn made an attack of the "inside", initially protected between the glass slides, possible.

When cuticle strips with rest-cellulose were examined microscopically after 2–3 months of exposure, we found that the degradation of the cellulose went faster than the decomposition of the cutin layer. Microorganisms tended to invade the cellulose layer first, starting the attack of the cutin layer again from the former "inside".

Light microscopy: Light microscopical examination of the "inside" surface of purified cutin strips showed a typical progression of the degradation (*Plate 1, a-g*). At first a swelling of the thickened wall-like boundaries, which in situ follow the limitations of the epidermal cells, is observed (*b*). On prolonged exposure (2 months) these boundaries appear much thinner (*c*) than in the unexposed reference strip (*a*). At the same time the soil remnants tend to stick to the surface which makes a cleaning of the entire surface impossible. The thin boundaries already show a tendency to break up into small arrays (*Plate 1 d*, arrows). This process continues further, eventually leading to total destruction (*e* and *f*). The break-up in perpendicular direction to the course of the boundaries is clearly seen on the enlargement given in *Plate 1 g*. The components of the "wall" seem to disengage in units or blocs of quite well defined dimensions, creating a uniformly spaced structure (arrows at center and lower left, and downward pointing lower right). The next step in the progressing destruction is the total disappearance of the boundaries, as can be seen on the same micrograph (downward pointing arrow at center, and upward arrow lower right), which precedes the final structureless appearance of the material.

Since these experiments had shown that the degradation of cutin could be

Plate 1: Progress of cutin decay in soil, as seen from inside surface views of purified cutin strips exposed in soil.

a) Unexposed reference material (100 ×).
b) Swelling of the boundaries, representing the former limitations of the epidermal cells, after 3 weeks of exposure (360 ×).
c) Progressing decay with the boundaries becoming thinner. Adhering soil remnants cannot be removed without rupturing the material (100 ×).
d) Enlargement showing the thin boundaries more detailed (360 ×). Arrows show beginning break-up of the structure.
e) Total decay to structureless material after 3 months; black objects are adhering spores (360 ×).
f) Detail of structureless mass, as seen with phase contrast after staining with 2% $KMnO_4$ (215 ×).
g) Disintegrating of the boundaries after 8–10 weeks of exposure. Disappearance of units from the structure, at arrows center left and lower right (downward pointing). Total destruction of the boundaries at center (downward pointing arrow) and lower right (upward arrow). Soil remnants are seen sticking to the surface (400 ×).

followed by simple light microscopical techniques, we tried to compare the destruction occuring in soils of different composition. The material was therefore buried in garden soil with high counts of microorganisms, or in soil and sand mixtures in a 1:1, 1:2 and 1:3 proportion. The progress of the degradation was checked every 3–4 weeks in all soil samples. We found that decomposition occured fastest in the pure garden soil. Some addition of sand (1:1) made no real difference, but the 1:2 mixture revealed a distinct slow-down of the process. In the 1:3 mixture almost no attack was visible, even after 11 months of exposure.

Electron microscopy: For more detailed information on the course of cutin degradation in garden soil, cross sections of the exposed material were examined with the electron microscope. For these experiments partly purified cutin strips, which had been separated by pectinase action and therefore containing rest-material of the pectic- and cellulose-layers, were used in order to offer more suitable conditions for the attack of the soil microorganisms.

The micrographs given in *Plate 2* demonstrate that microorganisms of the soil are capable to invade the cuticle components. The first photo (*2a*), given as a reference, shows a cross section through unexposed material, revealing the typical dotted bead-like structures arranged to parallel lamellae within the cutin layer. The soil organisms which penetrate into this layer are oriented along the course of the lamellated structure (*b*). Most of the organisms found in the cutin layer are yeasts (*Plate 2b*, lower part and foto c and d,) though in some cases tangential sections through mold hyphae would give similar pictures (*Plate* 2, center part). Bacteria have only been found within the rest-cellulose layer in close vicinity to the cutin layer. Most of them showed to contain accumulated lipids (globuli) within the cell, like bacteria which have been grown on fatty acids as sole carbon source (SCHAEFER & LEWIS 1965). Interestingly nearly all organisms found in the sections show these inclusions, at least during the stages of cutin decomposition. We therefore assume that these inclusions represent accumulated fatty acids which mainly originate from the degradation of the cutin poly-lipid. The observation that even those organisms which are still situated in the cellulose layer outside the cutin show this accumulation of lipid material suggests that exoenzymes, which are able to release fatty acids from the cutin, are formed, and dispatched into the environment.

Further observations revealed the structural alterations occuring upon the invasion of the cutin layer by microorganisms. After entering the cutin layer from the cellulose part the microorganisms settle within the cutin layer. During

Plate 2: Microorganisms in cross sections of soil-exposed cuticle layers.
- a) Electron micrograph of unexposed blanc material. Arrows show lamellated bead-like structure of cutin layer.
- b) Microorganisms within the cutin layer, oriented along the direction of the lamellae. Lower part shows yeast cells, and another one in the center. Elongated object in the center possibly is a part of a mold hyphae.
- c) Detachment of lamellated strands in close vicinity of cutin decaying yeast cells.
- d) Halo-formation around microorganisms within the cutin layer.

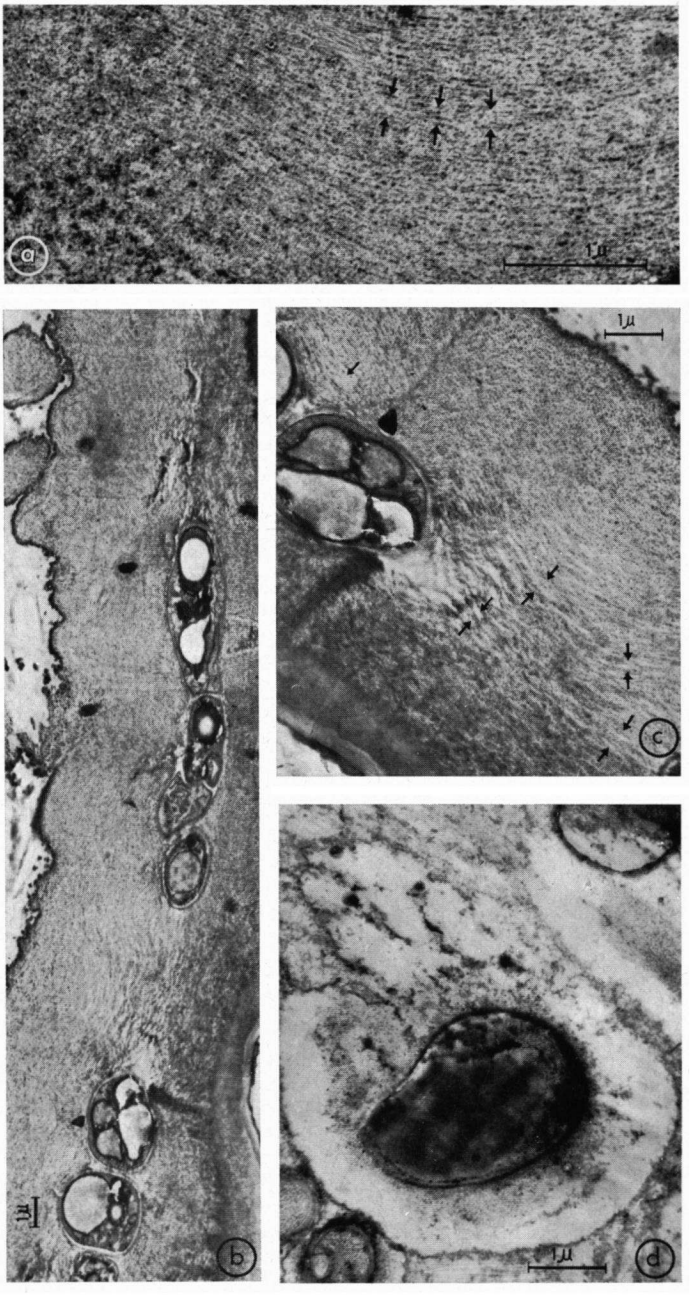
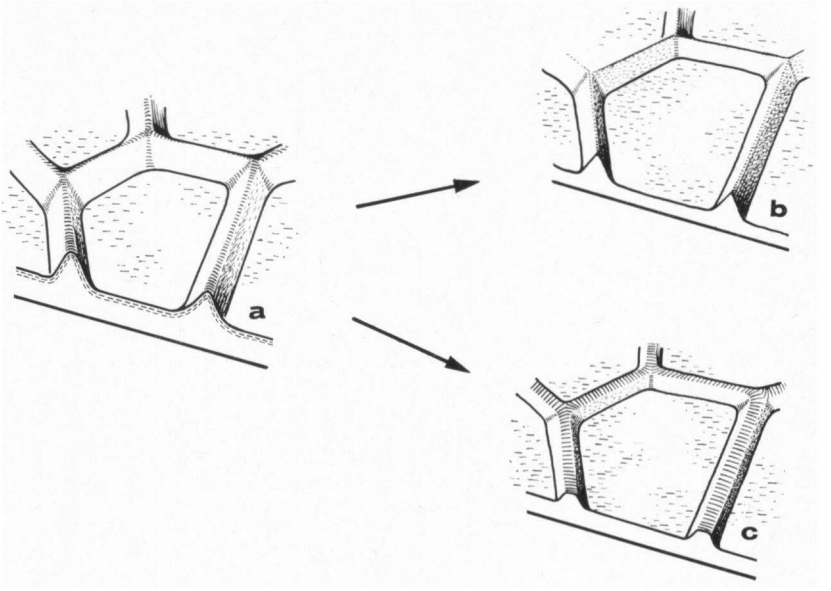


Plate 2



Scheme 2. Possible progression of destruction of boundaries, representing the former limitt tations of epidermal cells. If the material of the boundaries is weaker than the flapart, decay will procede from (a) to (c), leading to a broadening appearance of the boundaries. If sub-layers are successively removed (a → b), the boundaries will appear thinner with progression of cutin destruction.

the first weeks after entry the lamellated structure of the cutin layer still remains unchanged. On longer exposure however (6 months) the lamellae around the invading microorganisms start to desintegrate: The lamellae are detached (*2c*) and a halo of totally decomposed material, with no structure left, is formed around the cells (*d*), which are filled with accumulated lipids.

It was interesting to notice that the decomposition proceeded much slower in these experiments, which were started in late fall. Total decay was found to start no sooner than in late june, after 8–9 months, while strips exposed in midsummer appeared entirely structureless already after 3–4 months. Seasonal variations may therefore favour or temper the process of cutin decay under natural conditions.

4. DISCUSSION

The experiments show that the cellulose and cutin components of the cuticle are readily attacked by microorganisms of the soil. The decomposition of the pectic substances of the cuticle could not be shown by optical methods, but pectin is known to be degraded by molds like *Penicillium spinulosum* (HEINEN 1962) and a variety of soil microorganisms (MEYER 1959).

Examination of both the surface views and the cross sections reveal that the

destruction of cutin under natural conditions proceeds within several months. The primary disintegration of the former cell-limitations confirms that the decomposition starts at the former inside of the purified cutin strips, suggesting that this surface provides a less polymerized state of the poly-lipid, which favours the attack from this side. Two conclusions are possible in this context: Either the thickened boundaries themselves represent the weakest structure, so that their breakdown would precede the degradation of the flat part of the material (*scheme 2*, a → c). Or the entire material could be disintegrated by successive removal of sub-layers (*scheme 2*, a → b), leading to thinner boundaries. The latter is in accordance with our observations, and confirms earlier results obtained from cross sections of cutin layers, which had been incubated with *P. spinulosum* (HEINEN 1962).

The disengagement of the lamellated structure seen in the electron micrographs of the cross sections of the cuticle strips gives further evidence for the view that the destruction proceeds in a layer-by-layer removal. This process continues until whole blocs or units can be detached from the weakened material, followed by a total decay of the structure.

The disappearance of the relatively well defined units from the boundaries is a most intriguing process, the more so as the same picture appears upon incubation of cutin layers from *Aloe* and *Sanseviera* with *Cryptococcus laurentii*, *Rhodotorula glutinis*, or *Azotobacter chroococcum*. The progress of destruction within 3–5 days has recently been shown by RUINEN (1966), with the detachment of entire parts of the boundaries already visible after 48 hours. This step during the decay suggests that sub-units of the cutin polymer at least at the inside-surface of the layer may be interconnected by either non-lipid material or short-chain fatty acids. Small amounts of wax or pectic substances, not accessible to enzymic treatment within the intact cutin layer, could be responsible for this type of linkage, acting as a glue between the poly-lipid sub-units. The far-distance action of the microorganisms, and the halo-formation around most of them at later stages, gives convincing evidence that exo-enzymes with cutinolytic activity are produced and released by the invading organisms. Since the resulting accumulation of lipids was found in molds as well as yeasts and bacteria growing within or in close vicinity of the cutin layer, the extra-cellular attack of cutin seems to be a wide-spread property among microorganisms of the soil.

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