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# THE ISOLATION OF HAUSTORIA FROM CUCUMBER LEAVES INFECTED WITH POWDERY MILDEW[1]

*De isolatie van haustoriën uit komkommerbladeren die geïnfecteerd zijn met echte meeldauw*

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A procedure is described for the isolation of haustoria from cucumber leaves infected with powdery mildew *(Sphaerotheca fuliginea)*. It consists of a removal of the hyphae from the leaf surface and a release of the haustoria from the intact host cells by homogenization of the leaves, followed by purification of the haustoria by means of centrifugation of the leaf homogenate in glycerol and filtration through micro-mesh sieves of 15 and 5 μ with the use of ultrasonic vibration. Preliminary experiments showed that oxido-reduction processes still proceed in isolated haustoria.

## INTRODUCTION

Various attempts have been made to elucidate the nature of the obligate parasitism of mildew and rust diseases. The results have been reviewed recently by SHAW (1963) and by THROWER (1965).

WHITNEY, SHAW & NAYLOR (1962) showed that enlargment of the nuclei in rust-infected wheat cells was accompanied by an increase in ribonucleic acid (RNA) but not in desoxyribonucleic acid. It has been speculated that the metabolism of the affected host cells is so altered as to produce RNA and protein of a type compatible with the rust in a manner similar to that in which the metabolism of bacteria is shifted by infection with phages (SHAW, 1963).

More direct data on the metabolic potential of obligate parasites might be obtained if there were possibilities to study the metabolism of hyphae isolated from the host cells. However, it will be extremely difficult to isolate the ramified rust hyphae from the host. An attempt to isolate haustoria of rusts or powdery mildews from the host cells offers a better chance on success since methods for the purification of isolated metabolically active plant cell particles such as chloroplasts, nuclei, mitochondria and ribosomes are well known. They have been reviewed by JENSEN (1962) and SANWAL (1963). With the use of these methods it appeared possible to isolate haustoria from cucumber leaves infected with powdery mildew, *Sphaerotheca fuliginea* (Schlecht. ex Fr.) Poll. A preliminary investigation on the metabolism of the haustoria was undertaken.

## MATERIALS AND METHODS

### *Mildew culture*

Cucumber plants, variety 'Lange gele Tros', were grown under greenhouse conditions in flower pots containing steamed soil. An average temperature of $19 \pm 4°$ C was maintained. From October to April additional light was given

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from 4 to 10 p.m. The upper surfaces of the first and second leaves were inoculated with conidia of *Sphaerotheca fuliginea*. Hundred gram of green leaves covered with hyphae and conidia were harvested one week after inoculation. To obtain leaves free from starch grains the plants were placed in a dark room 16 hours before harvest.

*Apparatus*

Homogenization of the leaves was carried out in a Servall omnimixer with a stainless steel chamber (400 ml maximum capacity). The speed of the knife blades can be adjusted up to a maximum of 16,000 rpm.

The homogenate was filtered through woven phosphor-bronze wire sieves with meshes of 50, 40 and 30 μ. These sieves have diameters of 20 cm (Metaalgaas Twente, Hengelo, Holland). A nickel plate micro-mesh sieve of 15 μ diameter was used (Veco, Eerbeek, Holland). The holes of this plate are round and conical (COLON, 1965). The total diameter of this sieve was 13 cm. Finally the homogenate was filtered through a nickel plate micro-mesh sieve with square openings (Buckbee Mears Co, St. Paul, Minn., U.S.A.). The length and width of the meshes are 5 μ and the total diameter of the sieve 7.5 cm.

An ultrasonic cleaner has been used to facilitate filtration of the homogenate through the meshes of 15 and 5 μ. Clogged sieve meshes were also cleaned in this apparatus. The ultrasonic cleaner (Struers Scientific Instruments, Copenhagen) has a maximum capacity of 1600 ml. The electric energy from the generator (80 W) is transformed by a titanate transducer into sound wafes of 80 KC ± 8 KC/sec. The inside dimensions are: depth 10.2 cm, length 15.1 cm, width 13.7 cm.

*Oxygen uptake*

Oxygen uptake was measured by use of a CLARK electrode (Yellow Springs Instr., Yellow Springs, Ohio, U.S.A.). The electrode surface was covered by a teflon membrane and polarized at 0.6 V. The electrode was inserted into 3.5 ml of the haustorial fraction suspended in an air saturated 0.1 M phosphate buffer which was maintained at 24° C by use of a water jacket. The suspension was stirred with a magnetic stirrer. The oxygen uptake was recorded on an automatic micrograph BD 2 (Kipp, Delft, Holland) at 0.5 μA during 5 minutes.

## RESULTS

The procedure employed for the isolation of haustoria consists of three different steps. In the first step hyphae and conidia are removed from the leaf surface. Secondly the haustoria are released from intact host cells by means of homogenization of the leaves. The third step consists of the purification of the haustoria by centrifugation in glycerol and by sieving through micro-mesh sieves with special use of ultrasonic vibrations. The several steps will be described in detail.

*Removal of mildew hyphae and conidia from the leaf surface*

According to MILLERD & SCOTT (1963) the hyphae of *Erysiphe graminis* can be removed from the leaf surface of barley by brushing with a camel-hair brush. When, however, the leaf surface of cucumber is brushed, many hyphae,

conidiophores and conidia remain between the leaf hairs and in the nervature. Better results were obtained if the leaf, while lying flat on a petri dish, was brushed and simultaneously sprayed with fine tap water drops. Since the dimensions of the conidia vary approximately from 23–37 μ by 14–21 μ, the water is filtered through a 30 and a 20 μ mesh sieve. The bulk of the spores are collected from the sieve plates and stored at 1° C in phosphate buffer pH 6.5. The leaves are now freed from most of the spores, however, not from the hyphae (Scheme 1).

Further removal of the hyphae is achieved by dropping about 100 g of the leaves into a glass vessel filled with 2000 ml of tap water and 50 μl of a detergent (tween 20). The leaves are then first cut into pieces of about 1–2 $cm^2$ by mixing for 1 minute with the knife blades of the omnimixer at 4000 rpm. Subsequently conidia and hyphae are freed from the leaf surface by stirring water and leaf pieces in the same vessel for 60 minutes at low speed (500 rpm) with a stirring motor (Voss Instr. England). In contrast to the knife blades of the omnimixer the blades of the Voss motor are obtuse-angled, thus preventing further tearing up of the leaf pieces. Finally the leaf pieces are collected and put on a 400 μ mesh sieve and thoroughly rinsed with tap water for 30 minutes to liberate them from the released hyphae and spores. The resulting filtrate is filtered though a 50 μ mesh sieve. The bulk of hyphae threads remains behind on the sieve and are stored at 1° C in phosphate buffer whereas the spores which pass the meshes are discarded.

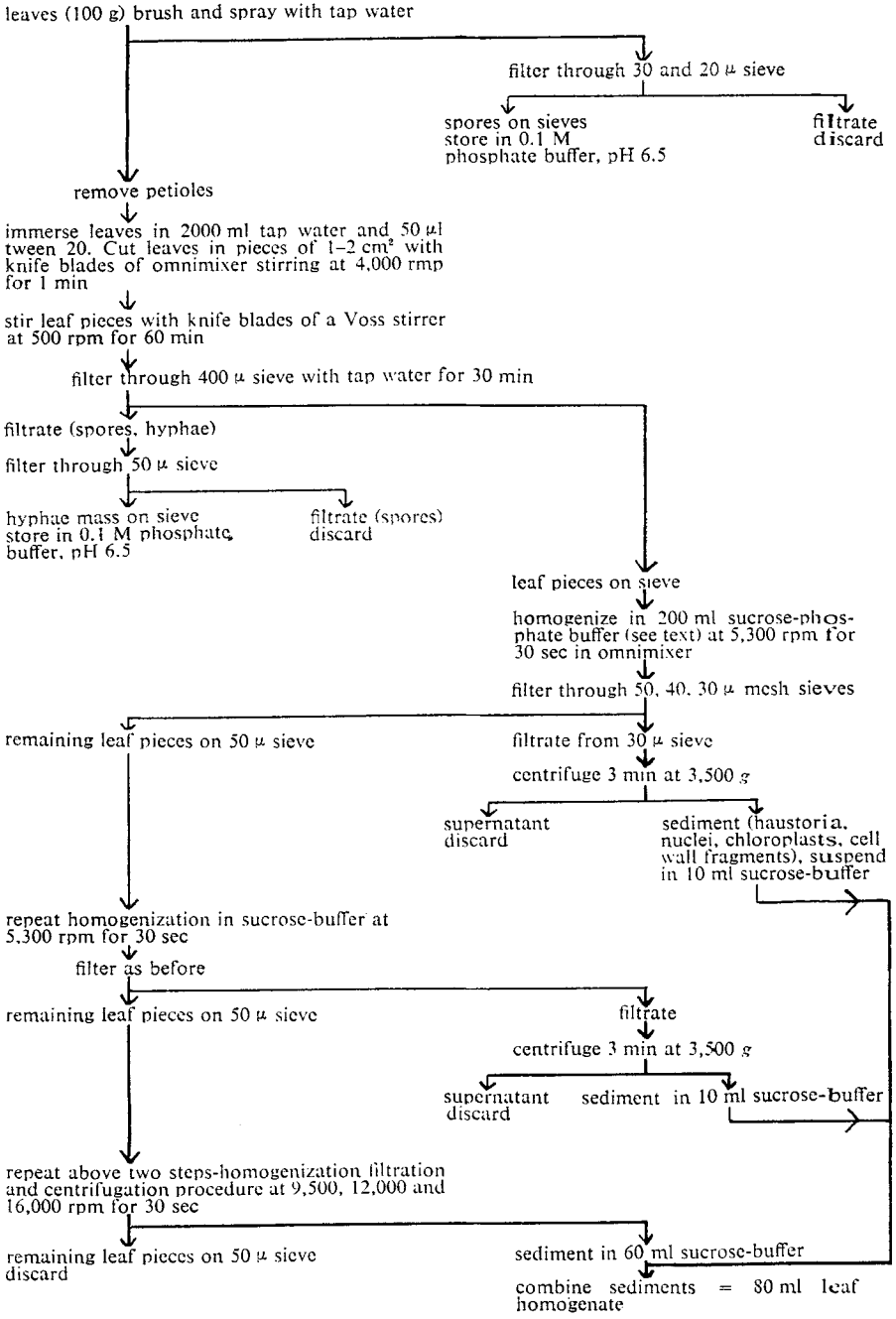
*Homogenization of host cells*

Haustoria could be released successfully from the host cells by homogenization of the cleaned leaf pieces in 0.1 M ice-cold phosphate buffer in an omnimixer. It was found, however, that a medium with a low osmotic value causes a disruption of the chloroplasts. This results in a clumping of the chloroplasts during centrifugation thus making a subsequent separation from the haustoria very difficult. Thus following JAMES & DAS (1957), 0.3 M sucrose was added to the buffer to keep the chloroplasts intact. Moreover, magnesium sulphate was added to the medium since bivalent ions are known to strengthen cell membranes. Thus the final medium contained 0.3 M sucrose, 0.03 M $Na_2HPO_4.2H_2O$, 0.07 M $KH_2PO_4$ and 0.003 $MgSO_4.7H_2O$ (pH 6.5).

The host cell walls tear up during homogenization of the leaf pieces and in most cases the haustoria are cut off from the penetration hyphae. In some cases, however, the penetration hyphae are still present (Figs. 2, 3, 5). Differential homogenization is applied to minimize further damage of those haustoria which already have been released. For this reason a standard procedure has been developed (Scheme 1).

Leaf pieces (100 g) were homogenized in 200 ml ice-cold medium at 5,300 rpm for 30 seconds in the omnimixer. The homogenate was filtered together with 200 ml ice-cold sucrose-buffer through 50, 40 as well as the 30 μ mesh sieves. The final filtrate of the 30 μ mesh sieve was centrifuged at 3,500 *g* for 3 minutes in a swinging out rotor (Hettich Silenta II) and the sediment containing the haustoria and other cell constituents were kept at 1° C in 10 ml sucrose-buffer. The remaining leaf pieces on the 50 μ mesh sieve were resuspended in 200 ml sucrose-buffer and again homogenized at 5,300 rpm for 30 seconds. The homogenate was filtered and centrifuged in the same way as after the first run.

SCHEME 1. The separation of spores, hyphae and haustoria from cucumber leaves infected with powdery mildew.



Subsequently the remaining leaf pieces on the 50 μ mesh sieve were homogenized in exactly the same way twice at 9,500 rpm, twice at 12,000 rpm and finally twice at 16,000 rpm for 30 seconds. The remaining leaf pieces on the 50 μ sieve were discarded. The sediment of each fraction was suspended in 10 ml sucrose-buffer. Thus finally the eight fractions yielded 80 ml of the homogenate which contained about $90 \times 10^6$ haustoria.

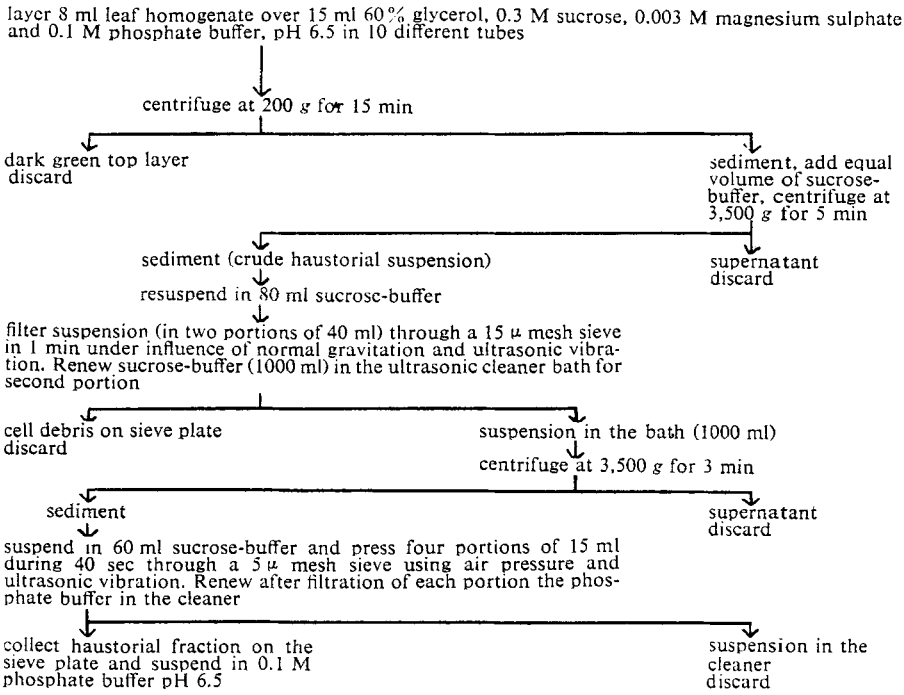
*Purification of haustoria by centrifugation in glycerol*

The fact that the rate of sedimentation of particles under influence of a field of centrifugal force depends upon their density; their size and shape is often used for separation of different cell particles (SANWAL, 1963). The dimensions of the haustoria vary from 14–21 μ by 7–14 μ, whereas the diameter of a cucumber chloroplast is about 3 μ. According to JAMES & DAS (1957) the densities of mitochondria and chloroplasts are 1.12 and 1.18, respectively, in spinach as well as in broad beans.

It has been tried to separate chloroplasts from haustoria in a medium with a density higher than 1.18. A suitable medium was obtained by mixing 60 ml glycerol with 40 ml of a solution containing phosphate buffer, sucrose and magnesium sulphate (density 1.22). The final concentration of the latter compounds was equal to that of the homogenization medium (pH 6.5).

The homogenate (8 ml) was layered over 15 ml of ice-cold medium in 10 tubes

SCHEME 2. The separation of haustoria from the leaf homogenate.



with maximum capacity of 80 ml and centrifuged at 200 *g* for 15 minutes (Scheme 2). A thick dark green band containing most of the chloroplasts, plastids and other small particles was visible on top of the glycerol layer. The glycerol layer itself contained almost all haustoria, some chloroplasts and some other small cell particles but also conidia, intact host cells, mycelium threads and numerous host cell wall fragments. The dark green layer on top of the glycerol layer was pipetted out with a hypodermic needle. The haustoria and other cell constituents were freed from the glycerol by adding 150 ml sucrose-buffer and centrifugation at 3,500 *g* for 5 minutes. The sediment (the crude haustorial suspension) was taken up in 80 ml sucrose-buffer.

*Further purification of haustoria with micro-mesh sieves*

The dimensions of the haustoria vary approximately from 14–21 μ by 7–14 μ and the dimensions of the conidia vary from 23–37 μ by 14–21 μ. From these dimensions it follows that most of the haustoria must be able to pass meshes of 15 μ but will stay behind on sieves with meshes of 5 μ. Conidia and relatively large cell wall fragments, however, will stay behind on the 15 μ mesh sieves, whereas chloroplasts and other small cell particles will pass all sieve openings. Thus, theoretically it must be possible to purify the haustoria further by filtration through micro-mesh sieves of 15 and 5 μ.

It was found, however, that the micro-meshes of 15 and 5 μ become quickly clogged during filtration of the crude haustorial suspension which has been obtained after centrifugation in glycerol. COLON (1965) used an ultrasonic cleaner to prevent a 5 μ mesh sieve from clogging by small glass beads. The sieve was held on the surface of the liquid bath of an ultrasonic cleaner. Vibration in the bath caused the formation of microscopic vacuum bubbles (cavitation) which travel at high speed through the solution and sieve openings, in this way preventing them from clogging by the glass beads.

This method appeared to be highly valuable for the filtration of the crude haustorial suspension through meshes of 15 and 5 μ. To minimize destruction of the haustoria by implosion of the cavitations, care must be taken to filtrate the haustoria as quickly as possible. The whole filtration procedure will now be described in more detail (Scheme 2).

The crude haustorial suspension is divided into two equal portions of 40 ml. The first portion is added to the 15 μ mesh sieve. Under influence of normal gravitation particles smaller than 15 μ pass the meshes in 1 minute and enter the ice-cold vibrating sucrose-buffer solution (1000 ml) of the bath of the ultrasonic cleaner. Thereafter the sieve is cleaned and the solution in the bath is again filled with 1000 ml sucrose-buffer. The second 40 ml portion of the homogenate is then filtered in exactly the same way as the first portion. Of both portions the spores, hyphae and cell wall fragments on the sieve are discarded whereas the haustoria and other cell particles present in the filtrate are collected by centrifugation at 3,500 *g* for 3 minutes. The sediment is taken up in 60 ml sucrose-buffer (pH 6.5).

This suspension is filtered through the 5 μ mesh sieve by means of vibration. It appeared, however, that particles smaller than 5 μ pass these meshes only very slowly under influence of normal gravitation and ultrasonic vibration. To avoid damage of the haustoria by a relatively long filtration period in the ultrasonic cleaner, this procedure is accelerated by air pressure. To this end air is pressed

through a hole of 0.5 cm diameter in the centre of a plastic sheet which is put over the rim of the sieve plate.

In this way the suspension (60 ml) is filtered through the 5 μ mesh sieve in four separate portions of 15 ml during 40 seconds. After each cycle of 40 seconds the fraction on the sieve which contains numerous haustoria is collected and stored in buffer at 1° C. The particles smaller than 5 μ are discarded.

Thus the total time during which the haustoria are exposed to the forces in the ultrasonic cleaner is 100 seconds.

*The yield of haustoria*

The area of 1 g of cucumber leaves which are routinely used in these experiments is about 46.4 $cm^2$. One $cm^2$ of the upper epidermal layer contains about 28,000 haustoria. From these data it follows that 100 g of leaves contain about $130 \times 10^6$ haustoria.

The numerous cell particles in the non-purified homogenate make an exact count of the isolated haustoria impossible. After centrifugation in glycerol, however, the amount of haustoria can easily be counted. It has been found that 100 g of leaves yield about $85 \times 10^6$ haustoria after this first purification step. Thus about 66% of all haustoria in the leaves have been recovered in the glycerol layer.

After filtration $65 \times 10^6$ haustoria have been recovered on the 5 μ sieve. The loss of about 25% during filtration is due to haustoria which remain on the 15 μ sieve ($3 \times 10^6$) and to the fact that the haustoria rather easily adhere to the glass walls of the centrifuge tubes as well as to that of the beakers which are used.

The dry weight of the fraction was found to be 5.9 mg after three hours at 105° C.

*Microscopic examination of the isolated haustoria*

A phase contrast photomicrograph of a suspension of haustoria collected from the 5 μ sieve is presented in Fig. 1. The suspension contains about 60% haustoria, about 38% host cell fragments, 2% spores and hyphae. Undamaged host cells have been observed only very rarely and plastids or other particles smaller than 5 μ are practically not present in the suspension.

About 10–15% of all haustoria in this fraction miss part of the cell wall (Fig. 7) or the whole wall (Fig. 8). This damage has been caused during homogenization of the leaves and not by the cavitation in the ultrasonic cleaner since the percentage of damaged haustoria after filtration with the micro-mesh sieves is not higher than after centrifugation of the homogenate in the glycerol.

Figs. 2–6 show some typical forms of isolated haustoria photographed by phase contrast microscopy or by bright field microscopy after staining with Janus green B or cotton blue lactophenol. Penetration hyphae are visible on about 1% of the haustoria (Figs 2, 3, 5). The haustoria are often characterized by a dense central part surrounded by a more diffuse part. Both parts contain many small granules of about 0.5–1 μ diameter (Figs. 2, 3).

*Oxygen uptake by the haustorial fractions*

In order to investigate if the isolated haustoria show any metabolic activities, oxygen uptake was measured during five minutes. Table 1 gives the results of an

TABLE 1. A comparison between the endogenous respiration of the haustorial fraction, spores and hyphae of *Sphaerotheca fuliginea* with that of non-germinated spores of some non-obligate fungi suspended in 0.1 M phosphate buffer pH 6.5 at 24°C. The oxygen uptake is calculated on mg dry weight basis.
*Een vergelijk tussen de endogene ademhaling van de fractie met haustoriën, sporen en hyfen van Sphaerotheca fuliginea met die van niet-gekiemde sporen van enige niet-obligate schimmels, gesuspendeerd in 0,1 M fosfaat-buffer pH 6,5 bij 24° C. De zuurstofopname is berekend per mg drooggewicht.*

| | $\mu$l $O_2$/mg dry weight/5 minutes<br>*$\mu$l $O_2$/mg drooggewicht/5 minuten* |
|---|---|
| Haustorial fraction<br>*Fractie met haustoriën* | 0.23 |
| Mildew hyphae<br>*Meeldauw-hyfen* | 0.10 |
| Mildew spores<br>*Sporen van meeldauw* | 0.50 |
| Spores of *Glomerella cingulata*<br>*Sporen van* Glomerella cingulata | 1.00 |
| Spores of *Cladosporium cucumerinum*<br>*Sporen van* Cladosporium cucumerinum | 0.42 |
| Spores of *Aspergillus niger*<br>*Sporen van* Aspergillus niger | 0.10 |

experiment in which the initial endogenous respiration of the haustorial fraction is compared on dry weight basis with that of mildew spores, mildew hyphae and non-germinated spores of some non-obligate fungi suspended in phosphate buffer, pH 6.5. The oxygen uptake of the haustorial fraction is about half of that of the mildew spores but twice as high as that of the hyphae. The endogenous respiration of the mildew spores is equal to that of the average of the non-obligate spores.

The haustorial fraction can be kept in buffer for at least three days at 1°C without detectable loss of activity. The oxygen uptake of the mildew spores, haustorial fraction and hyphae did not increase after addition of substrate (0.013 M), such as glucose, pyruvate, sodium acetate, or succinate.

As already described, a detergent is used to free the hyphae from the leaf surface. The possibility, however, cannot be excluded that tween 20 penetrates into the leaf and affects the metabolic activity of the haustoria. In this connection it is important that the concentration of tween 20 which was used did not change the oxygen uptake when added directly to the haustorial fraction. A fourty times higher concentration (2 ml tween in 2000 ml water), however, decreased the oxygen uptake to 50%.

*Reduction of Janus green B by the haustoria*

Although oxygen is taken up by the haustorial fraction, this does not prove that the haustoria are in fact the active particles in the fraction. In order to establish which component of the fraction is responsible for the oxygen uptake, reduction of Janus green B (Allied Chem. Co., New York, U.S.A.) was followed microscopically. Twenty ppm was added to the haustorial fraction suspended in phosphate buffer, pH 6.5. The fraction stood for 15 minutes in a shallow layer fully exposed to air. A drop, containing blue stained haustoria, was then mounted and examined microscopically after the cover slip had been put over. The

blue color at first evident in the haustoria became much paler after one hour at 24° C as anaerobiosis developed. Decolorization has also often been found in haustoria which had lost part of the cell wall. About 10% of all haustoria did not loose the blue color, whereas in the same period most of the host cell fragments did not decolorize.

Haustoria which had been heated for 2 minutes at 100° C before addition of Janus green B did not decolorize the dye.

These experiments show that in most of the isolated haustoria oxido-reduction processes proceed.

## DISCUSSION

The isolation of the powdery mildew haustoria presents various critical steps. First of all a detergent is necessary for a removal of hyphae from the leaf surface. Although the possibility cannot be excluded that the detergent has penetrated into the leaf it is important that the concentration applied has not been found to affect the endogenous respiration of the haustoria when added directly to the fraction.

In the next step the haustoria are released from the host. Homogenization of the leaf pieces must be carried out in a medium which is isotonic to that of the chloroplasts. Differential homogenization appeared to give the highest yield of undamaged haustoria. The fact that about 66% of the total number of haustoria in the epidermal layer can be released, indicates that they must have a strong cell wall. This view may perhaps be connected with the results obtained by EHRLICH & EHRLICH (1963). They carried out an electron microscope study of haustoria of *Erysiphe graminis* in wheat plants. The cytoplasm of the haustoria was found to be surrounded by a relatively thick sheath of unknown composition.

A successful separation between haustoria and chloroplasts has been obtained by centrifugation of the leaf homogenate in a mixture of glycerol and sucrose. The crude haustorial suspension collected from the glycerol layer has been purified further by sieving the fraction through a 15 and 5 μ mesh sieve. Filtration is facilitated by ultrasonic vibration. It is important that the total time of vibration (100 seconds) does not affect oxygen uptake of an isolated haustorial fraction.

Glycerol centrifugation can be omitted from the purification procedure. The disadvantage, however, of a purification procedure which consist only of filtration, is that filtration of the leaf homogenate takes far more time than that of the crude haustorial suspension. A longer period of filtration increases the chance of damage to the haustoria. The haustorial fractions used for respiration studies, appeared to be contaminated to about 40–50% with host cell wall fragments, some hyphae, spores and only extremely low numbers of undamaged host cells.

Microscopical examinations showed granules in the dense central part and in the more diffuse part of the haustoria. According to HIRATA (1937) and HIRATA & KOJIMA (1962) protrusions are formed at the ellipsoidal body of *Sphaerotheca fuliginea*. The finger-like protrusions grow convolutely. These authors point to the fact that the tangled finger-like protrusions are often misinterpreted as granules. Preliminary electron microscopic studies on slices

of isolated haustoria confirmed this view. These observations will be published in a next paper.

Oxygen consumption and reduction of Janus green B show that some catabolic processes still proceed in the isolated haustoria. The fact that the endogenous respiration does not rise after addition of substrate can be explained by assuming that the haustoria contain large amounts of food material. Addition of substrate will not cause an increase of oxygen uptake as long as substrate is not a limiting factor. Isolation experiments in which sucrose was replaced by 0.35 M NaCl, yielded haustoria with a high endogenous respiration which did not increase after addition of sucrose or glucose. This shows that the endogenous respiration of haustoria, isolated in sucrose phosphate buffer, is not due to the oxidation of sucrose taken up during isolation.

On dry weight basis the oxygen uptake of the haustorial fraction is lower than that of the mildew spores. Since the haustorial suspension contains also host cell wall fragments it may be assumed that the activity of a pure haustorial suspension is about equal to that of mildew spores. If that holds true the endogenous respiration on dry weight basis of haustoria, mildew spores and non-germinated spores of non-obligate fungi falls in about the same order of magnitude.

In preliminary experiments it has also been tried to isolate haustoria of powdery mildew *(Erysiphe graminis)* from wheat leaves. This was found, however, to be far more difficult than the isolation of haustoria from cucumber powdery mildew since the haustoria of *E. graminis* are branched. The finger-shaped protrusions appeared to be damaged easily during homogenization of the host cells.

Isolated haustoria may offer the possibility to obtain more information on questions regarding the obligate nature of mildew diseases. On the other hand the scope of its application may be very limited since many compounds may have leached from the haustoria into the isolation medium. In this case the metabolic activity of the isolated haustoria will be much smaller than their normal activity in the host cells.


## SAMENVATTING

Er wordt een methode beschreven voor de isolatie van haustoriën uit komkommerbladeren, geïnfecteerd met echte meeldauw *(Sphaerotheca fuliginea)*. Bij deze methode worden eerst de hyfen en sporen van het bladoppervlak verwijderd. Vervolgens worden door homogenisering van de bladeren de haustoriën vrijgemaakt uit de intacte waardplantcellen. De zuivering van de haustoriën geschiedt door centrifugeren van het bladhomogenaat in glycerol en filtratie door fijnmazige zeven van 15 en 5 $\mu$ met behulp van ultrasonore trillingen.

Uit een voorlopig onderzoek bleek, dat de geïsoleerde haustoriën nog in staat waren oxydatie- en reductieprocessen uit te voeren.



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