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## Morphology and *in vitro* cultivation of the bacterial symbiote of *Dacus oleae*\*

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The oesophageal diverticulum of the olive fruit fly, *Dacus oleae* (GMELIN), harboring the bacterial symbiote was investigated by scanning electron microscopy. It was shown that the organ was filled with a pure bacterial population. *In vitro* cultivation assays were successful. The symbiote was not identical with the causative agent of the olive knot, *Pseudomonas savastanoi*.

*Dacus oleae*, the olive fruit fly is one of the major pest insects of olive cultures. Despite a temporary overproduction of olives, the control of this insect is on a long term basis of primary importance.

*D. oleae* is closely associated during all the stages of development with a bacterial symbiote (PETRI, 1909). In the adult insect, the microorganisms are located in a special organ in the head designated as oesophageal diverticulum or cephalic cyst. The transmission from one generation to the other takes place by contamination of the eggs with the bacterium during oviposition. In the larvae which feed within the olive fruit the symbiote develops in the caeca situated near the anterior end of the midgut.

Although the exact role of the bacterium has not yet been established it is evident that we are faced with a highly developed symbiosis between an insect and a microorganism. This has been the subject of a number of early investigations which are summarized by HELLMUTH (1956). In later studies evidence was presented by FYTIZAS & TZANAKAKIS (1966) and TZANAKAKIS & STAVRINIDES (1973) that the symbiote was an essential factor in the development of *D. oleae*. Elimination of the microsymbiote with streptomycin intercepted the generation cycle of the insect.

The identity of the organism has not yet been established. PETRI (1909) as well as HELLMUTH (1956) suggested that a mixed culture consisting of *Pseudomonas savastanoi* and *Agrobacterium luteum* was involved in the symbiosis. It has to be added that *P. savastanoi* is a plant pathogen causing the olive knot disease. YAMVRIAS (1970), however, was not able to isolate *P. savastanoi* from *D. oleae*, collected in a region of Greece where olive knot was endemic. Thus, the real identity of the symbiote is still unknown. The aim of this paper was to confirm in a first step by scanning electron microscopy that only a single bacterium is involved. In a second part, *in vitro* cultivation of the symbiote is described.

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## MATERIAL AND METHODS

### *Insects*

Adults of *D. oleae* were used throughout the study. The specimens used in Zurich were shipped as pupae from Greece. After emerging the adults were kept at room temperature and fed with sucrose, yeast extract and water. The insects lived under these conditions up to three weeks.

### *Electron microscopy*

Head capsules of *D. oleae* were frozen in acetone at  $-95^{\circ}\text{C}$  (liquid/solid mixture). Following sagittal sectioning of the head capsules in this mixture, dehydration was carried out in 2,2-dimethoxypropane at  $20^{\circ}\text{C}$  for 60 min. The specimens were then washed twice during 15 min with acetone and subjected to drying (ANDERSON, 1951) in a Balzers-Union critical point dryer. Casting with palladium/gold was conducted in a Balzers-Union sputtering device. The preparations were examined in a Hitachi SEM-700 and Kodak Tri-X Pan TX 120 film was used for the micrographs.

### *Isolation of cephalic cysts*

The head capsules of the insects were dissected in sterile Ringer solution (0.85% NaCl) under the binocular. The intact cyst was transferred to 1 ml of the Ringer solution and gently homogenized. Each homogenate was checked for the presence of the symbiote under the phase contrast microscope before it was used as inoculum.

### *Microorganisms*

For comparative purposes a culture of *P. savastanoi* from the culture collection of the Benaki Phytopathological Institute, Kiphissia, Athens, was used. It was maintained on stock culture agar (Difco) and stored at  $4^{\circ}\text{C}$ .

### *Media*

Since the growth requirements of the symbiote were unknown different media were used for the primary cultivation assays.

*Medium No. 1: Egg yolk agar.* Eggs were surface cleansed for 2–3 h in 70% ethanol. The egg yolks were then separated and transferred to a sterile dish. With a disposable sterile syringe, the egg yolk was distributed into glass petri dishes in aliquots of 5 ml or into test tubes (2–3 ml). Subsequently, the egg yolk was hardened at  $80^{\circ}\text{C}$  whereby the tubes were layered out as slants. The egg yolk was then covered by an agar medium of  $50^{\circ}\text{C}$  which had the following composition (g/l): peptone (Bacto), 7.5; glucose, 5.0;  $\text{KH}_2\text{PO}_4$ , 6.8;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.123;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.002;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.014;  $\text{Fe}_2(\text{SO}_4)_3$ , 0.02;  $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.183; pH 7.2. This combined medium was left at room temperature over night and stored at  $4^{\circ}\text{C}$  until used for cultivation assays.

*Medium No. 2:* A medium containing glucose, yeast extract and mineral salts (g/l): glucose, 3.0; yeast extract (Difco), 2.0;  $(\text{NH}_4)_2\text{SO}_4$ , 2.0;  $\text{K}_2\text{HPO}_4$ , 0.5;  $\text{MgSO}_4$ , 0.2;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.08;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.05; pH adjusted with KOH to 7.3.

*Medium No. 3:* Peptone, glucose medium (g/l): Peptone, 20; glucose, 10; NaCl, 5.

*Medium No. 4:* YEPD medium used especially for the cultivation of yeasts (g/l): Peptone, 20; yeast extract, 10; glucose, 20.

*Medium No. 5:* Brain heart infusion medium, a very rich composition and suitable for the prolonged preservation of cultures (g/l): Brain heart infusion (BBL), 37; tryptone, 4; tri-sodiumcitrate · 2H<sub>2</sub>O, 3; proteose peptone No. 3, 0.2; pH 7.3.

Media Nos. 2 to 5 were solidified when necessary by the addition of 2% agar before autoclaving.

The different media will be referred to in the text by their respective numbers.

### *Inoculation*

The agar plates were subdivided into 40 squares and cyst homogenate was transferred by tooth picks and deposited into the middle of each square. A loop was used where liquid media in Erlenmeyer flasks or test tubes had to be inoculated.

## RESULTS

### *Prevalence of the symbiote*

All cysts investigated originated from insects collected in nature. Interestingly, the symbiote was not regularly present in the examined cysts. It was found that cysts of young adults which were a few days old and kept without feeding, were predominantly bare of bacteria or they were present in very small numbers. Such cysts appeared small and translucent. When supplied with nutrients, the insects developed cysts which were milky-white in color and filled with bacteria yielding a content of cream-like consistency. No difference between male and female specimens could be detected. Under the light microscope the bacteria appeared as homogenous cultures and motility was not observed.

### *Morphology of cyst and microorganism*

The cyst is a kidney-shaped organ, located anterior to the brain and connected to the foregut. The position of the cyst within the head capsule of *D. oleae* is shown in Fig. 1. The diameter of this organ is about 200 µm. In Fig. 2 the cyst is shown at a higher magnification revealing the strongly laminated inside. At a still higher magnification the bacteria which occupy the lamellae in thick layers appear (Fig. 3). Here, it can clearly be seen that the morphology of the bacteria is so homogenous that the presence of a mixed culture can be ruled out. Fig. 4 demonstrates the symbiote at high magnification which allows the observation of cell division and a view of the surface of the cell wall. Appendages of the bacteria within the cysts as described in an ultrastructural study by POINAR *et al.* (1975), were not visible. The size of the microorganisms averaged  $2.8 \pm 0.8$  µm in length and about 0.6 µm in width.

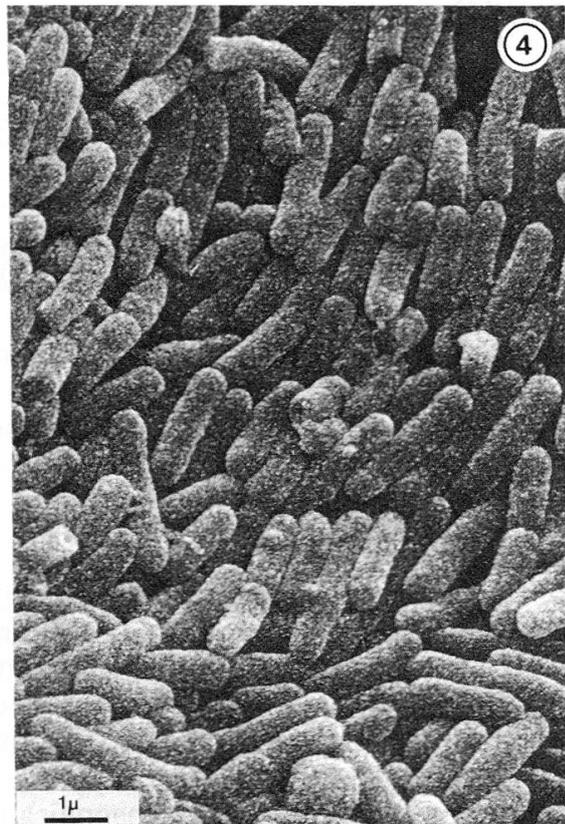
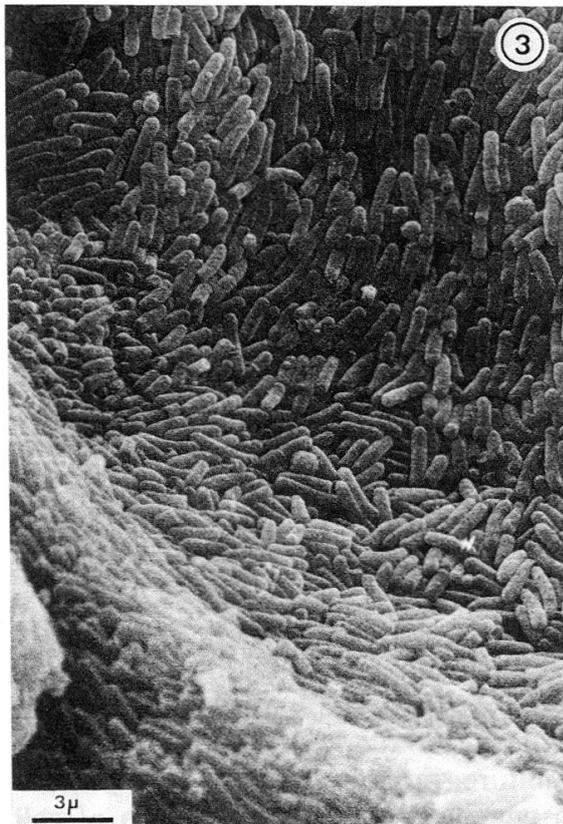
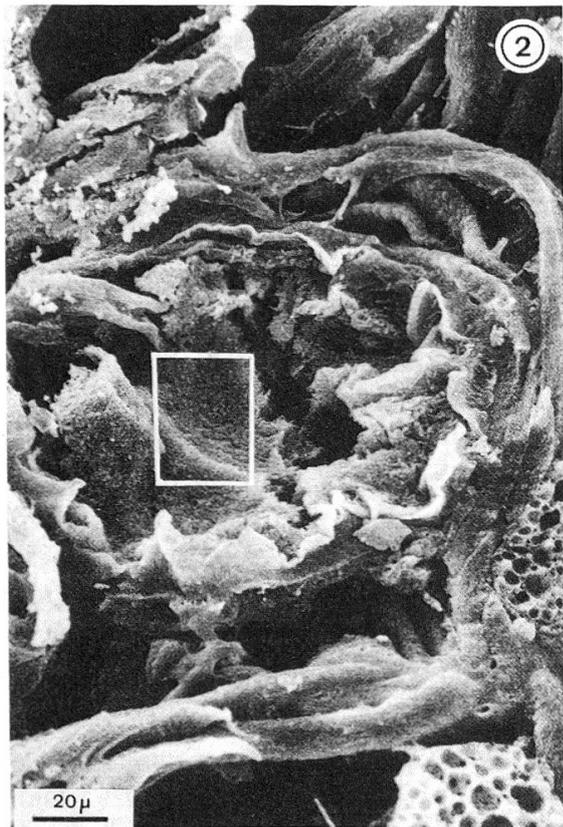
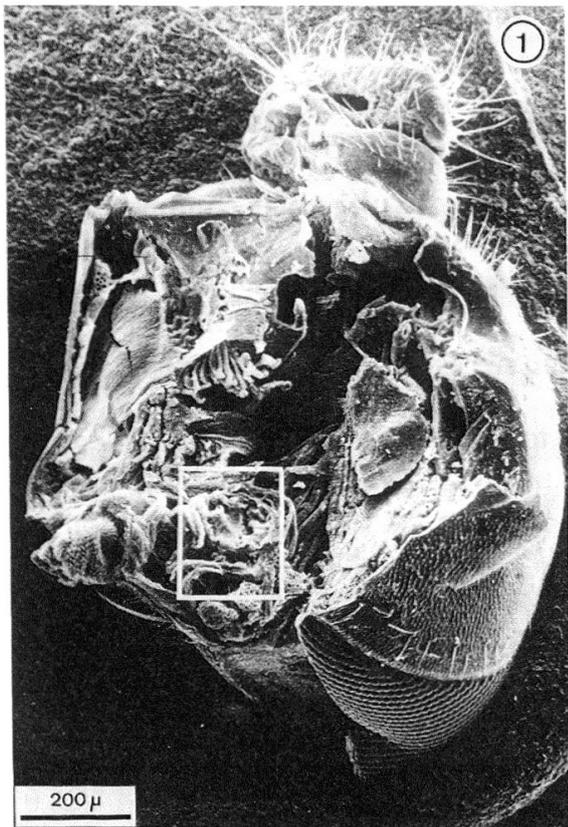


Fig. 1: Head capsule of *D. oleae* with the position of the cyst (marked area)

Fig. 2: The cyst of *D. oleae*. The marked area at higher magnification reveals the bacteria in Fig. 3 and 4.

### Gram reaction

Gram staining of the bacteria by different classic methods gave a clear Gram negative reaction.

### *In vitro* cultivation

The first cultivation experiments were carried out with the complex egg yolk agar (medium No. 1). The plates inoculated with tooth picks were checked daily for colony growth. It took 5 days until some of the inoculated spots developed visible growth. Colony transfer to egg yolk agar slants was successful. Compared to *in vivo* findings the bacteria grown *in vitro* were somewhat shorter with a tendency to become coccoid after a number of transfers.

A second series of cultivation tests were conducted with medium No. 2. Liquid stationary cultures incubated at room temperature developed growth after 3–5 days. Transfers grew within 2–3 days also on agar plates. No growth was observed under anaerobic conditions. The other media (Nos. 3, 4 and 5) were also suitable for growth and maintenance of the symbiote. Table 1 summarizes the growth experiments. On solid media the colonies had a diameter of 3–5 mm after one week and were translucent with a slight yellowish color.

Table 1: Summary of the growth experiments conducted with the symbiote of *D. oleae*.

Medium Nr.	Cultivation Temp. °C	on agar	Growth in liquid culture	anaerobic
1	28	+	ND	ND
2	20	+	+	-
2	28	+	+	ND
2	30	-	-	ND
3	20	+	+	-
4	20	+	+	-
5	20	-	ND	-

Legend: + growth positive; - no growth; ND not determined

Using medium No. 2, the organism was grown at different temperatures ranging from 20 to 37 °C. Growth was obtained up to a temperature of 28 °C. Already at 30 °C the bacteria did not grow. A comparison of the morphology of cultures grown at 20 and 28 °C showed that the bacteria were longer at the lower temperature whilst the majority of the cells were short rods or even cocci when grown at the higher temperature.

As a comparison *P. savastanoi* was cultured in the above-cited media. Growth was vigorous and complete within 24–48 h. The cells of *P. savastanoi* were motile whereas the symbiote of *D. oleae* never showed active movement. Furthermore, a diffusible fluorescent pigment was produced, optimum growth temperature was higher and the morphology of colonies was also distinct.

## DISCUSSION

With the successful *in vitro* cultivation of the symbiote of *D. oleae* the first step towards its identification has been achieved. *In vitro* cultivation is a prerequisite for a profound knowledge of the role of the symbiote and for an eventual attempt to control the olive fly by elimination of the bacteria.

The obtained results clearly demonstrate that only a single bacterial species is responsible for the symbiosis in the olive fruit fly and that this bacterium is not *P. savastanoi* as assumed by PETRI (1909) and HELLMUTH (1956). The latter author isolated a bacterium from *D. oleae* as accompanying organism of *P. savastanoi* and she named it *Agrobacterium luteum*, a species which is however not recognized by BERGEY (1975). We do not know yet if *A. luteum* of HELLMUTH is identical with the organism isolated and described here. Identification and systematic classification will be the subject of further research. According to the present knowledge it is unlikely that the symbiote will be placed within the genus *Agrobacterium*, because the cells are not motile, no slime is produced and the optimum temperature range is lower.

PETRI (1909) and HELLMUTH (1956) found *P. savastanoi* associated with *D. oleae* while this organism did not appear in our isolations. The results of the two authors mentioned above suggest however a frequent association of *P. savastanoi* with *D. oleae*. This bacterium could be a common and non-symbiotic inhabitant of the intestine and the insect may even be a factor in transmission of the olive knot disease from one tree to the other.

The question if there are means to suppress the development of the micro-symbiote to achieve control of the pest insect is open. Antibiotics are one possibility but their eventual use must be carefully evaluated because of predictable disadvantages. Only antibiotics can be considered which are not applied in human or veterinary medicine. Compounds have to be readily biodegradable. The problem of resistance will appear if only one antibiotic is used instead of a combination of two or even three.

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