

AN AMOEBA ISOLATED FROM THE LATEX OF *EUPHORBIA MARGINATA* PURSH. (EUPHORBIACEAE)

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ABSTRACT.—*Hartmannella castellanii* Douglas was isolated from the latex of subterranean portions of *Euphorbia marginata* Pursh. (Euphorbiaceae). Eighteen of 161 plants from Illinois and Minnesota harbored the amoeba. This organism, which has recently become famous as a contaminant and pathogen, is reported for the first time from a plant. Methods for obtaining bacteria-free amoebae and culturing them axenically are presented. A generation time of 25.5 hours was obtained. A description of the vegetative and cyst stages is given.

During a search for endophytic microorganisms of laticiferous plants, *Hartmannella castellanii* Douglas, an amoeba, and a bacillus resembling *Bacillus megaterium* DeBary were found in the latex of *Euphorbia marginata* Pursh. (Euphorbiaceae). Information concerning the latex bacterium has been published (Troll, 1966). Amoebae were reported previously from latex and non-latex plants.

Musgrave and Glegg (1904) described an amoeba found in the latex of *Lactuca* sp. These authors cultivated this organism in very simple nutrient broth (water and beef extract) and on solid medium. Schoevers (1918) recognized amoebae in the roots of diseased spinach plants. McWhorter (1922) isolated an amoeba, having many characteristics of *Entamoeba*, from cells of Fiji galls of sugar cane, *Saccharum officinarum* L. (Graminae). He proposed the name *Phytamoeba sacchari* for the organism. It can be cultured in cane juice.

Franchini, in his series of works on the protozoa of plants (1922-1923) has described some amoebae parasitizing a

large number of laticiferous plants, most of them tropical, growing in botanical gardens either in greenhouses or out of doors. Three of these amoebae were named *Amoeba chlorocodonis*, *A. cryptostegiae*, and *A. lactucae*. Franchini's works on the amoebae of plants including a list of all host plants is summarized by Nieschultz (1925) and Wenyon (1926).

Bruni (1925) found some protozoa of amoeboid nature in the latex of *Acanthaster venenata* (Apocynaceae). His diagrams certainly appear like amoebae but detail is lacking.

DuPorte (1925) describes some amoeboid protozoa from the latex of *Convolvulus sepium* L. (Convolvulaceae). No reports of amoebae in latex plants have appeared in the literature since the above mentioned paper.

MATERIALS AND METHODS

Latex occurs in the plant families Apocynaceae, Asclepiadaceae, Compositae, Euphorbiaceae, Moraceae, Papaveraceae, and Urticaceae. Latex samples were collected in Alabama, Florida, Illinois, Indiana, Iowa, Minnesota, Missouri, Tennessee, and Wisconsin. A minimum of 5 samples from different portions of each plant including subterranean structures were examined. Since microorganisms tend to abound at the surface of the plant, sterile techniques were used in extracting the latex.

A solution consisting of 0.25 gm mercuric chloride, 0.25 gm phenol, 1.25 ml concentrated HCl in 70% alcohol was used as a sterilizing agent. Sharp scalpels were kept in this solution for a minimum of 10 minutes and rinsed in sterile distilled water immediately prior to use. The plant parts were first

cleaned with water to remove the larger particles of dirt, etc., then the parts to be cut were swabbed with Lugol's iodine solution using a sterile cotton swab and allowed to dry. After the cut, the exuding latex was dropped onto a clean microscope slide. A thick smear was made with a sterile platinum loop which had been flamed using a cigarette lighter. After the smear was made, the loop was streaked over a nutrient agar slant. The smear was allowed to dry and was immersed in Schaudinn's fluid for 10 minutes. It was then rinsed with water and allowed to dry. The slides were marked with a notation indicating species and locality. Upon returning to the laboratory all smears were stained for 20 seconds with crystal violet and excess stain was removed with tap water. The slides were then dried using bibulous paper and thus were ready for microscopic observation. The use of this simple bacterial stain made it possible to stain many smears in a short time and those from infected plants could easily be distinguished at 430X magnification.

Nutrient agar slants that had been inoculated in the field were incubated for 48 hours at 25° C. and any growth on them examined under the microscope.

In order to isolate the amoebae from bacteria present, a sample of latex was taken aseptically, mixed with sterile saline solution, poured in a thin film on a nutrient agar plate, and incubated for 24 hours at 25° C. The superficial film of the plate was washed with sterile saline into a sterile centrifuge tube. Amoebic cysts were almost freed from bacteria by repeated centrifugation (five times) and resuspension in sterile saline. The sediment of the final centrifugation was streaked on a nutrient agar plate and incubated for 24 hours at 25° C.

For cultivation of the amoebae a liver-peptone-glucose (LPG) medium was prepared. The ingredients of the liquid phase are as follows: 50 g Bacto liver (Difco), 10g proteose peptone (Difco), 5g peptonized milk (Difco), 10g glucose, 2g NaCl, 0.2 Na₂HPO₄, 0.2g KH₂PO₄, 0.003g MgCl₂·6H₂O, 0.003g CaCl₂, 0.003g FeSO₄, and 1000ml distilled water. To prepare the solid phase 1.5% agar is added to the broth and slants prepared after autoclaving. Each slant is overlaid with 2ml of sterile nutrient broth. The medium was inoculated with one tenth ml of amoebic cyst suspen-

sion. Growth curves were determined by inoculating one half ml of washed 72 hour original culture into each of 10 tubes of fresh medium. The tubes were incubated at 25° C. Every 24 hours, up to 12 days, 0.1ml samples were withdrawn and pooled. Numbers of cells were determined by direct counts using the 'Improved Neubauer' type of hemocytometer.

Sterility tests for the detection of bacterial and fungal contaminants were carried out for all cultures using the media and techniques of Diamond (1961).

Most observations were made on living amoebae using the phase contrast microscope. Janus green B in absolute ethanol (1:10,000) was used to stain mitochondria and the standard Feulgen nuclear reaction was performed.

RESULTS

The amoebae were found only in the latex of subterranean parts of *Euphorbia marginata*. Eighteen of 161 plants from Illinois and Minnesota harbored the organism. Cyst stages of amoebae were recovered from the nutrient agar slants prepared in the field, while bacteria grew well. The final sediment of the centrifugation technique yielded individual bacterial colonies and isolated amoebic cysts. The latter were picked out with a sterile loop under the microscope. Cysts thus isolated were placed in a tube containing sterile saline and stored in a refrigerator.

The diphasic liver-peptone-glucose medium was conducive to the axenic growth of the amoebae. A generation time of 25.5 hours was obtained.

All descriptions are based upon observations of living and fixed materials using both conventional light and phase contrast microscopy.

Vegetative stage (Figure 1):

Size—Fifty trophozoites taken at random directly from the latex and from cultures varied from 11 to 34 micra in diameter with an average of 21 micra. It should be realized that size is difficult to measure accurately since the amoebae have variable shapes. A single amoeba may exhibit great variation in size and shape indicated by measurements according to its degree of extension owing to pseudopodial movement.

Pseudopodia—Three types of pseudopods are formed: lobopods, filopods, and

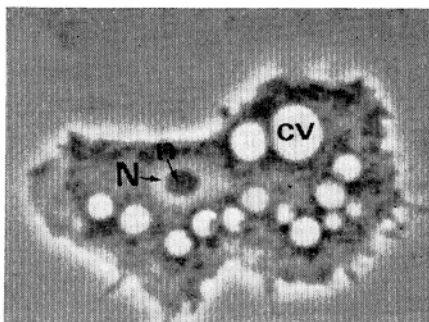


FIGURE 1.—*Hartmannella castellanii* Douglas, living trophozoite from latex of *Euphorbia marginata*. Nucleus (N), nucleolus (n), contractile vacuole (cv). Phase contrast, X 3000.

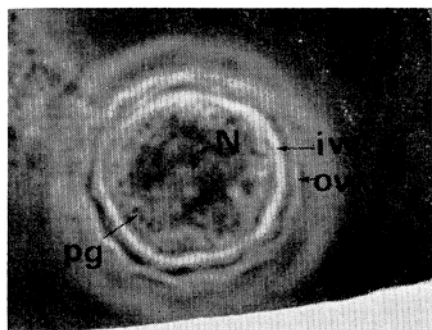


FIGURE 2.—*Hartmannella castellanii*, living mature cyst. Nucleus (N), inner cyst wall (iw), outer cyst wall (ow), peripheral granules (pg). Phase contrast, X 3000.

micropseudopods. All three may be present in the same individual at the same time.

Locomotion—Movement is comparatively sluggish. Differences are observed depending on the type of pseudopods present, the nutritional condition of the culture, and the stage of the life cycle. Prior to encystment, for instance, the amoebae withdraw their pseudopods, become rounded, and cease to move.

Intracellular structure—The cytoplasm, in most cases, is coarsely vacuolated and little cytological detail can be observed. There is a distinct, granular endoplasm and a homogeneous ectoplasm. Numerous filamentous mitochondria are observed upon staining with Janus green. One or more contractile vacuoles are present. No centrosome was observed.

The interphase nucleus is single, spherical, and varies from 3.5 to 4.5 micra in diameter. It possesses a distinct nuclear membrane, a large, very prominent centrally located nucleolus, and peripheral Feulgen positive chromatin granules along the nuclear envelope. The nucleolus is spherical, Feulgen negative, and 1.8 to 2.2 micra in diameter.

Reproduction occurs by mitotic division. No other reproductive phenomena have been observed.

Encystment:

In the laboratory on diphasic LPG medium cysts begin to appear 14 to 16 days after each transfer. The encystment process as observed on isolated

amoebae in LPG medium in depression slides lasts anywhere from 25 to 42 hours. At the onset of encystment the amoebae withdraw their pseudopods, become rounded, and cease to move. This precystic form resembles the one described by Ray and Hayes (1954) for *Hartmannella astronyxis*.

The morphology of the mature cyst is similar to that described by Volkonsky (1931) for *Hartmannella castellanii*. The cysts have two outer wrinkled walls and at various places these walls are in contact with each other (Figure 2). There usually is a single row of refractile granules at the periphery just inside the cell membrane. Fifty cysts taken at random from the same sources as the trophozoites varied in size from 11.2 to 13.4 micra with an average of 12.6 micra in diameter.

The description of the latex amoebae agrees very closely with that of an amoeba isolated by Castellani (1930) in a mixed culture of bacteria and *Cryptococcus parvulus* and now known as *Hartmannella castellanii*.

CONCLUSIONS

On the basis of this study and the excellent discussion by Adam (1964) concerning the taxonomy of hartmannellid amoebae, it is concluded that the amoeba isolated from the latex of *Euphorbia marginata* is *Hartmannella castellanii* Douglas 1930. A synonym is *Acanthamoeba castellanii* (Douglas 1930) Volkonsky 1931. It is noteworthy that this amoeba which in recent years has become a well-known pathogen of

mammalian tissues as well as a contaminant of microbial and tissue cultures is also a plant parasite.

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