STUDIES ON PURIFICATION OF RICE GRASSY STUNT VIRUS

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THE grassy stunt virus disease of rice was first reported[1] in India from West Bengal. This was subsequently confirmed on the basis of experimental findings in 1974[2,3], following its first appearance in epidemic proportions in Kerala during 1973 creating considerable concern. Ghosh *et al*[4], reported the differences in transmission efficiency of the different morphs of the plant hopper vector, *Nilaparvata lugens* (Stal). Mathew[5] studied the vector-virus relationship in elaborate details. This is incidentally the first attempt to purify the rice grassy stunt virus (GSV) in India, the technique and results of which are briefly presented here.

The cultures of GSV collected from South India were maintained on the rice cultivar, TN-1 by occasional inoculation through *N. lugens*. Leaves of GSV-infected rice plants were macerated in 0.2 M phosphate buffer (pH 7.0), containing 0.01 M diethyl dithio carbamate and 0.01 M ascorbic acid, using Waring blender. The sap was squeezed through a double layer of muslin cloth. The extracted sap was centrifuged at 10410 g for 20 min. The supernatant was again centrifuged for 3 hr at 65500 g and the pellet suspended in 0.01 M phosphate buffer and 20% chloroform and then centrifuged at 10410 g for 20 min. The top aqueous layer was separated and centrifuged at 89200 g for 3 hr. The pellet was then resuspended in 0.01 M phosphate buffer with 5% Triton X-100 and centrifuged at 89200 g for 3 hr. The resulting pellet was dissolved in small amount of 0.01 M phosphate buffer. The purified preparation was further analyzed with UV spectrophotometer (Pye Unichem S P5–800), indicating a typical nucleoprotein absorption (figure 1) and 260/280 ratio as 1.24.

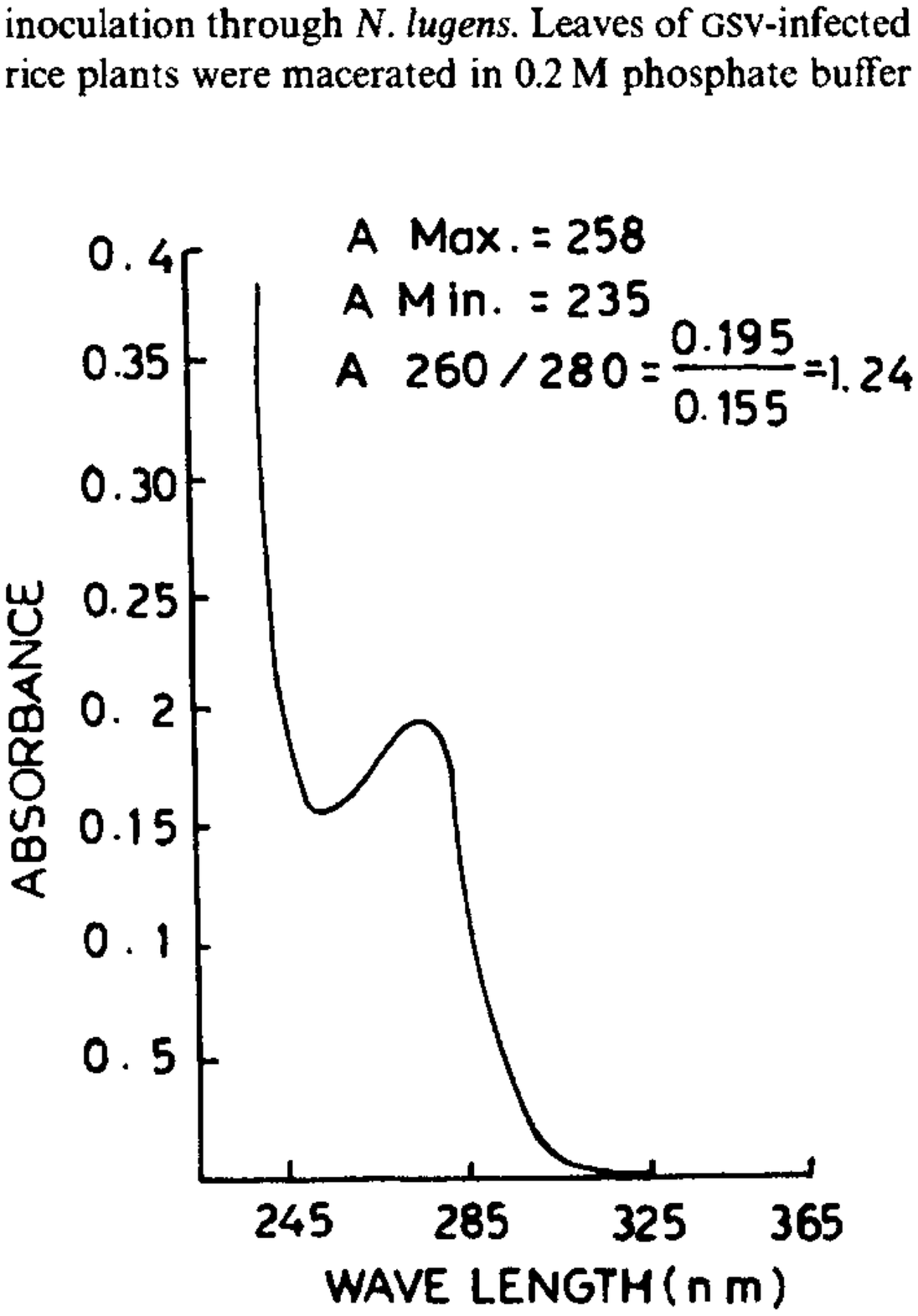

Figure 1. Absorption spectrum of grassy stunt virus

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HISTOPATHOLOGICAL STUDIES ON THE PROGRESS OF INFECTION OF *LEPTOLEGNIA* SP (SC-1) IN *ANOPHELES GAMBIAE* LARVAE EXPOSED TO ZOOSPORES IN THE LABORATORY

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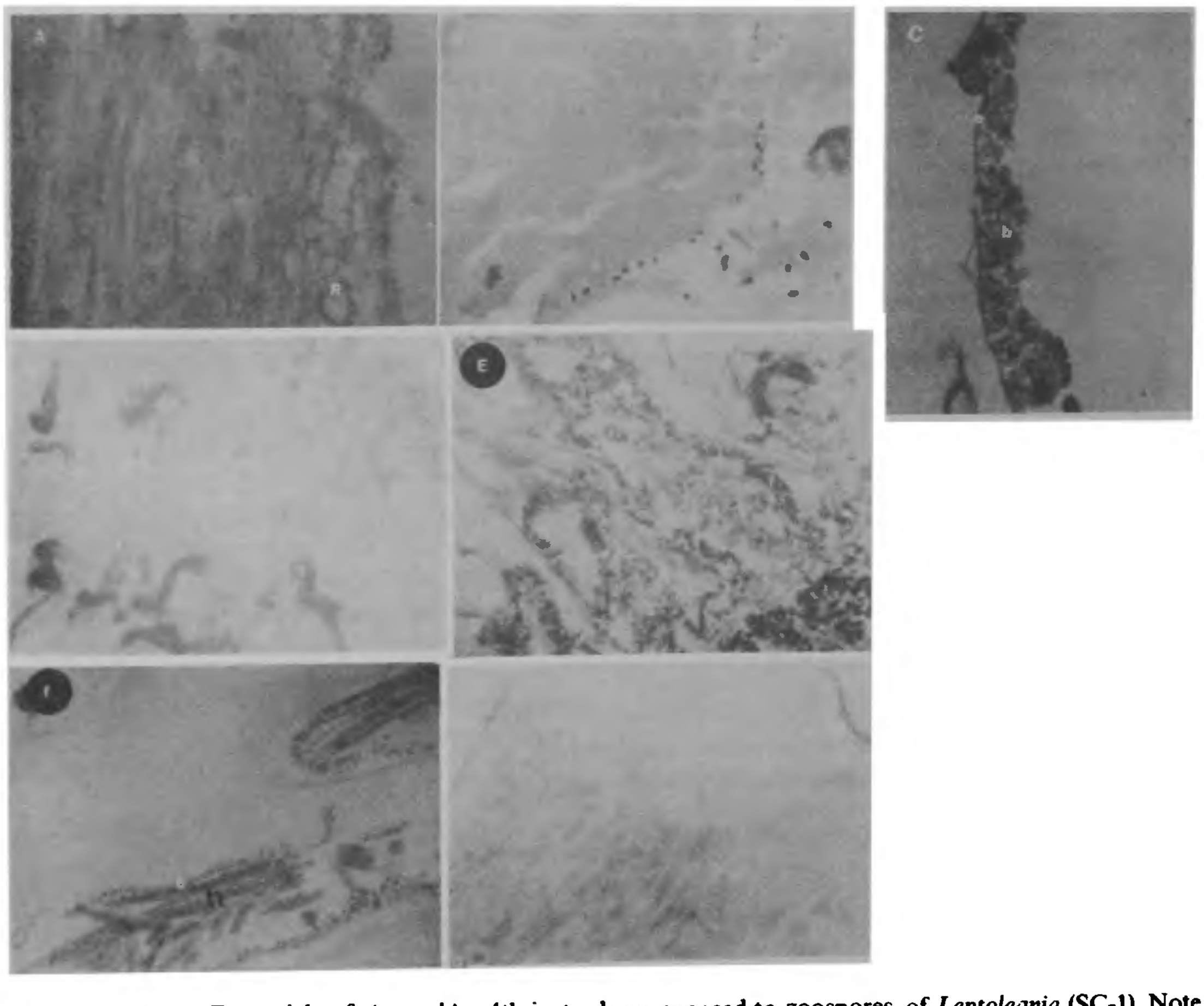
SEVERAL different types of fungi are being considered for use as biological control agents against vectors. These include: (i) the imperfect fungi *Culicinomyces clavosporus*, *Metarhizium anisopliae* and *Tolypocladium cylindrosporum*; (ii) fungi of the zygomycete group Entomophthoraceae; and (iii) aquatic fungi belonging to the genera *Coelomomyces Lagenidium*

and *Leptolegnia*[1]. The imperfect fungi and Entomophthoraceae reproduce and infect their hosts by non-motile conidia, whereas the aquatic fungi reproduce via motile zoospores that seek out hosts or other suitable substrates. *Leptolegnia* sp requires investigation for its potential in the biological control of mosquito larvae[2]. Although this new species was relatively easy to culture and manipulate *in vitro*, it was not considered sufficiently active to warrant further study as a mosquito larvicide, because it lost its larvicidal activity upon prolonged culture.

The objective and rationale of this study was twofold: (i) to report the medium used to enhance larvicidal activity of the fungus. *Leptolegnia*: (ii) to report the histopathological observations on the progress of infection of *Leptolegnia* sp in *Anopheles gambiae* larvae exposed to its zoospores in the laboratory.

Leptolegnia (SC-1) was cultured in Z medium (1 part Hemp seed extract, yeast glucose to 1 part wheat germ yeast glucose) on a water bath shaker for 24 hr at 28°C. Mycelia were collected, washed, zoospores produced



Figures A–G. **A.** Exocuticle of *A. gambiae* 4th instar larva exposed to zoospores of *Leptolegnia* (SC-1). Note route of entry (R) darkened from apparent host reaction to enzymatic action (× 320). **B.** Invasion of epidermis (e) by hyphae (big black dots) (t) 12 hr post exposure (× 80). **C.** Hyphal bodies (b) inhaemocoel of *A. gambiae*, below the epidermis (e) (× 320). **D.** A portion of larval mid gut of *A. gambiae* dissected from the host showing melanized germlings (g) invading the haemocoel (× 320). **E.** Extensive invasion of fatbody tissue by hyphae (f) 24–48 hr post exposure of *A. gambiae* larva in cross section (× 80). **F.** Extensive invasion of haemocoel by hyphae (h), fatbody (b) in longitudinal section of *A. gambiae* (× 320). **G.** Shows papillate Oogonia, the sexual stage in the fungus *Leptolegnia* (SC-1) in culture.

and counted on a haemocytometer per assay. Zoospores were added to sterile distilled water containing 100 each of second, third and fourth instar larvae of *A. gambiae* (which had been rinsed with 5% sodium hypochlorite) to a concentration of approximately 1×10^4 zoospores/ml. Samples of at least 4 larvae each were removed from the control and test larvae at 0, 1, 2, 3, 4, 5, 6, 9, 12, 15, 18, 24, 48, 72 hr after exposure to zoospores.

Larvae were fixed in 5% formal saline for 48 hr, dehydrated and embedded in paraffin wax which was serially sectioned at 5 μm thick in longitudinal and cross-section planes. These sections were stained with hematoxylin-eosin, mounted and photographed.

The subdivision of the cuticle and terminology are based on earlier studies[3]. The germination of zoospores was observed 6 hr after exposure. Zoospores attached and encysted on the cuticle and gut epithelium. The germ tubes passed directly into the epicuticle (figure A). The formation of appresoria was not observed but cannot be precluded. Evidence of direct penetration of the cuticle was observed 12 hr after exposure (figure B). Penetration of the cuticle by germ tubes was found to occur through the solid cuticle, pores of the sense organs and intersegmental areas. Areas surrounding the point of entry were darkened indicating lysis presumably due to enzymatic action (figure A). Lysis was observed in the epicuticle and exocuticle, but not in the endocuticle.

By 12 hr, the laterally branched hyphae penetrated the endocuticle. The hyphae grew parallel to endocuticular laminae. This agrees with the results reported on the penetration of *Nomuraea rileyi* through the cuticle of *Heliothis zea*[4]. Laterally-branched hyphae developed and penetrated the epidermis. The major route of infection was the gut (figure D) where many zoospores encysted on to the gut wall and germ tubes penetrated the gut wall to invade the haenocoele between 6 hr, 12 hr and 24 hr later. Hyphal bodies were formed by budding from pre-existing hyphae by obstruction of terminal pags (figure C). The hyphae bodies were short, thick, mostly 1, 2, or 3-celled filaments (figure C).

Blood cells were the first to be invaded and readily broken down. Hyphal penetration of the fat tissue started after 24 hr (figure E), and complete invasion occurred 24 hr later. By this time hyphal invasion occurred in the muscles and mesentrion (figure F). The larvae died between 24 and 72 hr after exposure to zoospores. There was no sign of infection in histological sections of the control larvae.

Prolonged culture of *Leptolegnia* sp on corn meal agar resulted in loss of larvicidal activity. This activity was enhanced when the fungus was transferred to a sterol-rich medium—hemp seed extract in Z medium. Previous studies indicated that sterol-rich media yielded 43850 zoospores more per ml than corn meal agar (1200 zoospores/ml, personal observations).

The mode of infection of most fungi pathogenic to insects, is usually through the cuticle. It was observed that this is achieved by a combination of mechanical and enzymatic action[4]. The results of this experiment with *Leptolegnia* (SC-1) showed that the cuticle was the minor route of infection for *A. gambiae*. The major route of infection was the insect midgut, where many zoospores cysted and germlings were found invading the haemocoele through the gut epithelium. The darkening of the germlings, hyphae and holes through which the invasion occurred was taken as evidence of some enzymatic action, *Culicinomyces* infects *C. fatigans* through the fore gut[5]. Empty zoospore cyst and the associated hypha penetrating wall of anal papilla of mosquito larva of *A. aegypti* has been reported[2]. A mass of zoospore cysts and germlings were also observed in the larval midgut[2].

The infection process was rapid. Many dead larvae were recorded 24 hr after exposure and 100% mortality was scored 72 hr after exposure. However, the extent of mycelial growth observed in the moribund and dead tissues was taken as evidence that this fungus kills by displacement of host tissues and possibly digestion of host haemolymph metabolites. *Lagenidium giganteum* kills the host larvae by digestion of haemolymph trehalose[6]. It is possible that the poor development of imaginary discs in the infected was due to the fungus competing with the host for nutrients.

The biological control potential of the fungus *Leptolegnia* has not yet been understood. It was earlier believed that the fungus lost its larvicidal activity upon prolonged culture on corn meal agar. It was observed that this drawback is easily overcome by growing the fungus on sterol-rich media which enhance zoospore production and pathogenicity. The information reported in this paper is merely to develop base line data as regards its larvicidal activity enhancement and its mode of infection. The fungus could establish itself in the environment as it is found growing profusely on the cadavers in the laboratory. *A. gambiae* was selected as test material because it is the major malaria vector in Uganda. The rapid growth of this fungus in the tissues of its host is an indication of the prospects of suppression of *A. gambiae* and therefore the reduction in the malaria incidence in the human population.

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A TECHNIQUE TO OBTAIN NAKED PUPAE OF RICE MOTH *CORCYRA CEPHALONICA* STAINTON

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RICE MOTH, *Corcyra cephalonica* Stainton is an important storage pest with a wide host range of food materials. As its culture can conveniently be maintained under laboratory conditions and reared continuously, it is being widely used as a laboratory host for multiplying some of the common parasites of insect pests. However, it is not popular as a test insect in entomology laboratories for physiological and toxicological studies as the larva passes its instars under dense gallaries and pupating in silken cocoon. This phenomenon forms a hurdle in determining the age of the insect. In the process of utilizing this insect for physiological studies, a simple technique was developed to prevent concealment and utilize pupae of known age.

Rearing Corcyra cephalonica

Adults were collected from the laboratory stock by using suction aspirator and transferred to breeding jars. Since *C. cephalonica* prefers to lay eggs on rough surface[1], a wire mesh was introduced at the bottom of the breeding jar for egg laying. The eggs laid on bottom wire mesh were automatically collected in a pan kept below the breeding jar.

Clean eggs, 0.1 cc (approximately 2,200 eggs[2]) were mixed with 750 g of sterilized (80°C for 3 hr) broken grains of sorghum, which is known to be superior to all other food materials for this insect[3]. Sorghum grains were fortified with 1% yeast powder to improve the nutritional quality[4]. The larvae of *C. cephalonica* were maintained in battery jars (15 × 20 cm) at 30 ± 2°C.

Preventing grain cocoon formation by pupa:

The method described for *Galleria mellonella*[5] was adopted with suitable modifications to prevent cocoons with grain and frass around pupae of *C. cephalonica*. Pieces of transparent teflon tubes, 2 cm long and 3 mm diameter, were kept in a petridish so that the entire petridish was covered. The size of the teflon tube is so fixed that its length is sufficient for pupation of a single larva and the internal diameter, a little more than that of mature larva, just sufficient to permit construction of a thin silken cocoon only. The mature larvae, dirty white in colour throughout the body[1], were separated from the culture jars and released in the petridishes containing teflon tubes for pupation. Teflon tube acted as an artificial gallery and the larvae pupated inside the tube in a very thin transperant silken cocoon in 3–4 days. The petridishes were closely observed regularly at short intervals under illumination and those tubes showing light yellowish brown pupae were separated. Due to transparency of thin cocoon and teflon tube, the pupation time could be easily fixed. The pupae with thin transparent silken cocoons were carefully removed from teflon tubes with the help of forceps.

Setting free the pupae from silken cocoons:

The technique described for setting free the pupae of great wax moth *G. mellonella*[6] from their thin cocoons could also be successfully utilized for *C. cephalonica*. Groups of 10–20 pupae (less than 24 hour old) with cocoons were immersed in 1 N NaoH solution with the help of a nylon sieve for 15–30 seconds to dissolve the thinnest parts of the silken cocoon. Then the pupae