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CULTIVATION OF SPIROPLASMA FROM SEASAMUM AFFECTED BY PHYLLODY IN CHICK EMBRYOS

N. SRINIVASAN & J.J. SOLOMON

Central Plantation Crops. Research Institute, Regional Station, Kayangulam
KRISHNAPURAM-690 533

ABSTRACT

A *spiroplasma* (helical mycoplasma) from the tissues of sesamum plants affected by phyllody disease was successfully cultured in embryonated hen's eggs. The organism adapted to the egg system and grew in the yolk and the allantoic fluid. It readily readapted from eges to as serum enriched cell-free culture medium. Helical, filamentous, pleomorphic bodies were constantly observed in allantoic fluids of inoculated eggs under electron microscope. The egg inoculations triggered faster adaptation of the mollicutes *in vitro*.

INTRODUCTION

Sesamum is an important oil-seed crop, cultivated in different parts of India. Phyllody is a serious disease of this crop. Mycoplasma-like organisms (Motlicuter) are associated with the phyllody affected sesamum plants, as several other plant diseases. Among the organisms with a helical morphology (*spiroplasmo*) have been successfully cultured and biologically characterized (Lee & Davis, 1986). Helical structures were observed by the present authors while examining phyllody affected sesamum tissues under an electron microscope. In a preliminary experiment the organism was tried to be cultured *in vitro*. As the nutritional requirements of mollicutes are exacting, chick embrpos have been reported to be an appropriate system for cultivating such fastidious organisms (Liao & Chen 1982). Hence the culturing of *spiroplasma* from phyllody affected sesamum plants in embryonated hen's eggs was attempted. This paper presents the findings of this study.

MATERIAL AND METHODS

Spiroplasma from sesamum phyllody plants was extracted aseptically into a serum enriched C-3 G liquid medium (Lee & Davis, 1986) and a diluted inoculum was prepared. Hatching eggs of white leghorn hens were obtained and embryonated (Blaskovic & Styk, 1967; Clark & Rorke, 1975). The exprerimen t as detaile are as follows :

The hatching eggs were incubated at 38-39°C (r.h. 55-60%) and were turned twice daily for symmetric development of embryos. Embryo development and viability

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inside the eggs were monitored in a candler and the fertile eggs were monitored in a candler and the fertile eggs were selected. The embryonated eggs were arranged in clean trays and their air sacs were delineated. The area of the shell over the air sac was surface sterilized with 70% ethanol. The eggs were inoculated with the spiroplasmal inoculum through yolk sac and the allantoic cavity with due aseptic precautions. Sixty embryonated eggs were inoculated in two replications with the extract of sesamum phyllody plants. Extracts from healthy sesamum plants were equally used as controls.

Eggs incubated for seven days were inoculated by the route of the yolk sac. For this pupose, a hole was drilled over the air space on top of each egg. The inoculum from diseased sesamum tissues (0.1 ml/egg) was introduced through the hole directly into the yolk sac using a hypodermic syringe filted with a No. 23 gauge. Eggs incubated for 10 days were inoculated by the route of allantoic cavity. For this purpose, a hole was drilled just above the base of the air sac over the position of the embryo in the egg. The inoculum (0.2 ml/egg) was introduced into the allantoic eavity below the air sac by inserting a hypodermic needle through the hole. The extracts from healthy sesamum tissues were similarly introduced into eggs by the routes of the yolk sac and the allantoic cavity.

The punctured sites of the eggs were swabbed with 70 per cent ethanol after inoculation and sealed with cellophane tape. The inoculated eggs were incubated at 32 ± 1°C for 10-12 days and periodically candled to see the viability of the embryos. After the desired incubation period, the eggs were chilled overnight in a refrigerator, the seal was removed and the air chamber area surface was sterilized. The egg shell over the air sac was chipped off, the shell membrane below the air sac and also the chorio-allantoic membrane underneath the shell membrane were carefully torn to expose the allantoic fluid. The allantoic fluid and yolk from the yolk sac of experimental eggs were aseptically harvested separately for further studies.

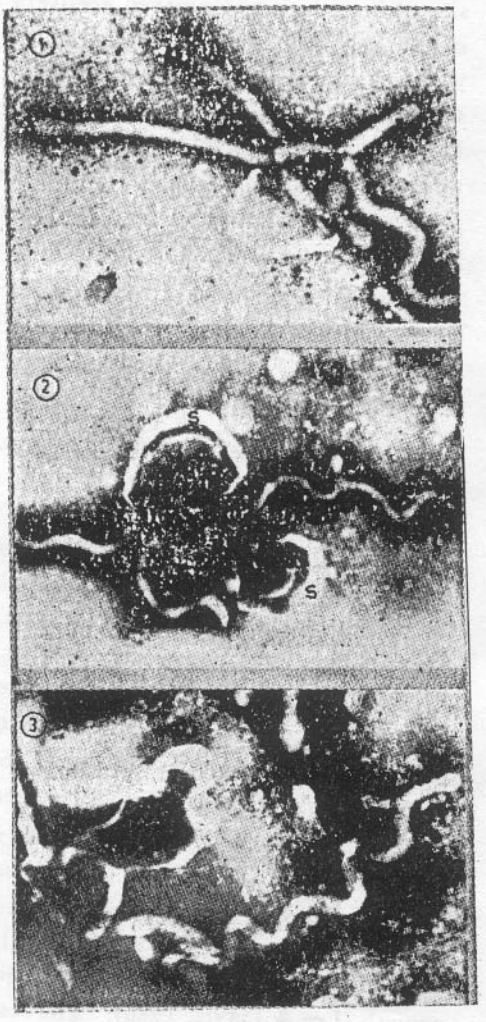
Samples of allantoic fluid were centrifuged at 18,000 rpm for 1 hr and the sediments suspended individually in two drops of sterile 1% ammonium acetate. A droplet of each of the suspension was transferred to carbon coated copper grids and stained with 5% ammonium molybdate and examined the electron microscope. Simultaneously, alliquots of allantoic fluid and yolk were cross inoculated into the serum enriched C-3G liquid medium, incubated at 30 ± 1°C and monitored for the growth of the organism *in vitro*. The liquid culture samples were also centrifuged and the sediments exomined electron microscopically.

RESULTS & DISCUSSION

The spiroplasma from phylloid sesamum was cultivable in the embroyonated eggs. Electron microscopy of the allantoic fluid collected from several of the inoculated eggs showed numerous pleomorphic bodies and depicted cells haging a variety of morphological forms (Fig. 1). The pelletted egg samples contained elementary bodies, round or irregularly shaped small to large main bodies from which slender filaments with unit membrane were connected. Branched filaments were also observed. Most of the filaments and their branches had bulbous ends and sacklike blebs (Fig. 2). Among cellular bodies the spiral form of filaments were invariably observed confirming the spiroplasmal nature of the pleomorphic organism (Fig. 3). The differential cellular morphology of the organism appeared to be due to its growth in embryonated eggs. Changes occur in cellular morphology during growth and reproduction of *Spiroplasma* (Fudl-Allah & Calavan, 1973). The helical, filamentous, pleomorphic organism observed in inoculated chicken eggs resembled the *Spiroplasma* isolated by the authors in a preliminary experiment from the phylloid sesamum tissues into a cell-free medium.

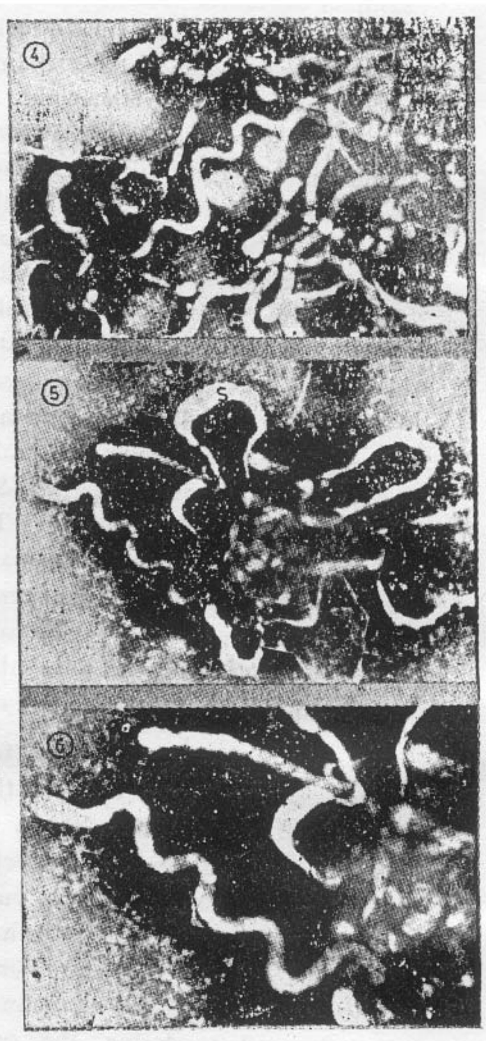
Yolk and allantoic fluid from inoculated eggs frequently changed the colour of the liquid medium from red to yellow accompanied by turbidity The changes were faster than that by direct culturing of *spiroplasma* from plant tissues into medium. It is likely that in the *in vitro* system of embryonated eggs the adaptive mechanism is triggered rendering the organism to readily adapt to grow in the culture medium. Similar instance of egg adaptation of fastidious spiroplasma and later their better growth in a culture medium has been reported (Stiller *et al.*, 1981; Tully *et al.*, 1981). Yolk and allantoic fluid from eggs inoculated by either of the two routes, developed growth in the medium confirming internal movement of *spiroplasma* in eggs from the site of inoculation. However, recovery of the oaganism from egg yolk (inoculated by the yolk sac route) was more frequent and yielded strong turbidity in the liquid medium. Liquid cultures developed from yolk and allantoic fluid also showed under the electron microscope pleomorphic, helical organisms (Figs. 4-6). In the culture medium, inoculated with samples from control eggs no comparable changes were of served and no organism could be observed under the electron microscope.

Chick embryo inoculation was first reported for the isolation of a fastidious *spiroplasma* from SMCA in ticks (TULTY *et al*, 1976). Plant pathogenic, flower and honey bee associated spiroplasmas have since been cultured in embryonated chick eggs (Liao & Chen, 1982; Lee & Davis 1986). Nienhaus *et al.* (1978) reported cultivability of plant pathogenic Rickettsia like organisms in chick embryos. Spiroplasmas of ticks, flowers and honey-bees were pathogenic to chick embryos (Tully *et al.*, 1976; ROSE *et al.*, 1979). In contrast, the plant pathogenic spiroplasmas did not exert pathogenicity to chicken eggs and the inoculated chicks remained viable despite their growth (Lee & Davis, 1986). The sessamum phyllody associated *spiroplasma* also did not exert any obvious pathogenicity to eggs and the inoculated chicks remained viable. Recovery of the organism from yolk sac indicates a unique role which the egg yolk might play for culturing of such fastidious organisms. Chang & Impson (1987) found egg yolk etract substituting serum for spiro-



Figs. 1-3 : Election micrographs showing spiroplasma from allantoic fluid of eggs inoculated with extract of phyllody affected sesamum plants (x40,000).

1. A colony of the pleomorphic bodies.
2. Branching filament of the organism with bulbous end (B) and sack like blebs (S).
3. Typical spiral filament of the organism appeared in allantoic fluid.



Figs. 4-6 : Electron micrographs showing sesamum phyllody associated spirplasma in serum enriched culture medium, adopted to grow fron eggs.

4. Colonies of pl:omorphic bodies sedimented from culture (x24,000).
5. Young cell of *Spiroplasma* from culture Note spiral filament and sack like blebs (x40,000).
6. Enlarged view of this typical spiral filament (x80 000).

plasmal growth *in vitro* and suggessed egg yolk to be a nutritional factor for culturing spiroplasmas. Our observations omisolation of *spiroplasma* from sesamum phyllody affected plant tissues by the chick embry model system broadly strengthens the usefulness of the technique and form the first report from India on this line. There is no evidence of plant mycoplasmas (other than spiroplasmas) being tried or reported to be cultivated in chick embroys. However the chick embryos offer scope for cultnring fastidious prokaryotes (Mollicutes).

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