ISOLATION, CULTIVATION AND CHARACTERIZATION OF MYCOPLASMA-LIKE ORGANISMS FROM PLANTS

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Mycoplasma-like organism from plants affected with citrus greening disease was cultured on artificial media. PPLO agar medium supplemented with culture filtrate of *X. malvacearum*, horse serum; RNA, DNA or cholesterol supported better growth than on media with plant or soil extract. The organism could grow, in media with *p*H ranging from 6.0 to 7.8 and at temperatures from 25 to 35°C; best growth was obtained in alkaline *p*H and at 30° ± 1°C. Addition of carbohydrates in the medium did not improve growth. Growth of the organism was inhibited by tetracycline antibiotics at 10 ppm; benlate and penicillin were inhibitory at 200 and 250 ppm respectively.

INTRODUCTION

Electron microscopy and tetracycline therapy are two main evidences presented for the mycoplasmal etiology of most of the yellows type of plant diseases. For proper identification and classification of these disease agents, it is essential to culture these organisms and transmit them to susceptible plants. Several workers have attempted cultivation of mycoplasma-like organisms from plants but only a few have reported success (Hampton *et al.* 1969; Chen and Granados, 1970; Faivre-Amiot *et al.* 1970; Lin *et al.* 1970; Lombardo and Pignattelli 1970; Ghosh *et al.*, 1971; Gianotti and Vago 1971; Saglio *et al.* 1971; Fudl-Allah *et al.* 1972). Here we are reporting the behaviour of citrus greening mycoplasma when cultured under various conditions.

MATERIALS AND METHODS

Organisms were cultured as described by Ghosh *et al.* (1971) and maintained in PPLO Broth supplemented with 2 per cent horse/rabbit serum.

Inoculated plates were incubated in moist chamber (desiccator) at 30°C. Plates were observed for at least two weeks. Plant extracts, soil extracts and bacterial cultural filtrates were sterilized by filtration and then incorporated in media.

RESULTS AND DISCUSSION

Effect of media—A number of media (Table I) were tested for isolation and cultivation of mycoplasma from plants affected with citrus greening. Recognisable mycoplasma-like colonies were obtained only on Morton's PPLO medium. Growth of the organisms was studied on PPLO agar medium supplemented with plant sap, soil extract or bacterial cultural filtrate (Table II). No growth was obtained in media containing plant sap extracted in solvent ether, chloroform or

TABLE I

Media used for the isolation of mycoplasma

I. Media for the isolation of bacteria (Pelczar *et al.* 1951)
   (*a*) Nutrient Agar/Broth
   (*b*) Nutrient Dextrose Agar/Broth
   (*c*) Nutrient Sucrose Agar/Broth

II. Media for the plant tissue culture
   (*a*) White's Medium (White, 1963)
   (*b*) Murashige and Skoogs' Medium (Murashige and Skoog, 1962)
   (*c*) Above medium supplemented with coconut milk

III. Mycoplasma media (Morton *et al.* 1951)
   (*a*) PPLO Agar/Broth (Difco) + serum
   (*b*) PPLO Agar/Broth (Difco) + serum + yeast extract

TABLE II

Effect of plant extract (PE), soil extract and bacterial culture filtrate on the growth of the organism

S.No.	PPLO agar medium supplemented with	Growth of the organism	
1.	PE* in cold water	++	Ill-developed colonies
2.	PE in cold phosphate buffer (*p*H 7.0)	++	-do-
3.	PE in solvent ether	—	
4.	PE in chloroform	—	
5.	PE in ether + chloroform	—	
6.	Soil extract in cold water	+	A few colonies developed
7.	Culture filtrate of *X. malvacearum*	++	Nice colonies developed
8.	Horse serum	++	-do-
9.	RNA (0.02%)	++	-do-
10.	DNA (0.02%)	++	-do-
11.	RNA + DNA	++	-do-
12.	Cholesterol (0.01%)	++	-do-
13.	Cholesterol + RNA	++	-do-
14.	Cholesterol + DNA	++	-do-
15.	Cholesterol + RNA + DNA	++	-do-
16.	Control (PPLO agar)	+	Only a few ill-developed colonies appeared

*PE: Plant extract

mixture of ether and chloroform. Distinct colonies were developed on media containing plant sap extracted in cold water or phosphate buffer or soil extract. Good colonies were obtained when the media contained seven days old bacterial (*Xanthomonas malvacearum*) cultural filtrate. In medium supplemented with cholesterol nice colonies developed.

TABLE III

Effect of different carbohydrates on growth of the organism

PPLO agar medium supplemented with serum and 1% carbohydrate	Growth of the organism
Ribose	++ Well-developed colonies
Glucose	++ –do–
Fructose	++ –do–
Mannose	+ Colonies not well-developed
Sucrose	++ –do–
Rhamnose	+ –do–
Sorbitol	+ –do–
Mannitol	+ –do–
Cellubiose	+ –do–
No additional carbohydrate	++ Well-developed colonies

TABLE IV

Effect of different chemicals on growth of the organism

Concentration of chemicals	Penicillin (units/ml)	Achromycin (ppm)	Terramycin (ppm)	Aureomycin (ppm)	Ledermycin (ppm)	Benlate (ppm)
1	+	+	—	—	+	+
5	+	+	—	+	—	+
10	+	—	—	—	—	+
25	+	—	—	—	—	+
100	+	—	—	—	—	+
125	+	—	—	—	—	+
200	+	—	—	—	—	?
250	?	—	—	—	—	?
500	—	—	—	—	—	—

— No growth; + Growth; ? Doubtful growth

*Effect of p*H—The organisms could grow in media with *p*H ranging from 6.0 to 7.8, but better growth was obtained in alkaline *p*H. This is expected as the phloem sap is generally alkaline (Street and Opik 1970). Fudl-Allah and Calavan (1973), however, reported optimum growth of the citrus stubborn mycoplasma at neutral *p*H.

Effect of temperature—Cultural plates were incubated just after inoculation at various temperatures ranging from 0° to 40°C. The organism could grow from 25° to 35°C but best colonies developed at 30°±1°C. At 35°C colonies remain restricted

and at 40°C no colony developed. The temperature requirement of citrus stubborn mycoplasma-like organism isolated by Fudl-Allah and Calavan (1973) is also similar (Fudl-Allah *et al.* 1972), although *Spiroplasma citri* grows best at 32°C (Freundt 1973).

Effect of carbohydrates—Organism was grown in medium supplemented with various carbohydrates to find out the effect of these compounds on the growth of the organism. None of the carbohydrates provided an advantage for development of colonies. Colonies in media containing glucose, fructose, ribose and sucrose were similar to that of control whereas those containing other carbohydrates, supported poorly developed colonies (Table III).

Effect of antibiotics—Various antibiotics (Table IV) and a systemic fungicide, benlate, which was found to be effective against sandal spike disease (Raychaudhuri *et al.*, 1972) were incorporated into the medium to test their sensitivity *in vitro*. At 10 ppm and above all the four tetracyclines inhibited the growth of the organism. Benlate and penicillin were inhibitory at concentrations 200 and 250 ppm respectively. These inhibitions are difficult to explain although some mycoplasma species are known to be inhibited by penicillin even at a very low concentration (Taylor-Robinson 1967).

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References

Chen, T. A., and Granados, R. R. (1970). Plant pathogenic mycoplasma : *in vitro* maintenance and transmission to *Zea mays* L. *Science*, **167**, 1633–1636.

Faivre-Amiot, A., Moreau, J. P., Cousin, M. T., and Staron, T. (1970). Essai de mise en culture de l'agent de la Phylloidie du Trèfle. *Ann. Phytopathol.*, **2**, 251–258.

Ghosh, S. K., Raychaudhuri, S. P., Varma, A., and Nariani, T. K. (1971). Isolation and culture of mycoplasma associated with citrus greening disease. *Curr. Sci.*, **40**, 299–300.

Giannotti, J., and Vago, C. (1971). Role des mycoplasmes dans l'étiologie de la phylloidie du Trèfle : Culture et transmission expérimentale de la maladie. *Physiol. Veg.*, **9**, 541–553.

Fudl-Allah, A. E., Calavan, E. C., and Igwegbe, E. C. K. (1972). Culture of a mycoplasma-like organism associated with stubborn disease of citrus. *Phytopathology*, **62**, 729–731.

Fudl-Allah, A. E., and Calavan, E. C. (1973). Effect of temperature and *p*H on growth *in vitro* of a mycoplasma-like organism associated with stubborn disease of citrus. *Phytopathology*, **63**, 256–259.

Hampton, R. O., Stevens, J. O., and Allen, T. C. (1969). Mechanically transmissible mycoplasma from naturally infected peas. *Pl. Dis. Reptr.*, **53**, 499–503.

Hottle, G. A., and Wright, D. N. (1966). Growth and survival of *Mycoplasma neurolyticum* in liquid media. *J. Bacteriol.*, **91**, 1834–1839.

Lin, S. C., Lee, C. S., and Chin, R. J. (1970). Isolation and cultivation of, and inoculation with a mycoplasma causing white leaf disease of sugarcane. *Phytopathology*, **60**, 795–797.

Lombardo, G., and Pignattelli, P. (1970). Cultivation in a cell-free medium of a mycoplasma-like organism from *Vinca rosea* with phyllody symptoms of the flowers. *Ann. Microbiol.*, **20**, 83–88

Morton, H. E., Smith, P. F., and Leberman P. R. (1951). Investigation of the cultivation of pleuro-pneumonia-like organisms from man. *Amer. J. Syph.*, **35**, 361.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, **15**, 473–497.

Pelczar, M. J. (1951). General Bacteriology Lab. Exercises. Burgess Publishing Co. Minnesota.

Raychaudhuri, S. P., Chenulu, V. V., Ghosh, S. K., Varma, A., Rao, P. S., Srimathi, R. A., and Nag, K. C. (1972). Chemical control of spike disease of sandal. *Curr. Sci.*, **41**, 72–73.

Saglio, P., Laflèche, D., Bonissol, C., and Bové, J. M. (1971). Culture *in vitro* des mycoplasmes, associés au 'Stuborn' des agrumes et leur observation au microscope électronique. *C. R. Hebd. Seances Acad. Sci.*, Ser. D, **272**, 1387–1390.

Street, H. E., and Opik, H. (1970). The physiology of flowering plants : their growth and development. E. Arnold Ltd. London pp. 263.

Taylor-Robinson, D. (1967). Mycoplasmas of various hosts and their antibiotic sensitivities. *Postgrad. Med. J.*, **43**, Suppl., 100–104.

White, P. R. (1963). The cultivation of animal and plant cells. Ronald Press, N. Y.