STUDIES ON NECTRIA GALLIGENA BRES.

Part II. Bacterial Association

A.J. Al-Zarari

Department of Plant Protection, College of Agriculture and Forestry,
University of Mosul, Hammam Al-Alil, Iraq

(Received October 30, 1977; revised March 22, 1978)

Abstract. Lytic phenomenon in the fungus *Nectria galligena* Bres. caused by bacteria has been observed for the first time. Various media have been used for the isolation of the fungus and bacteria in pure culture. It was observed that the bacteria lived in close association with the fungus and was difficult to separate.

INTRODUCTION

During a study of different aspects of the fungus *Nectria galligena* Bres., fungal hyphae and spores lysis was observed. This lysis was suspected to be due to unidentified bacteria which was constantly found with different fungus cultures obtained from different sources. Lahoz et al. [4] observed fungal lysis and attributed it to autolytic changes. No work appears to have been done on bacteria-fungus lysis relationship as yet. However, there are several reports on the autolysis of *Aspergillus* species[1,3,6].

MATERIAL AND METHODS

To obtain pure cultures of the fungus the following media were used:

(a) Malt agar with different concentration of Chloramphenicol. Fifty mg of chloramphenicol was dissolved in 100 ml distilled water. 33.6 g of malt agar (Difco) was dehydrated and dissolved in 900 ml distilled water. Both solutions were mixed well and heated at 100°C for 20 min to melt the agar. The hot medium was dispensed into 20 ml quantities in universal bottles, autoclaved at 15 psi for 20 min, cooled to 55°C and poured aseptically into plates. The same procedure was repeated with other concentrations i.e. 100 mg and 500 mg/litre.

Preparation of the Inoculum. Perithecia were taken from fresh shoots of diseased apple trees, washed ten times with sterile distilled water, then crushed into 2 ml sterile distilled water to obtain ascospore supension. Five plates of each concentration were inoculated with spore suspension (streaking method). After 24 hr incubation (20°C), hyphal tips were removed and plated out on 5 plates of MA for each concentration; each with one tip. This procedure was repeated three times; each after 48 hr of incubation at 20°C. The first two were on MA with chloramphenicol but the third time it was on MA alone. The fungus after one week of incubation at 20° C was examined microscopically for all treatments.

(b) *Malt agar with streptomycin.* The previous procedure was repeated and only chloramphenicol was replaced by 10 mg streptomycin, added aseptically to the autoclaved, melted and cooled medium.

(c)*Malt agar with streptomycin and chloramphenicol.* The same procedure as in previous experiments but with a mixture of 100 mg each of the streptomycin and chloramphenicol.

(d) *OEAS Medium.* The fungus was inoculated on OEAS medium, which has been reported to inhibit the bacterial growth as it contains very high doses of both streptomycin and chloramphenicol (500 mg). The fungus was retransferred after one week of incubation each time and incubated at 20°C for 1 week for each, then examined under the microscope.

(e) *Inoculation of Bramely apple fruits.* Five apple fruits were inoculated aseptically with 0.5 cm core taken from the colony of *Nectria galligena* grown on MA for 10 days. The inoculation was done by removing a piece of the same size from the apple fruit and replacing it by the fungus growth, and covering. Inoculated apples were put in polythene bags to keep the moisture constant and were incubated at 20°C for 15 days. The fungus was then transferred from the deep flesh of the diseased apple fruits to five petridishes with MA medium and incubated for 1 week at 20°C. The mycelium of all plates was examined under the microscope.

To obtain pure cultures of the bacteria the following media were used:

(a) Cultures were inoculated into nutrient broth

Table 1. Growth of the fungus, bacteria and lysis in different media.

Characters	MA with chloramphenicol			MA with streptomycin	MA with chloramphenicol and streptomycin	OEAS medium with chloramphenicol and streptomycin
	50 mg/l	100 mg/l	500 mg/l	100 mg/l	100 mg/l each	500 mg/l
Growth of fungus	+	+	+	+	+	+
Growth of bacteria	+	+	+	+	+	+
Lysis of fungus	+	+	+	+	+	+

medium in McCartney bottles and incubated at 20°C for 1 week. The medium was prepared according to manufacturer's directions (Difco). From these cultures streaking was done on two media namely nutrient broth agar and nutrient broth agar with 2% dextrose. Plates were incubated at 20°C and checking for bacterial growth was done every day.

(b) Bacterial medium with Actidione. Nutrient agar medium was prepared according to the manufacturer's directions (Difco). 0.2 ml of 1% actidione was added to the melted, cooled medium, then dispersed into plates aseptically. Five plates were streaked with fungus suspension grown on nutrient broth for 1 week, and then incubated at 20° C for 5 days.

RESULTS AND DISCUSSION

All the inoculated plates showed the presence of living bacteria with the fungus. Although chloramphenicol is reported to inhibit bacterial growth at such low doses as 35 mg/l, it had no inhibitory effect even at such high doses as 500 mg/l. Results with other antibiotics alone and in combination had also given similar results (Table 1).

It seems likely that bacteria may prefer to live on the fungus than on the media and may derive its nourishment for growth and metabolism from the fungus alone or the fungus may protect the bacteria by releasing chemicals which inactivate the antibiotics. This aspect, however, needs further research and evidence before a definite conclusion can be reached. Similar results were obtained when the fungus was inoculated in Bramely apple fruit. Inspite of the fact that apples contain malic acid, which is reported to inhibit bacterial growth it failed to stop its growth. This illustrates that bacteria live in close association with the fungus and is dependent on it for its growth.

It was also observed that all the cultures of the fungus showed lysis during the period of their growth. It is assumed that the lysis may be the result of bacterial association with the fungus and not due to autolytic changes as reported by Lahoz *et al.* (Figs. 1 and 2).

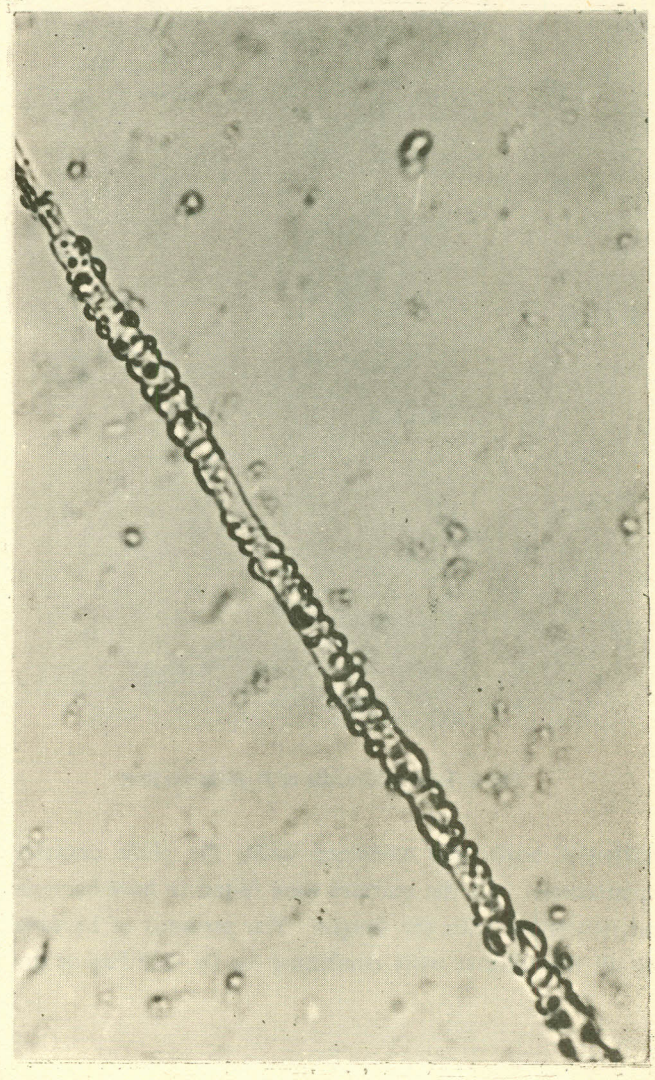

Fig. 1. Lysis of hypha of *N. galligena* Bres.

To gather more evidence, cultures of the same fungus were obtained from different parts of the world and from

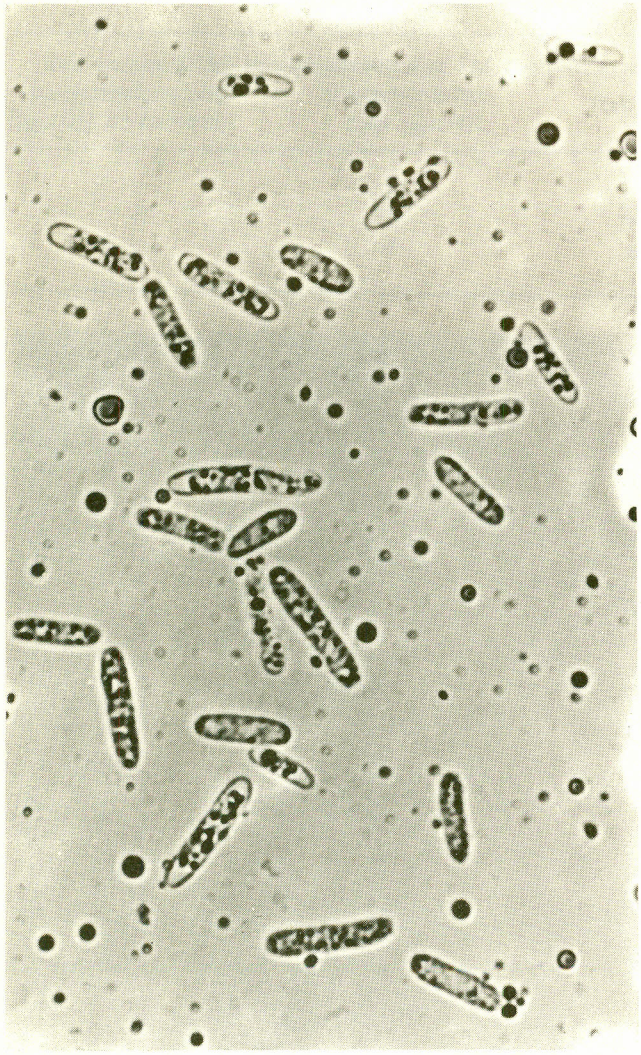

Fig. 2. Lysis of Conidia of *N. galligena* Bres

different hosts, and examined under the phase contrast microscope. All the cultures were found to have bacteria in association with the fungus. The presence of bacteria in all the cultures were confirmed by Dr. G.F.Peg of the Department of Biological Sciences and Dr. E.H. Wilkinson and Dr. W.W.Schwabe of the Department of Horticulture, Wye College, University of London (personal communication).

Attempts to grow and isolate the bacteria on NBA, and NBA with 2% dextrose failed to show any growth. Further cultures were made on bacterial medium containing Actidione. No fungal or bacterial growth on any of the plates was observed. This provides further evidence that bacterium prefers the fungus to the NBA medium, which is recommended media for bacteria and as the fungus was killed by the Actidione the bacteria died too. This suggests that bacteria is closely associated with the fungus for its nutrition and growth. More work is needed in this direction for isolating the bacteria in pure culture by using different media and environmental conditions. So also it is pertinent to locate the position of the bacteria, whether it is carried extracellularly or intracellularly by the spore or the fungus mycelium.

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