

COMPARATIVE STUDY OF THE HISTOCHEMISTRY
AND PHYLLOSPHERE MICROFLORA
OF CERTAIN LEAF DISEASE

BY

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The nature of causal agents of little leaf disease of brinjal, red varenda and chili was investigated using histochemical techniques. Dienes' staining reaction of brinjal leaf, which is known to be caused by mycoplasma, was compared with that of little leaf diseases of red varenda and chili. Infected phloem tissues of brinjal and red varenda but not of chili, showed positive reaction to Dienes' reagent. Phloem tissues, especially the sieve elements, of infected brinjal and red varenda, but not that of chili plants, exhibited bright-orange-yellow fluorescence when treated with fluorochrome aniline blue. The results suggested mycoplasma nature of the causal agent of little leaf of brinjal and red varenda plants but not of chili.

The population distribution pattern of phyllosphere microflora of the infected and healthy plants were compared. The populations of microorganism including the free-living nitrogen fixers were significantly more on the phylloplane of infected than in the healthy plants. Leaves of all the three infected plants leached out significantly more amount of intracellular electrolytes than that of the healthy plants.

INTRODUCTION

In the recent years certain histochemical techniques have been developed which can effectively and quickly differentiate mycoplasma or virus nature of plant diseases showing symptoms of yellowing, witches, broom, or reduction of leaf lamina (Maramorosch, 1974). Dienes' staining (Hayflick, 1967) originally applied for mycoplasma infected animal tissue has been successfully used for identification of mycoplasma infected plants (Dealey *et al.*, 1979; Ghosh *et al.*, 1974; Raju *et al.*, 1981). Hiruki and Shukla (1973) demonstrated that mycoplasma infected phloem tissue exhibited strong fluorescence in presence of fluorochrome aniline blue. Little leaf diseases of brinjal (*Solanum melangena* L.), red varenda (*Jatropha gossypifolia* L.) and chili (*Capsicum annum* L.) are of widespread occurrence in and around Kalyani. Although the little leaf of brinjal is known to be caused by mycoplasma (Verma *et al.*, 1969), the nature of the casual agent of little leaf of red varenda and chili is not known.

Virus infection of plants results in increased leaching of intracellular metabolites from the leaves and consequently such leaves harbour more phyllosphere microorganisms (Tukey *et al.*, 1958; Beute and Lockwood, 1967; Preece and Dickinson, 1971). Little is known regarding the phyllosphere microflora and leaching of metabolites from mycoplasma infected leaves.

The objective of this investigation was to ascertain the mycoplasma or viral nature of the casual agents of little leaf of red varenda and chili as compared to little leaf of brinjal. An attempt was also made to compare the distribution pattern of phyllosphere microflora and leakage of electrolytes from the healthy and infected leaf samples.

MATERIALS AND METHODS

Healthy and naturally infected little leaf symptoms bearing plants of brinjal, and red varenda and chili were used. Brinjal and chilli plants were collected from farmer's field and red varenda plants were obtained from Kalyani University campus.

The procedure of Deely *et al.* (1979) was followed for Dienes' staining of the petioles of healthy and infected leaves. Sections were stained with 0.2% Dienes' reagent for 5 to 6 minutes. For fluorescent microscopy the sections of petioles were treated with 0.1% aqueous solution of fluorochrome aniline blue as described by Hiruki and Shukla (1973). The fluorescence of the treated section was monitored by using a Carl Zeiss (East Germany) model No. 10001-R fluorescent microscope.

Phyllosphere microflora of healthy and infected leaf samples were isolated and enumerated following the method of Dickinson and Preece (1976). Leaf washings were serially diluted with sterilized distilled water and plated on Nutrient agar (NA), Potato dextrose agar (PDA) and Thronton's agar (TA) media for bacteria, fungi and actinomycetes respectively. For each dilution replicate plates were incubated at 37°C (for NA medium) and 28°C (for PDA and TA media). Population of microorganism were expressed as number/g of fresh weight of tissue.

Leaf leachates were collected following the method of Tukey *et al.* (1959). Electrolyte leakage from healthy and infected leaves were determined by measuring the electrical conductivity of the ambient solution of the leaves floated on glass distilled water following the method of Samaddar and Scheffer (1968). The specific conductance of the leachates was measured with a conductivity bridge (Philips Model PR 9500) using a dip type electrode cell ($K=1.25$).

RESULTS

Histopathology by Dienes' staining: The infected plants of three species produced numerous little leaves instead of healthy leaves of usual dimension. The infected

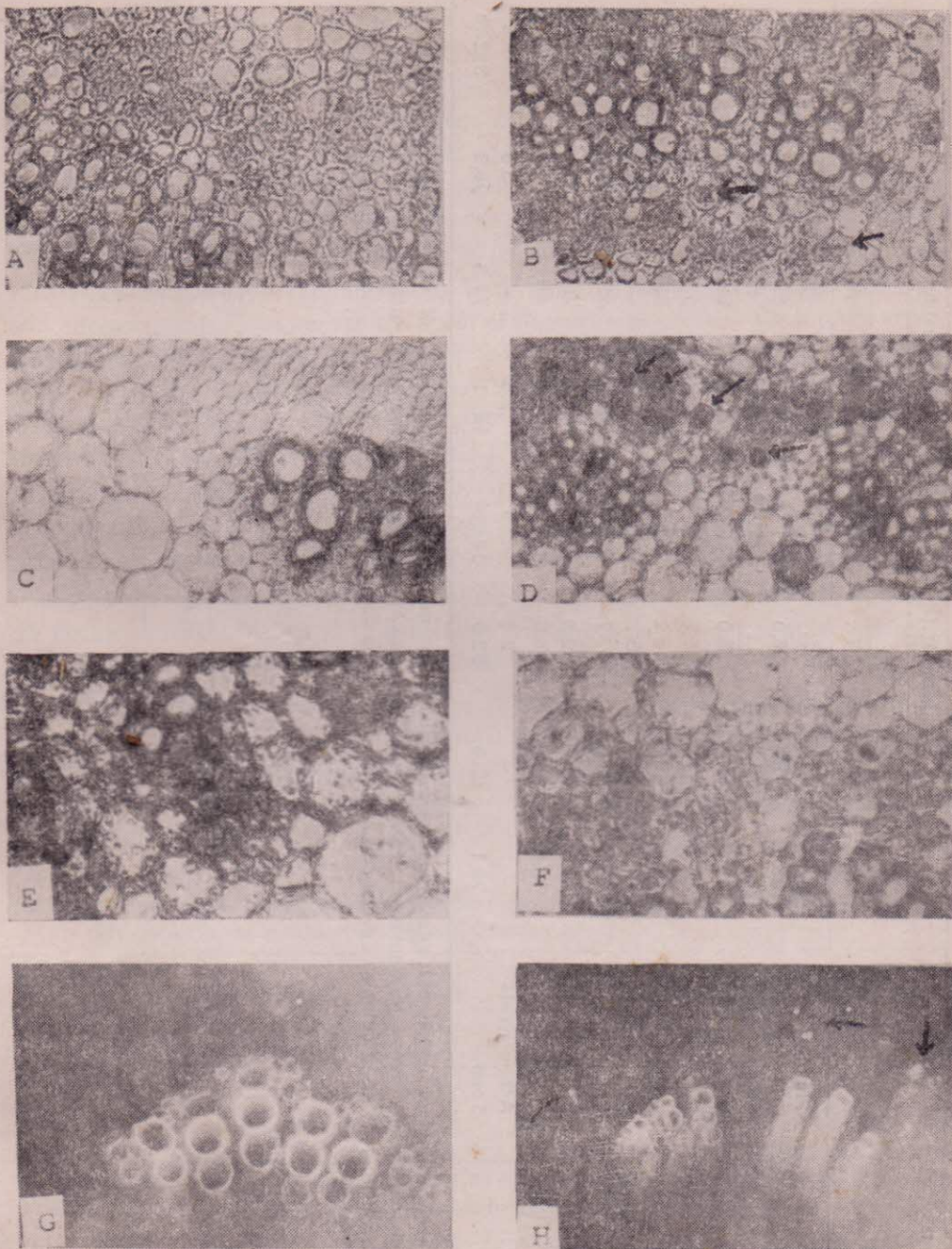


Fig. 1. Photomicrographs (A to F) and Fluorescent micrographs (G, H) of little leaf disease. A=Health brinjal ; B=Infected brinjal ; C=Healthy red varenda ; D=Infected red varenda ; E=Healthy chili ; F=Infected chili ; G=Healthy red varenda ; H= Infected red varenda.

Table 1 : Comparison of population dynamics of phyllosphere microorganism of healthy and little leaf infected plants

Test Plants	Type of Organisms	Number ($\times 10^3$)/g of fresh weight of leaf on media ^a											
		Healthy						Infected					
		NA	PDA	TA	Total	NA	PDA	TA	Total				
<i>Brinjal</i>	Fungi	—	2.5	—	2.5	3.8	8.8	2.5	15.1				
	Bacteria	105	18.1	35.0	1581	237.5	87.5	125.6	450.6				
	Actinomycete	—	—	—	—	2.5	—	2.5	5.0				
	Total	105	20.6	35.0	160.6 ^b	243.8	96.3	130.6	470.7 ^b				
<i>Red Varenda</i>	Fungi	—	12.5	12.5	25	—	25	12.5	37.5				
	Bacteria	212.5	46.3	66.2	325	247.2	68.6	121.2	437				
	Actinomycete	12.5	—	25	37.5	—	—	25	25				
	Total	215	58.8	103.7	387.5 ^b	247.2	93.6	158.7	499.5 ^b				
<i>Chili</i>	Fungi	2.5	20.3	2.5	25.3	6.3	26.3	5	37.6				
	Bacteria	70.6	35.6	48.8	155	156.3	45	87.5	288.8				
	Actinomycete	—	—	2.5	2.5	2.5	—	3.8	6.3				
	Total	73.1	55.9	53.8	182.8 ^b	16.1	71.3	96.3	332.7 ^b				

a. Leaf washings of healthy and infected leaves were planted on nutrient media and the No. of colonies of microorganisms was determined; NA = Nutrient Agar; PDA = Potato Dextrose Agar; and TA = Thronon's Agar.

b. Grand total of No. of colonies developed on all the test media combined.

leaves of brinjal were reduced to 1/30 or 1/50 of the healthy leaves and were leathery and crinckled. The infected leaves of red vareda and chili were reduced to 1/10 and 1/20 times of the healthy leaves and were papery and chlorotic in colour. Free hand sections of the petioles of healthy and infected leaf samples were treated with Dienes' reagent and observed under a light microscope. It is apparent (Fig. 1, A-F) that bluish black bodies were present in the phloem region of brinjal and red vareda. No such inclusion bodies could be detected in the phloem of infected plant of chili or the healthy plants of the test plant species.

Fluorescent microscopy—The fluorescent behaviour of phloem tissues of healthy and infected plants following the treatment of fluorochrom aniline blue as compared to that of healthy plants was investigated. The phloem cell elements of little leaf infected brinjal and red vareda plants showed strong yellow orange fluorescence whereas, phloem elements of all healthy and little leaf infected chili plants showed little or no such fluorescent (Fig. 1 G, W).

Phyllosphere microflora of healthy and infected plants—The quantitative and qualitative distribution patterns of phyllosphere microflora on healthy and infected plants were examined. It is apparent that the total microflora per unit weight of leaf tissues were significantly more on the phyllosphere of infected leaves than the healthy leaves (Table 1). Furthermore, bacteria were the dominant microflora on the phyllosphere of healthy or infected nature of leaves.

In a separate series of experiments the population density of phyllosphere N_2 -fixing microorganisms on the healthy and infected leaves were compared. It is evident that (Table 2) the population of N_2 fixing organism capable of growing in Burks' Agar medium was significantly more on the phyllosphere of infected leaves than healthy leaves.

Table 2. Comparison of population density of phyllosphere inhabiting N_2 -fixing microorganisms on the leaves of healthy and 'little leaf' infected plants

Test plants	Number ($\times 10^3$) of N_2 -fixers ^a / g of fresh wt of leaf of	
	Healthy	Infected
Brinjal	2.5	17.5
Red vareda	6.3	31.2
Chili	0.8	5.0

a. leaf washings from healthy and infected leaves were plated on Nitrogen-free Burks' Agar Medium and the colonies developed were considered as N_2 -fixers.

Leakage of intracellular metabolites—Leakage of intracellular materials as measured by loss of electrolytes from the infected and healthy leaves was determined. The results were expressed as specific conductance of leachates. It is apparent (Table 3)

that the infected leaf samples leached out more electrolytes irrespective of mycoplasma or other type of causal agents.

Table 3. Comparison of specific conductance of leachates from the leaves of healthy and 'little-leaf' infected plants

Test plants	Time intervals (hr)	Specific conductance (μ mole) of leachate/g of fresh wt of leaf of	
		Healthy	Infected
Chili	0	43.86	40.00
	1	44.44	43.86
	2	46.94	50.00
	3	46.94	53.48
	4	46.94	53.48
Red varenda	0	31.94	31.94
	1	43.86	43.86
	2	46.94	50.00
	3	50.00	53.48
	4	50.00	53.48
Brinjal	0	24.21	23.53
	1	30.76	33.33
	2	31.94	34.84
	3	33.33	36.36
	4	33.33	38.17

DISCUSSION

It is evident from the results of this work that the phloem tissue of little leaf infected plants of brinjal and red varenda gave positive reaction to Dienes' stain. All the test healthy plants and the little leaf infected plants of chili did not respond to Dienes' stain. Positive Dienes' staining of the phloem tissue is considered as an indication of presence of MLOs by several workers (Deeley *et al.* 1979, Raju *et al.* 1981). Mycoplasma nature of little leaf of brinjal has been established previously by electron microscopy of phloem tissue (Verma *et al.* 1969). Positive Dienes' staining of the phloem tissue of infected brinjal plants in this study further confirmed the mycoplasma nature of the disease.

The results suggest that the little leaf of red varenda but not that of chili is caused by the mycoplasma like organism. Apparently Dienes' staining can successfully differentiate mycoplasma infections from the healthy or viral infections.

The positive fluorescences of mycoplasma infected phloem cells in presence of fluorochrome aniline blue as proposed by Hiruki and Shukla (1973) has been

found to be true in case of phloem tissue of little leaf infected plants of brinjal and red varenda, but not that of chili. The data suggest mycoplasma nature of causal agents of brinjal and red varenda little leaf disease, but not of chili. The data further indicated that the staining with fluorochrome aniline blue and fluorescent microscopy technique can also be successfully employed for differentiation of mycoplasma infections from that of healthy or other type of infections. The histochemical staining procedures, however, cannot differentiate between *Mycoplasma* and *Spiroplasma* infection (Raju *et al.* 1981).

The results obtained with respect to the distribution pattern of phyllosphere microflora of the healthy and infected plants used in this investigation clearly indicate that the infected plants, irrespective of mycoplasma or other type of infections, harboured significantly more number of microorganism than the healthy plant. No significant difference could be observed with respect to mycoplasma or other type of infections. Furthermore, the rate of leakage of electrolytes from all the three infected plant types was comparable. Evidently the ecological niche provided by macoplasma or viral infections on the phylloplane was more or less similar. The qualitative difference of the phylloplane inhabitants and the type of leachates of mycoplasma and virus infected plants, however, could not be ascertained during the tenure of this investigation.

It is apparent from this investigation that the Dienes' staining and fluorechrome aniline blue technique could be effectively used for rapid identification of mycoplasma or viral nature of the causative agent of little leaf diseases of plants.

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