A study of halo blight of beans (*Phaseolus vulgaris*)

by

Mohamed El Hilo Omer
B.Sc. (Agr.) Khartoum, D.I.C.

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Department of Botany and Plant Technology,
Imperial College of Science and Technology,
London, S.W.7.

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ABSTRACT

The investigation carried out in this thesis dealt with translocation and population trends of the halo blight organism (Pseudomonas phaseolicola) in susceptible and resistant varieties of beans.

Race-1 of the pathogen is capable of substantial multiplication in resistant variety Red Mexican. The rate of multiplication in the resistant is much lower than that in susceptible variety. The susceptible reaction is a two step process starting with water-soaking then necrosis of the tissues while the resistant reaction is essentially necrosis of inoculated region. Population maxima depend on bacterial number inoculated as well as maturity of the leaf. Host reaction was unrelated to buffering capacity of leaf extracts and bacteria multiplied equally well in resistant and susceptible leaf extracts. Preinoculation of the avirulent race at high concentration reduced the number of lesions caused as a result of inoculating the virulent race in the same place. When high proportions of non-virulent Race-1 were mixed with virulent Race-2 the rate of multiplication was greatly reduced compared to that of the virulent.

Clear cut chlorosis was produced by filtrates from cultures containing rich organic source of nitrogen but the activity of the toxin was unrelated to ~~host~~ specificity of parasitism. The toxin was thermostable, dialysable and its production in liquid media was favoured by temperatures of 12-22°C. Chlorosis of infected plants or culture filtrate treated leaves was associated with reduction in soluble protein content and an increase in free amino acids. Sprays of benzimidazole, kinetin or zeatin had no effect on degree of chlorosis.

INTRODUCTION

Studies of host-parasite relationship provide useful contribution to the understanding of phenomena associated with resistance of host tissues to plant pathogenic micro-organisms. Unlike fungi, bacteria causing necrotic spots on leaves are suited for quantitative assessment of such a relationship in terms of bacterial numbers capable of multiplying within the tissues.

In this thesis the two races of Pseudomonas phaseolicola (Burk.) Dows., causal organism of halo blight disease of beans, Phaseolus vulgaris, had been selected to study differences between resistant and susceptible reaction. Two main varieties of beans were used, Canadian Wonder to which both races are pathogenic and Red Mexican which is resistant to Race-1 and susceptible to more virulent Race-2.

The disease causes lesions of small size surrounded by haloes and the leaves produced above infection are normally chlorotic. This is assumed to be due to a toxin that diffuses in the plant tissues away from infection area. The nature of this toxin, its role in pathogenicity and the factors affecting its production remain largely unknown. The present work is an attempt to inquire into some of these aspects.

LITERATURE SURVEY

General

Halo blight disease of beans (Phaseolus vulgaris L.) is characterized by general dwarfing of the plants, and by necrotic spots of variable size surrounded by light coloured or greenish yellow areas as haloes The causal organism was isolated and recorded by Burkholder (1926) as Phytomonas phaseolicola (Burk), and later renamed Pseudomonas phaseolicola (Burk.) Dows. (Dawson, 1943). A bacterial disease of Kudzu (Pueraria thumbergiana) with symptoms identical to those of halo blight on beans and caused by Bacterium purariae (Hedges) was described at about the same time as Burkholder characterized the halo blight organism (Hedges, 1927). Comparative studies and cross inoculation revealed that the two species were identical (Hedges, 1928, 1930).

When newly infected, susceptible leaves show dark green water soaked lesions which later turn brown and usually become surrounded by a halo margin. The halo, however, is a variable character, because isolates differ in their ability to produce this symptom.

Many workers stated that the pathogen may also cause a more generalized infection (Burkholder, 1926; Zaumeyer, 1932; Stapp, 1961; and Zaumeyer and Thomas, 1957).

Zaumeyer (1932) using infected seeds found that the bacterium does not move to a great extent in a downward direction; and is seldom found in tissues of hypocotyl below the cotyledonary nodes. The bacteria occur in intercellular spaces as well as in the xylem vessels of cotyledons. When infection is severe enough to cause breakdown of tissues large cavities may develop.

Waitz and Schwatz (1956) found no obvious connection between the appearance of Chlorotic symptoms and the presence of bacteria in leaves situated above infection point. Chlorotic leaves may or may not contain any bacteria and that bacteria sometimes occur in apparently healthy leaves. Bacteria do not penetrate to greater distances in large masses from point of inoculation, but single bacteria probably do so in the transpiration stream and then multiply locally. The spread is usually upwards and for longer distances is usually somewhat erratic.

Goss (1940) reported that temperature affects the development of disease symptoms because at high temperature the typical halo does not appear although there is more infection thanat lower temperature. In such conditions the symptoms simulate those of common blight and it is seldom possible from superficial examination to differentiate between diseases caused by the two organisms (Zaumeyer and Thomas, 1957).

Jensen and Livingston (1944) studied 13 isolates of Ps. phaseolicola and found that although all isolates failed to produce halo symptoms at 28°C. and above, at lower temperatures, 16-22°C., some isolates did whereas others did not. Patel and Walker (1963) found that the halo is large and pronounced at 16°C. and 20°C. and decidedly smaller and less obvious at 24°C. and 28°C. At high temperature the actual number of infections is not high as reported by Goss (1940). As for most other bacterial diseases, the youngest leaves on a plant are the more susceptible and resistance of leaves increases with age.

Reaction of the plant and races of pathogen.

The search for varieties resistant to halo blight has proceeded for some time, and sources of resistance have been reported by various workers. Jensen and Goss (1942) showed that the varieties Red Mexican and Schwart No. 27 respond to infection with small, inconspicuous, brown, circular necrotic lesions with little if any halo, suggesting that these varieties were resistant. Zaumeyer and Thomas (1957) found that Michelite, Great Northern, Pinto, Pinks and Red Mexican are also highly resistant. Burkholder and Zaleski (1932) found no differences in the susceptibility of a number of varieties to two American and one European strain of Ps. phaseolicola. Ferguson et al., (1955) noticed in the

vicinity of Ottawa, Canada, that Red Mexican is susceptible and suggested that the strain in this area is different from that occurring elsewhere. But it was until 1964 that the discovery of a new race was announced by Patel and Walker (1964). It was called Race-2 and was found to be pathogenic to Red Mexican and a large number of new lines under development at that time.

Patel and Walker (1965) recently screened many phaseolus spp. and varieties for their reaction to the two races in an attempt to find new sources of resistance. Among snap bean varieties tested only Siminole and black seeded Blue Lake showed resistance as a tolerant reaction to Race-1 and Race-2. Comparison among line introductions from different counties revealed that about 22 percent from Asia were uniformly resistant or tolerant. Similar figures for America, Europe and Africa were 8, 3 and 4 percent. All lines from Australia were susceptible to Race-1. None of the lines were hypersensitive to Race-2. Reactions of other species of Phaseolus and related genera fell into several groups as follows:-

1. Species susceptible to both races - P. radiatus, P. lathyroides, P. lunatus, P. coccineus, P. bracteatus, P. polystachus, P. polyanthus, P. atropurpureus and P. acutifolius.

2. Species giving hypersensitive reactions - P. calcaratus, P. mango, P. acontifolius and two unidentified species.

3. Vigna sinensis, V. sesquipedalis, Vicia faba, Pisum sativum and Trifolium pratense showed no signs of infection when inoculated with Race-1 and 2.

4. Pueraria hursuta and Glycine max were susceptible to both races.

Coyne, Schuster and Shaughnessy (1966) studied the inheritance of the reaction to Race-1. Their results suggest that a major gene is partially involved and that the tolerant reaction is dominant. Disease reaction is probably affected by many modifying genes because not all tolerant plants have the same tolerance. Schuster et al., (1965) describe isolates of Ps. phaseolicola more virulent than Races 1 and 2 and with a wider host range because they infect lines of Phaseolus vulgaris and other legumes tolerant to Races 1 and 2.

Hypersensitive reactions

Incompatible combination of host and potentially parastic fungi usually results in a limited necrosis, and very limited development of mycelium, reaction usually described as hypersensitive. Similar mechanisms operate in plants infected by certain avirulent strains of bacteria

(Müller, 1959). Thiers and Black (1951) observed that lesions caused by Xanthomonas malvacearum were much smaller in resistant than in susceptible varieties of cotton, although no morphological structure which restricts spread of the bacteria is present; microscopic examination reveals no histological difference between resistant and susceptible tissues. X. malvacearum and cotton plant were reported to share common antigens. Susceptibility or resistance of a variety of cotton to a particular race of X. malvacearum depends on "antigenic compatibility". Fewer antigens are shared with the bacterium in resistant than susceptible varieties (Schnathorst and De Vay, 1963). Rudolph and Stahmann (1964) found catalase of bacterial origin in the necrotic area and the halo of lesions in bean leaf infected with Ps. phaseolicola. They suggest that catalase interferes with host peroxidase and that this may account for the retardation of browning of tissues characteristic of hypersensitive reactions to the bacterium. Deverall and Walker (1963) found that homogenates of bean leaves resistant to Race-1 have a higher oxygen uptake than preparations from susceptible leaves.

Bacterial growth in Vivo

It is generally accepted that the severity of disease symptoms in diseases of leaves caused by bacteria is

correlated with the number of bacteria that develop in intercellular spaces. When a plant is repeatedly inoculated with bacteria and no symptoms of disease develop that particular plant is regarded as resistant and the bacterium as non-pathogenic.

Several workers have investigated resistance and susceptibility to plant pathogenic bacteria by studying the multiplication of bacteria in resistant and susceptible plants. Allington (1945) states that the water soaking of leaf tissues for a short period, especially by beating rain, greatly facilitates penetration of Ps. tabaci and also its subsequent spread within the plant. Thus, in soybeans, soaking leaves with water before immersion in a bacterial suspension gave a population of over 10^4 viable cells per leaf disk in 24 hours, whereas in non-soaked controls there were fewer than 15 cells. Allington and Camberlain (1949) did similar experiments with leaves of Red Kidney (Phaseolus vulgaris), and soybean, (Glycine max) and Xanthomonas phaseoli and Pseudomonas glycinea. A water soaking method of inoculation was used. In both hosts there was at first a considerable and similar multiplication of bacteria but after a few days there were many more in susceptible hosts. Allington and Chamberlain suggest that the resistant reaction does not begin to operate until

bacteria in the intercellular spaces affect adjoining living cells. Others have also investigated the multiplication of bacteria forcefully introduced into plant tissues. Thus, Diachun and Troutman (1951, 1954) studied Bacterium tabacum in leaves of resistant Nicotiana longiflora and susceptible N. tabacum. In both populations the number declined very quickly after reaching a peak which was higher in susceptible plants.

Sharen (1959) compared populations of X. phaseoli in susceptible P. vulgaris var. Greath Northern, P. coccineus, Scarlet Runner which exhibits field tolerance and highly resistant Tepary bean, P. acutifolius var. latifolius. The expected geometric multiplication did not occur. The population of X. phaseoli in susceptible and resistant leaves increased steadily at about equal rates for several days after inoculation. This was followed by temporary decline, then an increase to a maximum. The temporary decline in resistant tissue was ascribed by Sharen to a host reaction that developed only after considerable numbers of pathogen were present. Klement and Lovrekovich (1961) studied growth of various pathogenic and non-pathogenic Pseudomonas spp. and Xanthomonas spp. introduced into bean pods by means of a hypodermic syringe. They divided the infection process into three phases:

1. Pathogenic phase where only pathogenic bacteria were able to multiply. In this phase antibacterial substances are able to operate against avirulent strains and host defence mechanisms are activated; it is described as "the inductive phase".

2. Static phase where the defence reactions are activated and consequently the pathogen reaches a "static phase". The host is apparently in a "reaction phase", and an equilibrium is set up between host and pathogen.

3. Inactive phase characterized by progressive decline in host resistance. Non-pathogenic strains are able to multiply in the host and this may be regarded as saprophytic in nature. This phase is also described as the "saprophytic phase".

Klement and Lovrekovich (1962), using bean pods, also compared the multiplication of bacteria in susceptible and resistant plants, the parasites used were Pseudomonas phaseolicola, Xanthomonas phaseoli, and X. phaseoli var. fuscans; and as non-parasites of bean, Ps. syringae, Lilac strain, X. juglandis and X. malvacearum and the saprophytic Ps. aeruginosa. The numbers of bacteria pathogenic to beans were increasing geometrically and the maxima did not depend on initial cell number. The largest numbers developed in pods infected by virulent bacteria.

The next largest in pods infected by pathogenic but avirulent species, and the lowest in pods infected by Ps. aeruginosa. Klement, Farkas and Lovrekovich (1964) found that resistance of tobacco to Ps. tabaci was also associated with lower rate of multiplication.

Skoog (1952) reported that increases in population of Pseudomonas phaseolicola within leaves of resistant Red Mexican and susceptible Red Kidney were about the same for the first three days. In the susceptible variety this was followed by further increases accompanied by necrosis and collapse of leaf tissue on the 6th day. The population increased much less in resistant Red Mexican and later there was a decrease. Recently Perry (1966) found a lower multiplication rate of X. malvacearum in resistant compared with susceptible leaves, and Schuster et al., (1964) much the same for Corynebacterium flaccumefaciens var. aurantiacum in tolerant and susceptible bean stems.

Toxins

The production of metabolic products by parasites which can reproduce part of the natural syndrome has been demonstrated by many workers. The literature on the subject has been reviewed by Dimond and Waggoner, 1953; Ludwig, 1960; Braun and Pringle 1959; Wheeler and Luke 1963; Deverall, 1964; and Pringle and Scheffer, 1964.

The earliest evidence for a toxin in a bacterial plant disease was provided by Hutchinson (1913) who noted that in wilted tobacco plant infected by Pseudomonas solanacearum there were too few bacteria to account for disease symptoms. Feder and Ark (1951) isolated wilt inducing polysaccharides from Agrobacterium tumefaciens, Xanthomonas phaseoli and Erwinia carotovora, but the wildfire toxin produced by Ps. tabaci is perhaps the most important bacterial plant toxin so far characterized. The symptoms produced in tobacco by this organism closely resemble those produced by Ps. phaseolicola in beans. The disease and the bacterium were studied by Johnson and Murwin (1925), and Clayton (1934) who demonstrated that sterile filtrates can reproduce typical disease symptoms. But culture filtrates are non-specific because a wide range of species not related to tobacco develop characteristic symptoms when treated with the toxin. Braun (1950, 1955) showed that the toxin inhibits growth of Chorella vulgaris in liquid media. The toxin was isolated by Woolley, Pringle and Braun (1952) and later identified as the lactone of α - lactylamino - B hydroxyaminopimelic acid (Woolley et al., 1955) which is a structural analogue of methionine.

Burkholder (1930), and Harter and Zaumeyer (1944) assumed that toxin was responsible for chlorosis of the leaves and the haloes around infection centres in halo blight.

Waitz and Schwatz (1956) found the toxin in cultures on sterile filtered press juice of bean plants, but not in cultures on synthetic media. They conclude that:

1. Toxin formation is dependent on certain substances in the leaves.

2. During the systemic infection only young leaves are affected. Old leaves show little if any halo around the infection point, and do not become chlorotic in systemically infected plants.

3. There is no toxin in sterile press juice from infected plants, or from chlorotic leaves not carrying the bacterium.

Muller quoted by Patel and Walker, (1963) reported that the bacterium produces a thermolabile toxin in nutrient broth, but not in a synthetic medium, but Skoog (1952) obtained a thermostable toxin from cultures in glucose broth.

Effect of toxin on metabolism of the host tissue

Microbial toxins may affect metabolism of host plants. Victorin from Helminthosporium victoriae increases respiratory activity and permeability of susceptible tissue (Romanko, 1959; Krupka, 1959; Grimin and Wheeler, 1963; Wheeler 1961; and Wheeler and Black, 1962). Lycomarasmin, from fusarium oxysporum var. lycopersici, disturbs water balance and reduces turgidity of the cells (Gaümann, 1951).

Both fusaric acid from Fusarium oxysporum and lycomarasmin chelate with heavy metals such as Fe^{+++} and Cu^{++} and their biological activity may depend on this property (Waggoner and Dimond, 1953). Braun (1955), using Chlorella vulgaris as a test organism, showed that the wildfire toxin of Ps. tabaci is an antimetabolite of methionine presumably because it is a structural analogue of this amino acid. Methionine sulphoximine another structural analogue simulates the effect of the toxin in tobacco plant; it also prevents growth of Chlorella vulgaris at low concentrations. Other structural analogues of methionine were ineffective. Farkas and Lovrekovich (1963), Lovrekovich and Farkas (1963) and Lovrekovich, Klement and Farkas (1964), using sterile culture filtrates of Ps. tabaci, showed that the toxin increases the concentration both of ammonia and free amino acids and this is associated with a reduction in the soluble protein and the RNA of treated tissue. Patel and Walker (1963) working with halo blight found that samples from the halo and from Chlorotic leaves of infected susceptible plants have considerably more free amino-acids compared with healthy plants. A striking effect is the accumulation of ornithine and B-alanine. There are however, no qualitative differences in the free amino-acids of healthy susceptible and resistant. Rudolph and Stahmann (1964) reported a

reduction in soluble protein from the halo region and from chlorotic leaves compared with healthy leaves.

Counteraction of toxicity

Woolley (1946) nullified the effect of lycomarasmin with small amount of strepogenin (a substance promoting the growth of Lactobacillus casei and obtained by tryptic digestion of protein). Waggoner and Dimond (1953) showed that the toxicity of the lycomarasmin iron complex can be reduced with 8 - quinolinol which preferentially chelates iron, or by adding copper which is preferentially chelated by lycomarasmin. Farkas and Lovrekovich (1963) counteracted the effect of wildfire toxin by spraying leaves with kinetin at 10^{-5}M. Treated leaves retained their green colour and their protein content increased compared with leaves treated with culture filtrates only. Hoitink, Coulson and Pelletier (1964) reported a reduction in halo size induced when leaves infected by Ps. phaseolicola are sprayed with kinetin. Luke and Wheeler (1966) on the other hand reported that the phytokinins 6 - furfuryl - aminopurine, 6 phenyl-aminopurine, and 6 - benzyl - aminopurine, act synergistically with victorin on oat tissue. This is interesting because the two materials have opposite effects in the senescence of detached leaves; victorin accelerates

senescence whereas phytokinins retard it. In this connection it is interesting to note that Osborne (1962), Richmond and Lang (1957), and Sugiura, Umemura and Oata (1962) have shown that in senescent leaves kinetin shifts the balance of protein synthesis in favour of increased decomposition of protein. The development of chlorotic symptoms and the changes of protein metabolism caused by wildfire toxin are similar to those occurring in senescent leaves as in the latter both chlorosis and breakdown of protein can be counteracted by kinetin.

MATERIAL AND METHODS

Plant material

(a) Source of seeds:- The two varieties of French beans (Phaseolus vulgaris L.) mostly used throughout this work were Canadian Wonder, obtained from Suttons and Sons Ltd., Reading and Red Mexican UI-34 kindly supplied by Bean Research Laboratory, College of Agriculture, Idaho University. Other varieties used for disease reactions were either from Suttons and Sons Ltd., or Carters Ltd., London.

(b) Growth of plants

(1) Soil - Plants were grown in John Innes potting compost in earthenware pots (14 cm. top diameter) or as single seedlings in plastic pots (9 cm. top diameter) in a heated greenhouse maintained at a minimum temperature of 16°C. Artificial light was provided by 80 w fluorescent tubes above plants.

(2) Vermiculite - For population studies plants were grown in vermiculite , No. 3 size (from Pan Britannica Industries Limited), in plastic pots (9 cm. top diameter). The vermiculite was kept moist with tap water at first; this was replaced with nutrient solution (see 3 below) after germination. Plants selected for population studies were usually inoculated 3-4 days after emergence and transferred

to growth chambers provided with fourteen hours artificial light and controlled temperature of 17-18°C.

(3) Cut shoots - These were normally transferred to nutrient solution when plants reached the fully developed unifoliate stage.

The nutrient solutions used were those of Long Ashton Research Station described by Hewitt (1952). For 100 litres of nutrient 33.6 g. of KNO_3 and 54.7 g. of $Ca(NO_3)_2$ were used. Iron was supplied in the form of iron ethylene - diamine - tetraacetic acid complex (Fe-EDTA).

Media

The following were commonly used:-

(1) Nutrient broth - Oxoid nutrient broth No. CM1 was used. This was supplied as powder and only required to be dissolved in the appropriate quantity of distilled water to give the complete medium.

This medium consists of:-

'Lab lemco'	Beef extract	10g./Litre.
Peptone	(Oxoid)	10g./Litre.
Sodium chloride		5g./Litre.

The final medium has a pH of 7.4

(2) Nutrient agar - This was nutrient broth to which 15 g. of bacteriological agar were added to a litre.

(3) Dextrose-peptone

Bacteriological peptone	(Oxoid)	20g./Litre.
Dextrose		10g./Litre.
Sodium chloride		5g./Litre.

(4) Glucose-Casaminoacids

Glucose		10g./Litre.
Casaminoacids - 'acid'	(Oxoid)	4g./Litre.
KH_2PO4		1g./Litre.
MgSO4		0.5g./Litre.

All media were sterilized for 15 minutes at 15 lb. pressure.

(5) Leaf extract - A known weight of bean leaves was frozen and then thawed and macerated in a mortar. Washed carborundum powder, 220 mesh, was added to facilitate maceration. The crude macerate was filtered through layers of muslin and filtrate was centrifuged twice at 15,000 r.p.m. for 30 minutes to remove larger particles. The supernatant was then sterilized by passing through an Oxoid membrane filter.

Bacterial cultures

(a) Source of cultures

Isolates used throughout this work were obtained from The National Collection of Plant Pathogenic Bacteria, Harpenden.

These were classified according to physiological races by Epton and Deverall (1965). Isolate 604 obtained from the Commonwealth Mycological Institute was found to be Race-1. The original accession numbers are retained in this thesis. The classification according to disease reactions of these isolates is given in Table 1.

(b) Maintenance of stock cultures

Type cultures from single colonies on dilution plates were grown on slopes of nutrient agar in McCartney bottles. Cultures were kept at - 20°C., 4°C., and also under mineral oil at room temperature. Type cultures were subcultured every 6 months.

(c) Harvesting of cells for inoculation

Bacteria for plant inoculation grown for 48 hours at 25°C. in liquid media were separated by centrifugation at 10,000 r.p.m. for 30 minutes. The centrifuged cells were resuspended in sterile distilled water and recentrifuged again at 10,000 r.p.m. for 30 minutes. The clear supernatant was decanted and the cells were resuspended in sterile distilled water. When solid media were used, about 15 ml. sterile distilled water was added to each medical flat. The cells were suspended by slight rocking before centrifugation.

Table 1. Race classification of isolates

Accession No.	Origin of culture	Reaction of differential variety	Race
380	N.C.P.B.	S	2
605	N.C.P.B.	R	1
1057	N.C.P.B.	R	1
1098	N.C.P.B.	R	1
1103	N.C.P.B.	R	1
1321	N.C.P.B.	S	2
1341	N.C.P.B.	S	2
604	C.M.I.	R	1

S = Susceptible R = Resistant

(d) Standardization of inoculum

(i) Using a nephelometer

An Eel nephelometer head attached to an Eel Unigalvo type 20 was used for this purpose. Suspensions were measured relative to a standard.

(ii) Bacterial counting chamber

Bacterial suspensions were mixed with 4 per cent formaldehyde solution (1:1 v/v) and allowed to stand for 10 minutes before counts were taken.

Surface sterilization of plant material

A sodium hypochlorite solution containing 0.2 per cent available chlorine was used for surface sterilization. Plant material was immersed for 10 minutes in this solution followed by washing in several changes of sterile distilled water. A drop of Tween 80 was added to the sodium hypochlorite solution when necessary to wet the material thoroughly.

Methods of inoculation

(1) Needle inoculation:- A dissecting needle was flame sterilized before dipping in a bacterial suspension which was then pricked into the plant.

(2) Spraying:- Under surfaces of the leaves were sprayed to run off with a bacterial suspension before transferring to a humid chamber.

(3) Water-soaking method:- This method was used for tracing bacterial growth in vivo. The method usually adopted by previous workers was to water-soak leaves from the under surface with a standard bacterial suspension using a spray-gun and then removing bacteria on the surface with several changes of water. Alternatively the suspension has been injected under the lower epidermis from a hypodermic syringe. The method used in this work is a modification of the infiltration technique. The under surface of the leaf was shielded except for standard areas 5 mm. diameter which were exposed to the spray Fig. 1. The sprayer used was obtained from Colour Paint Co. Ltd. It was attached to an air cylinder at a pressure of 15 lb/sq.in. Only plants at the monofoliate stage were used for population studies and they were selected for uniformity before inoculation. Spraying was continued until the exposed surface was uniformly dark green which meant that the leaf below was water-soaked. The inoculated leaves were then washed in sterile distilled water to remove surface bacteria. The first sample in every case was taken immediately after inoculation, other samples were taken periodically usually over a period of 12 days.

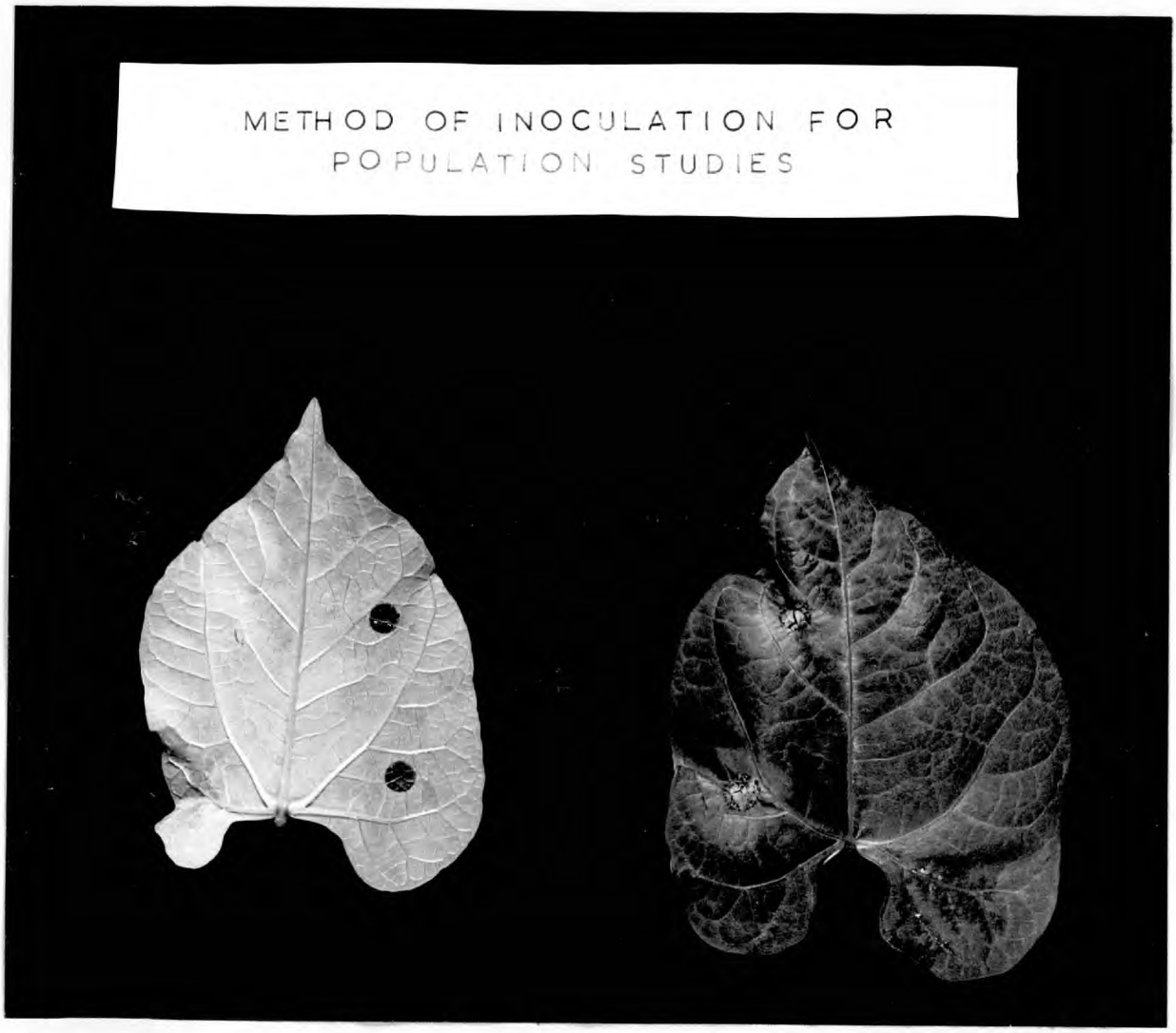


Figure 1. Left leaf immediately after inoculation of defined area. Right leaf 15 days after inoculation.

Each inoculated region was removed with a sterile cork borer 10 mm. in diameter, macerated thoroughly in 0.2 ml. of quarter-strength Ringer solution (sodium chloride 2.25g., potassium chloride 0.105g., calcium chloride 0.12g. and sodium bicarbonate 0.05g. in 1 litre water to give a solution pH 7.0 approximately) and then made up to 10 ml. Suspensions were allowed to stand for 10 minutes with shaking at intervals and then serial 10 fold dilutions were prepared. 1 ml. of suspension was added to 12 ml. of nutrient agar in a test tube, shaken and poured very quickly into sterile Petri dish. The temperature of the tubes was kept at 40-41°C. before pouring. Viable counts were taken after 3-4 days incubation at 25°C.

Evaluation of plate counts

Optimum range:- This is defined as Petri dishes containing 30-100 colonies. Colony counts falling within this range formed the basis of the results, ignoring adjacent 10 fold dilutions. The average of a replicate series was accepted when it fell within the optimum range.

Sub-optimum range:- This is defined as Petri dishes giving 10-29 and 101-300 colonies (both inclusive). When the counts for adjacent 10 fold dilutions fell within the sub-optimum ranges, these counts were adopted after applying

the following formula

$$\text{Log } yo = \frac{\Sigma \text{Log } yi + \Sigma i}{n}$$

where yo = Counts/ml.
i = Dilution index.
yi = No. of colonies per plate.
n = No. of determinations.

Counts of 301 and above were only used when the counts on the next dilution showed a number below the sub-optimum range or counts on all dilutions exceeded 301.

Counts of 9 and below were only considered where plates of 10^{-1} dilutions were concerned and no higher counts were obtained.

Preparation of plant material for microscopic examination

For the preparation of permanent mounts the following methods were adopted:-

Fixation

Fresh plant materials were fixed in a mixture of

Formaldehyde	(10 per cent saturated solution v/v)
Glacial acetic acid	(5 per cent v/v)
90 per cent ethyl alcohol	(50 per cent v/v)

Plant materials were infiltrated with the above (FAA) for at least 3 days before dehydration.

Dehydration

Because the material was fixed in FAA containing 50 per cent alcohol, it was already partially dehydrated, the material was transferred to corresponding alcohol concentration following Johansen's tertiary butyl method (1940).

Embedding

Plant material was embedded in wax (melting point 56°C.). This was done by the method described by Johansen (1940).

Sections 5-10 μ thick were prepared using a Cambridge rocker microtome. Haupt's adhesive mixture (Johansen, 1940) was used to fix the serial sections to slides which were air dried for 48 hours before passing through the following solutions.

Xylol
Xylol - absolute alcohol (1:1 v/v)
Absolute ethyl alcohol

This was followed by 90,70,35 and 5 per cent alcohol, then water. Slides were allowed 5 minutes in each solution.

Staining

The following staining methods were tried.

(1) Carbol fuchsin

(a) Slides were stained in 1 per cent carbol fuchsin for 1 minute.

(b) Washed in water.

(c) Washed briefly in 95 per cent ethyl alcohol.

(d) Cleared in a 1:1 ethyl alcohol, xylol solution before xylol.

(2) Carbol thionin - orange G

Stoughton's (1930) method was used. Slides were left in carbol thionin (10 ml1 saturated alcoholic solution in 100 ml. water) for 30 minutes. This was followed by differentiation in orange G for 1 minute. Sections were washed well in absolute alcohol and cleared in xylol.

(3) Safranin - fast green

/Modification of Quadruple stain, see Johansen, 1940/

(4) Giemsa stain

The method used was modification of Hrushovietz (1956). The preparation obtained from Gurr's Ltd. (R66) was diluted twenty times in 0.1 M phosphate buffer (pH 6.9). A fresh preparation was used every time. Slides were stained in Giemsa for 25 minutes, washed briefly in phosphate buffer followed by ethyl alcohol, ethyl alcohol and xylol (1:1), then xylol. Permanent slides were mounted in Euparol or Canada balsam.

Preparation of culture filtrates

100 ml. of sterile liquid media in 500 ml. flasks were inoculated with 1 ml. 24 hour old bacterial culture. Still cultures were incubated at 18°C. and after 10-12 days growth liquid cultures were centrifuged twice at 15.000 r.p.m. for 30 minutes at 5°C. The supernatant was filter - sterilized through an "Oxoid" membrane filter in a Seitz filtration apparatus and cell-free culture filtrates were stored at-20°C. until required.

Testing activity of culture filtrates

1. Infiltration method:- Cell free culture filtrate was injected into stomatal chambers and intercellular spaces of half unifoliate leaves. The spray used for population studies (see method of inoculation) was found to be satisfactory for this purpose. The sprayer was held an inch from the under surface of leaves and the filtrate applied at a pressure of 15 lb. p.s.i. Spraying was continued until the whole half unifoliate was uniformly dark green and, therefore, water-soaked.

2. Cut shoots:- These were at the monofoliate stage when allowed to take up culture filtrates for 24 hours in growth cabinets before being transferred to nutrient solution.

Estimation of culture filtrate activity

Quantitative reduction in total chlorophyll content was taken as a measure for assessing activity of culture filtrate. Samples of known fresh weight were stored at - 20°C. for 24 hours or longer, then the chlorophyll was extracted in 80 per cent cold acetone in the dark. The chlorophyll solution was separated by centrifugation at 1,000 r.p.m. for 10 minutes. The supernatant was collected and the total chlorophyll content assessed optically in a Beckman spectrophotometer (Model DB) by measuring absorption at a wave length of 652 mu. The total chlorophyll was calculated by the formula of Bruinsma (1961).

$$\text{Total chlorophyll in mg./l.} = \frac{A_{652} \times 1000}{36}$$

where A is absorption.

EXPERIMENTAL

PART I VARIETAL TESTING

Two isolates, 604 (R-1) from C.M.I., and 1341 (R-2) from N.C.P.B., were used in this and subsequent experiments unless otherwise stated.

The following varieties of dwarf and runner beans were tested for their reactions to the two races:-

1. Michelite
2. Blue Lake
3. Saints best of all
4. Prince
5. Canadian Wonder
6. Masterpiece
7. Carters Cookham Dean
8. Tender and true
9. Carters lightning
10. Carters Merton
11. Carters white monarch
12. Carters purpetual
13. Carters streamline
14. Carters Grand stringless
15. Wax or golden butter
16. Red Mexican

Plants were grown in non-glazed earthenware pots, four to each pot, and tested at the monofoliate stage. Each monofoliate was infiltrated at 4 points with standardized washed bacterial suspension, three replicates of each variety. Inoculated plants were kept in the greenhouse for 12 days and observations on presence or absence of water-soaking, halo and chlorosis of developing trifoliates were recorded. All varieties inoculated with Race-1, except Red Mexican, invariably showed water-soaking, and marginal chlorosis; Michelite, however, failed to show the chlorosis of developing trifoliates which was apparent in other varieties. Red Mexican showed necrosis of inoculated areas, marginal chlorosis was practically absent and systemic chlorosis did not appear with concentration of inoculum used.

All varieties including Red Mexican gave susceptible reactions to R-2.

PART II TRANSLOCATION OF BACTERIA IN SUSCEPTIBLE AND RESISTANT PLANTS

A. - Stem Inoculation

Previous workers have indicated that though the bacterium is essentially a mesophyll parasite it may reach the xylem and be carried through it to tissues distant from the point of inoculation.

In this experiment an attempt was made to see how far the bacterium travels within tissues of susceptible and resistant plants. Race-1 to which Canadian Wonder is susceptible and Red Mexican gives resistant reactions was used.

Plants of these varieties grown singly in plastic pots were inoculated at the cotyledonary node with a dissecting needle using a dense bacterial suspension (approx. 10^9 bacterial cells/ml.). The inoculated region was sealed with a sterile vaseline wax mixture and the plants were kept in the greenhouse. Eight were periodically sampled at different heights along the main axis, petiole and mid-ribs for plate inoculation; other samples were deposited in FAA for anatomical study.

Stem and petiole segments were covered at both ends with the sterile vaseline wax mixture before being surface sterilized. Samples from leaves, stem and petioles were immersed in sodium hypochlorite solution containing 0.2 per cent chlorine for 10 minutes then washed in several changes of sterile distilled water, blotted between sterile filter papers and segmented aseptically. Segments were plated on poured sterile nutrient agar plates. After a period of 4 days incubation plates were inspected for presence or absence of bacterial colonies. No attempt was

made to confirm the pathogenicity of all isolations obtained in this way, a practice, though preferable was not possible owing to the large number involved. A number of tests that were made showed that the plant material contained the parasite and that surface sterilization with sodium hypochlorite was satisfactory. The experiment has given a number of useful results shown in Table 2.

(1) In both susceptible and resistant variety bacteria were isolated from regions away from infection site in a matter of few days.

(2) Whereas the organism was isolated from regions above the monofoliates, the movement below the cotyledons was restricted to about 4 cm. and more samples above infection point gave positive result than corresponding samples taken below the cotyledons. This confirms that movement of bacteria is dependent on direction of flow of xylem fluids.

(3) Although the number of samples giving bacterial colonies over short distance increased with time, translocation for long distances was inconsistent and only few plants gave positive result.

(4) In the susceptible variety the bacteria were translocated to much higher regions of the plant and always a greater number of samples gave colonies compared with corresponding samples from resistant variety.

Table: 2 Distribution of bacteria in plants inoculated at the cotyledonary node

Time in days	Variety	Length in cm. along the axis						Primary leaf node	Primary leaf petiole	Primary leaf mid-rib	1st trifoliate node	1st trifoliate petiole	1st trifoliate mid-rib of leaflet
		Above			Below								
		2	4	6	2	4	6						
2	C.W.	6	0	0	1	0	0	-	-	-	-	-	-
	R.M.	2	1	0	0	0	0	-	-	-	-	-	-
3	C.W.	6	3	0	2	2	0	0	-	-	-	-	-
	R.M.	6	1	0	3	0	0	0	-	-	-	-	-
6	C.W.	8	6	4	3	1	0	4	3	1	-	-	-
	R.M.	7	2	1	3	0	0	1	0	0	-	-	-
8	C.W.	8	8	8	7	2	1	6	6	0	0	0	0
	R.M.	8	2	2	5	1	0	4	0	0	0	0	0
10	C.W.	8	8	8	8	3	0	8	5	3	2	0	0
	R.M.	8	7	2	4	0	0	4	2	0	0	0	0
12	C.W.	8	8	8	8	4	0	8	8	6	3	1	0
	R.M.	8	8	6	7	1	0	5	1	0	1	0	0
15	C.W.	8	8	8	8	5	0	8	7	7	2	1	1
	R.M.	8	6	6	6	1	0	6	1	1	0	0	0
18	C.W.	8	8	8	8	2	0	8	8	7	4	1	0
	R.M.	8	4	4	8	2	0	4	4	3	0	0	0
21	C.W.	8	8	8	8	5	1	8	8	8	2	1	0
	R.M.	8	8	6	8	1	0	7	1	1	1	0	0

C.W. = Canadian Wonder
R.M. = Red Mexican
Each figure shows number of times bacterium isolated out of eight possible plants.

Although the organism was isolated from main veins in the leaf, distant from inoculation point, in no case there was spread from the xylem vessels to the neighbouring mesophyll of expanded leaves; but when susceptible plants were inoculated in the same manner while the cotyledons were still plumb small microscopic lesions developed in very young leaves infolding the terminal bud. Growth of these plants was very much arrested and one or the two primary leaves remained permanently flaccid.

Microscopic examination confirmed the presence of bacteria within xylem vessels and that only in very young tissues did it infect the mesophyll Figures 2, 3, 4, 5 and 6.

Attempts to infect plants, grown under sterile conditions by dipping roots in bacterial suspension then washing excess of bacteria before transferring to nutrient solution did not succeed but cut shoots allowed to take up suspensions of the bacterium developed the characteristic chlorosis of the developing trifoliates and remained stunted.

B. - Mesophyll Inoculation

Samples taken from the halo region surrounding infection points and away from necrotic area gave very low numbers of bacteria after being surface sterilized and macerated for viable counts. An average of 50-150 viable cells/5 mm. disk from halo surrounding necrotic area and less number from chlorotic leaves has been obtained.

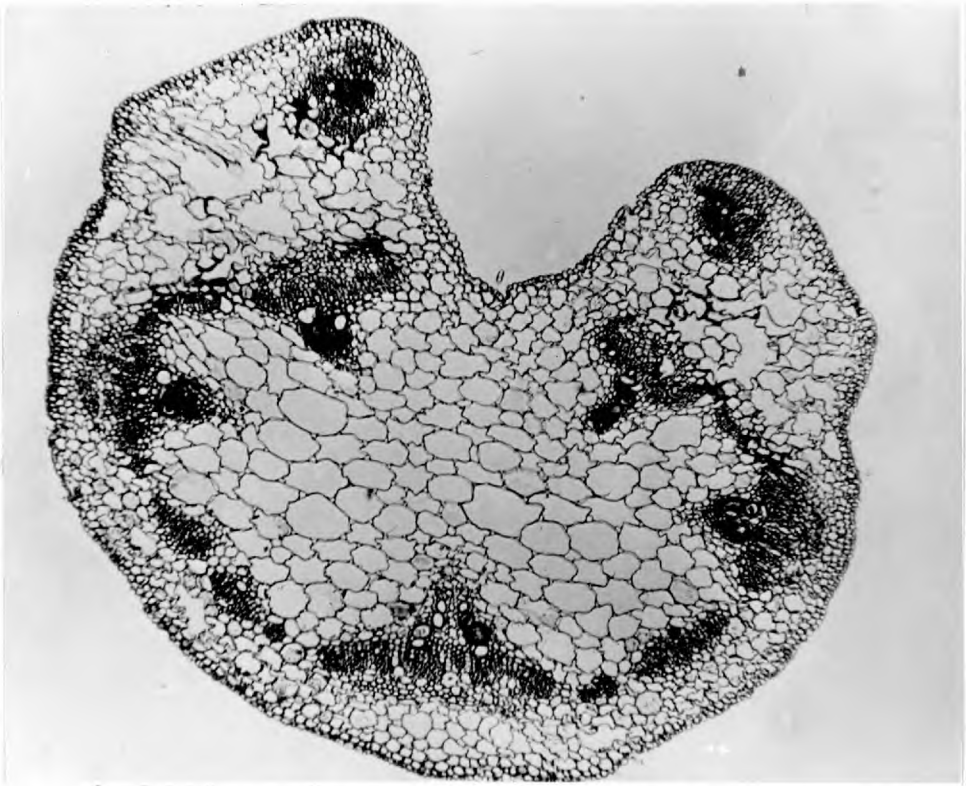


Figure 2. T.S. petiole showing distribution of bacterial masses in xylem vessels x 20

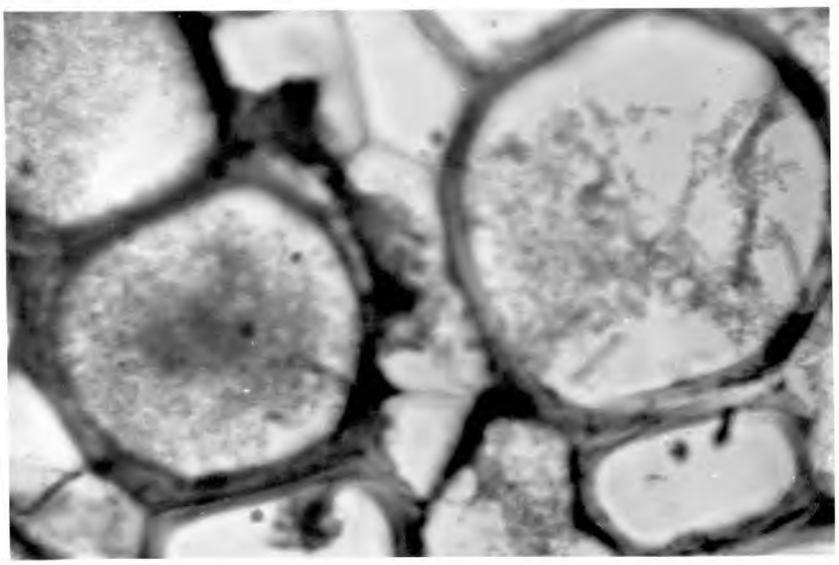


Figure 3. T.S. stem showing bacteria in xylem vessels x 1520

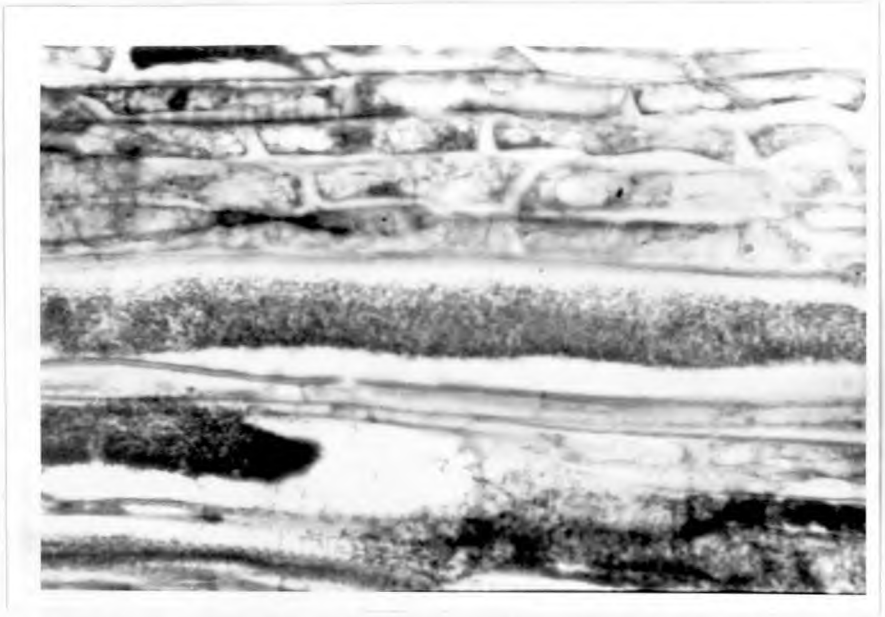


Figure 4. L.S. stem showing bacteria in xylem vessels x 1140

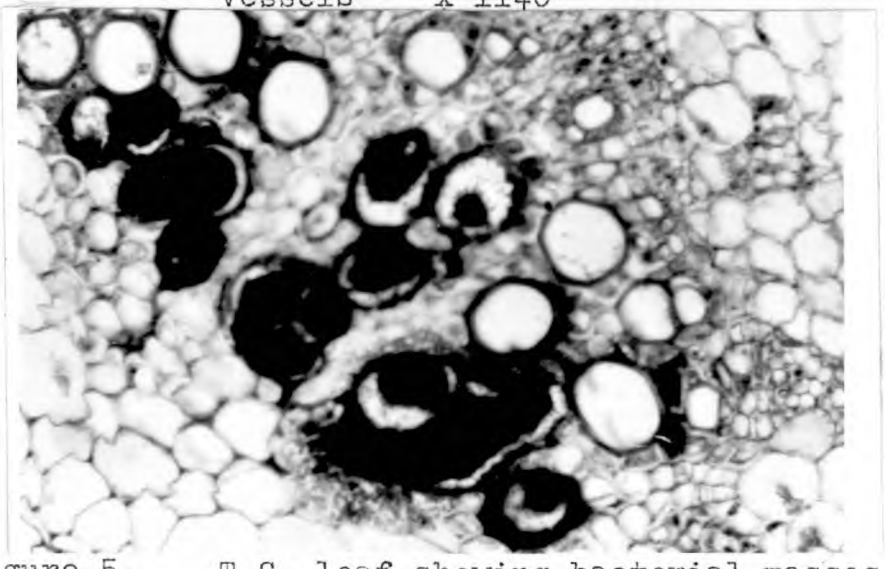


Figure 5. T.S. leaf showing bacterial masses in xylem vessels x 480



Figure 6. L.S. growing point showing systemic mesophyll infection in young leaves x 16

/Infected regions have taken darker stain/

Sections taken through leaf lesions few days after inoculation show that the bacteria multiply in the intercellular spaces of leaf mesophyll forming zoogleal masses where they presumably draw nutrients from the neighbouring cells. These cells are eventually cut off and crumble at a stage when the lesion becomes necrotic (Figures 7 and 8). There is an intercellular penetration of densily staining material in which bacterial rods could only be discerned at high power. No qualitative difference between infected susceptible and resistant tissues was observed.

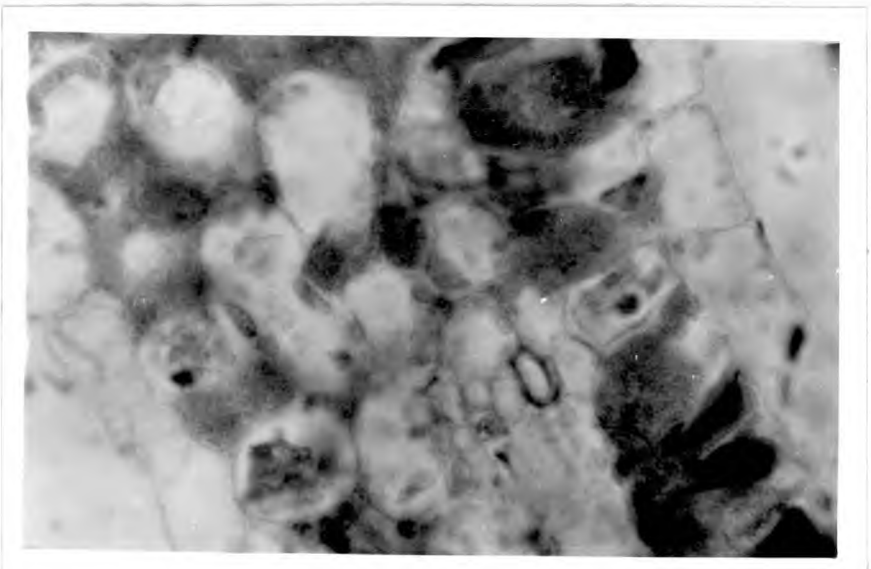


Figure 7. T.S. leaf, 4 days after infection showing bacteria in the intercellular spaces x 480

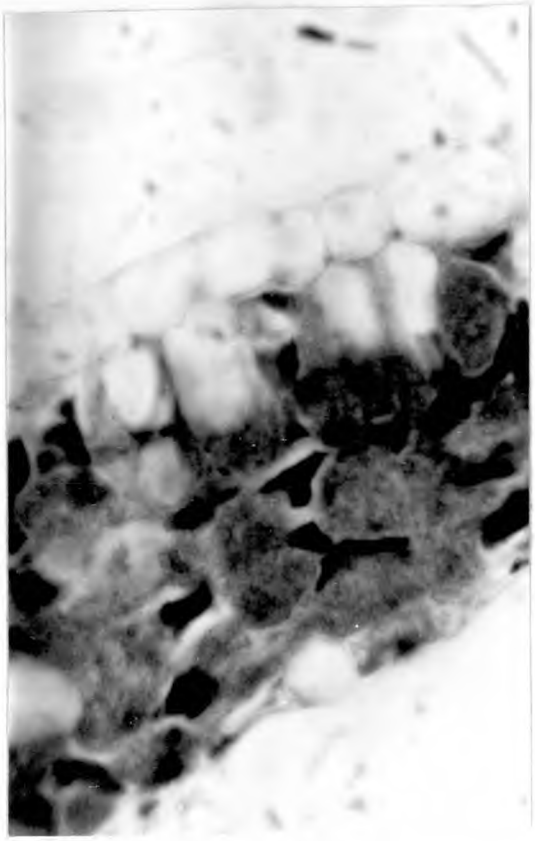


Figure 8. T.S. leaf late stage of infection when lesion became necrotic x 480

PART III BACTERIAL GROWTH IN VIVO

1. Growth behaviour of Race-1 in susceptible and resistant leaf tissue

Single seedlings of susceptible Canadian Wonder and resistant Red Mexican were grown in vermiculite. Three days after emergence, when the monofoliates were still not fully expanded prescribed areas 5 mm. in diameter were inoculated with standardized, washed suspension of bacteria in sterile distilled water from 48 hour cultures on dextrose-casaminoacids. Each monofoliate was inoculated at two points away from the main veins. Inoculated leaves were washed in several changes of sterile distilled water before the plants were transferred to growth chamber.

Samples of four disks from each variety were taken immediately after inoculation and at intervals over a period of 12 days.

Fig. 9 shows the growth curves of Race-1 in the two varieties. Bacteria in both susceptible and resistant leaf tissue started multiplication immediately after inoculation, entering a logarithmic phase and reaching a maximum in 3-5 days. Afterwards there was not much change in the total number and the bacterial population remained in a stationary phase. The maximum number reached in the susceptible host was much higher than in the resistant host.

Figure 9.

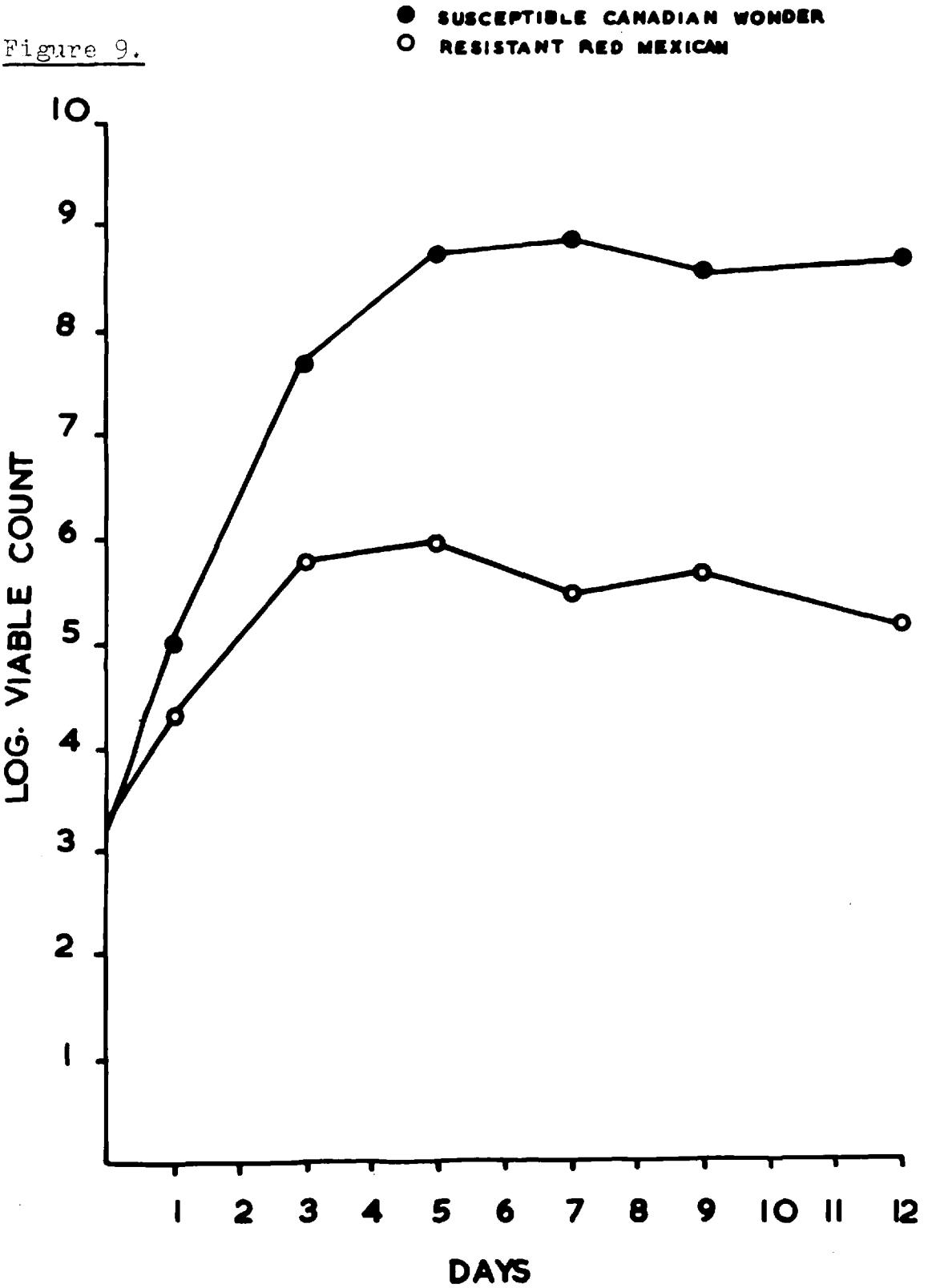

GROWTH CURVES OF RACE-1 IN SUSCEPTIBLE AND RESISTANT LEAVES
SUSCEPTIBLE CANADIAN WONDER
RESISTANT RED MEXICAN
LOG. VIABLE COUNT
10
9
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12
DAYS

Figure 10.

GROWTH CURVES OF R-1 IN SUSCEPTIBLE CANADIAN WONDER

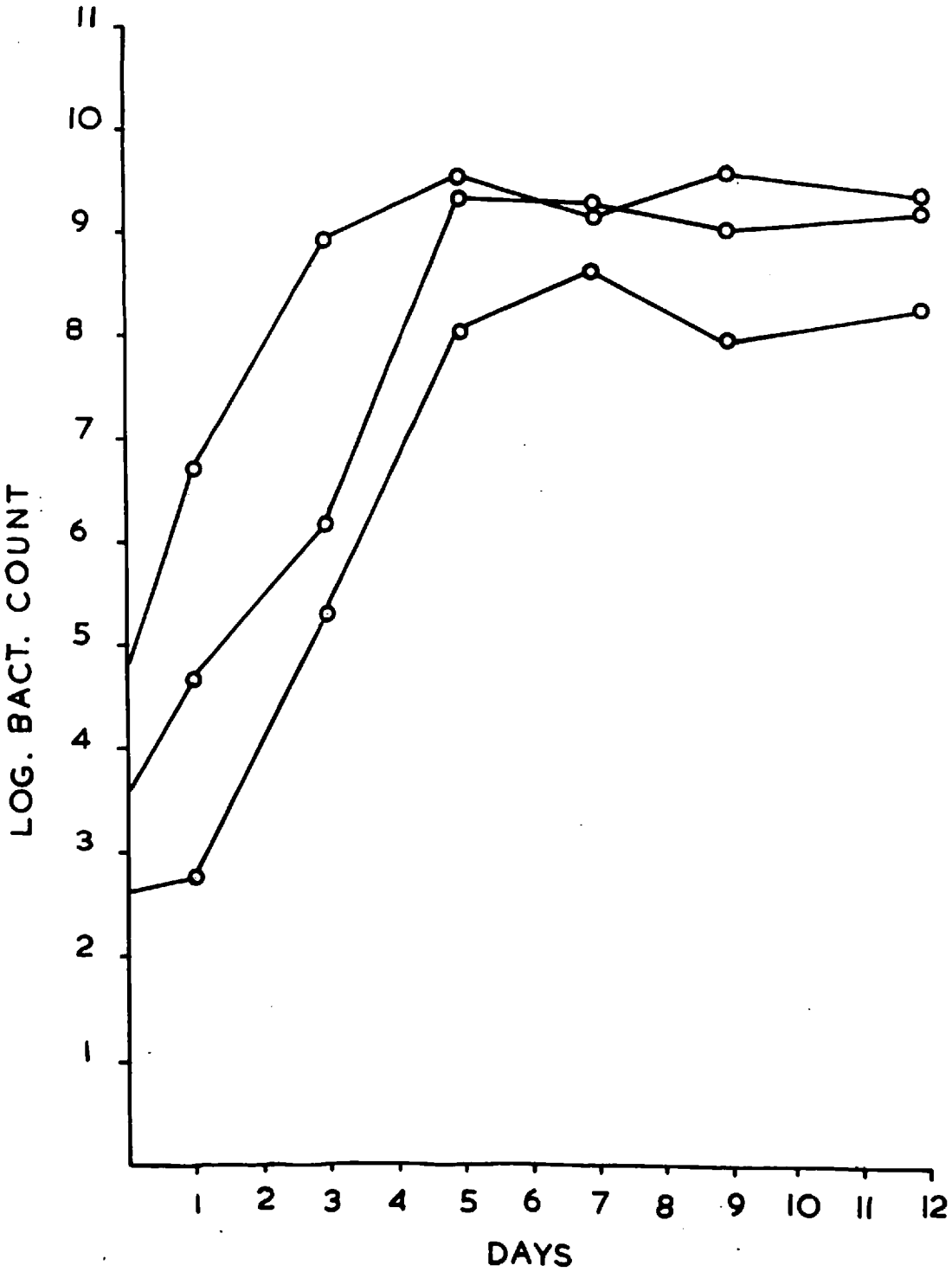
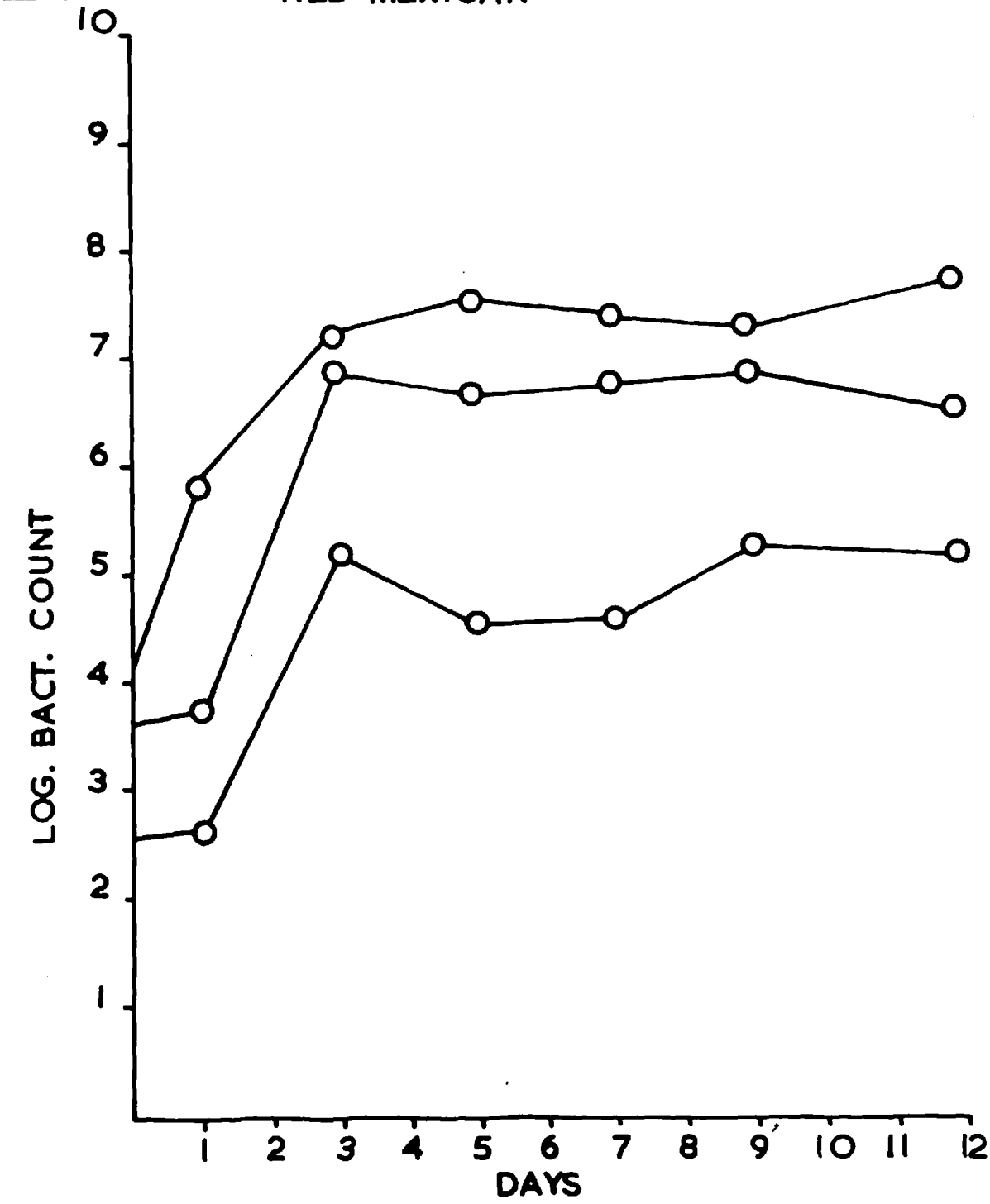


Figure 11.


GROWTH CURVES OF R-1 IN RESISTANT RED MEXICAN
LOG. BACT. COUNT
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
DAYS

2. Effect of increasing initial bacterial cell number in susceptible and resistant plants

Three bacterial suspensions in the ratio of 1:10:100 of Race-1 were used to study the effect of increasing the inoculum concentration on bacterial multiplication. Canadian Wonder and Red Mexican plants were inoculated in the manner outlined for previous experiment. Here again samples were taken over a period of 12 days. The results are shown in Figures 10 and 11.

From previous graphs one can now conclude the following about the multiplication of the bacterium in susceptible and resistant leaves.

1. In general, bacteria whether inoculated into susceptible or resistant plant tissue immediately enter a phase of multiplication building up to a peak and reaching a more or less stationary phase.

2. In resistant plants, bacteria enter the stationary phase in 3 days while in susceptible host this normally occurs one or two days later.

3. The rate of multiplication and peak reached in susceptible leaves are much higher than in those of resistant leaves.

4. The time of symptom development is independent of the amount of inoculum whereas the maximum population reached

is a function of the number initially injected into the leaf. The latter is, however, much the case of resistant than the homogenous plant bacterial combination.

5. The entry to the stationary phase in susceptible leaves coincides with the appearance of water-soaking of the infected tissue, while in the resistant plant browning of the tissues occurs when the population reaches its maximum. Disease symptoms in susceptible plants were initiated by all concentrations of inoculum applied, none of the concentrations injected into resistant leaves induced typical halo blight symptoms.

6. The intensity of both water-soaking in susceptible plants, and browning or necrosis of resistant tissue is a function of initial inoculum concentration. The higher this is the more prominent are both reactions.

7. The growth behaviour of bacteria in leaf tissue resembles that which might be expected from discrete colonies in solid media.

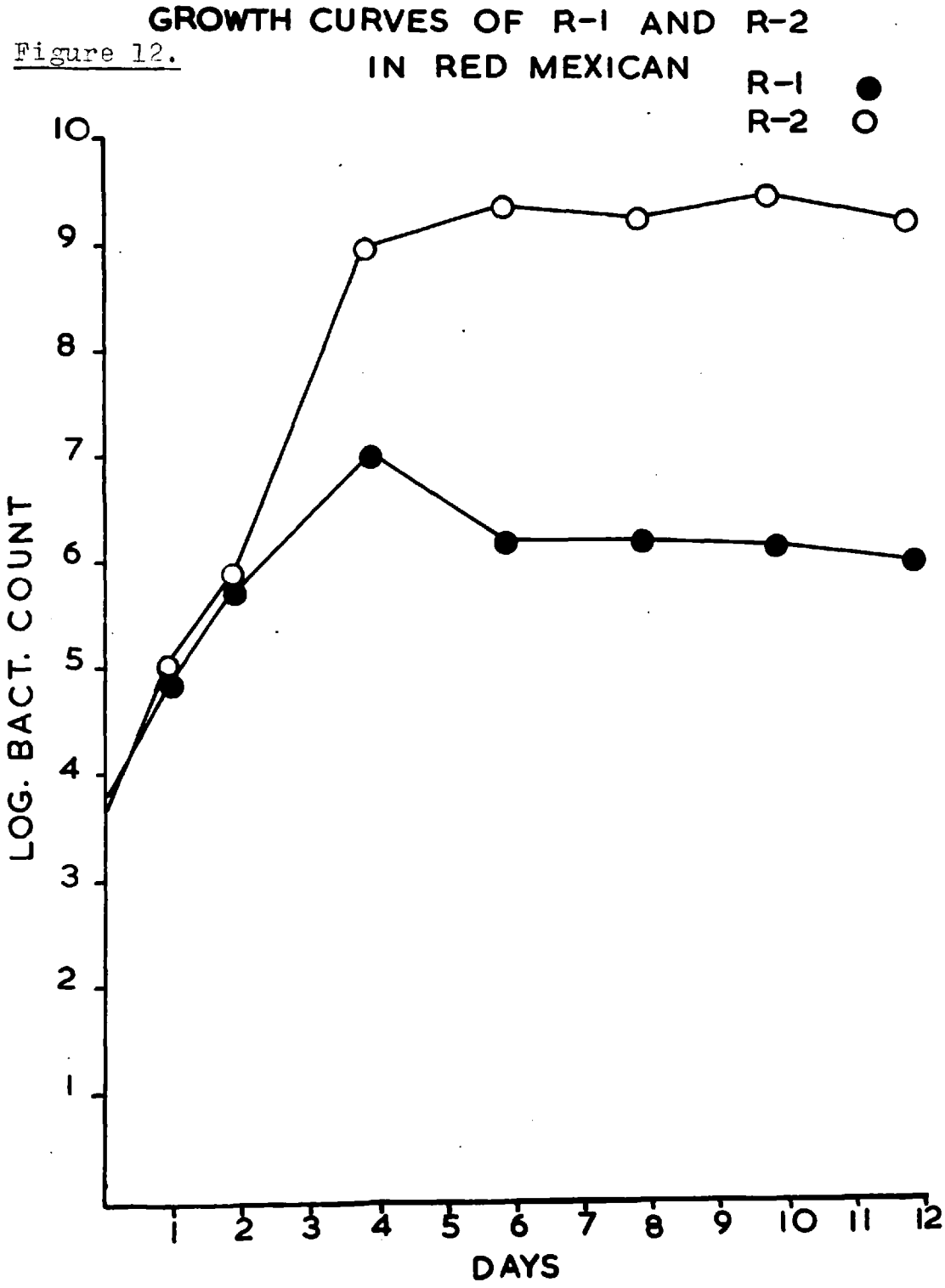
3. Growth behaviour of the two races in Red Mexican

The previous experiment shows that Race-1 population maximum in susceptible plants is 10^3-10^4 times that in resistant variety.

To study the behaviour of Race-2 in Red Mexican two groups of plants were grown and inoculated with suspensions of the two races as outlined above. Washed bacterial suspensions were standardized to equal turbidity measured in a nephelometer. Here again monofoliates were used. Four samples of each race were taken immediately after inoculation and over a period of 12 days. Growth curves of the two races are represented in Figure 12.

Multiplication of the two races was very much similar during the first two days; after four days, however, a much wider difference in population was observed. As far as the rate of growth is concerned, Race-2 in Red Mexican behaved much the same as Race-1 in susceptible plants and the water-soaking symptom was apparent 4-5 days after inoculation.

Figure 12.


GROWTH CURVES OF R-1 AND R-2
IN RED MEXICAN
R-1
R-2
LOG. BACT. COUNT
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
DAYS

Reactions of two new lines to the two races

Two new lines of Phaseolus vulgaris were kindley offered by Mr. Kovachich of Unilever Ltd. These varieties were reported to be tolerant to the two races and they were given accession numbers Ab 116 and Ab 395.

Plants from these two varieties were tested for their reactions to the two races and multiplication of bacteria in the leaves was determined. Reactions and multiplication of bacteria in the two varieties were compared with those of Canadian Wonder and Red Mexican.

Both varieties reacted similarly to the two races. A few days after inoculation lesions started as transparent, water-soaked zones but these soon turned necrotic becoming brownish purple in colour and surrounded by a halo. No systemic chlorosis developed in younger, uninoculated leaves. Figures 13 and 14 illustrate the reactions of these two varieties, Red Mexican and Canadian Wonder to the two races.

Multiplication of bacteria per leaf disk taken 5 days after inoculation is shown in Figure 15. The two races grew to about the same extent in Ab 116 but Race-2 grew slightly more than Race-1 in Ab 395. Differences in growth in Canadian Wonder and Red Mexican were as expected.

Figure 13.

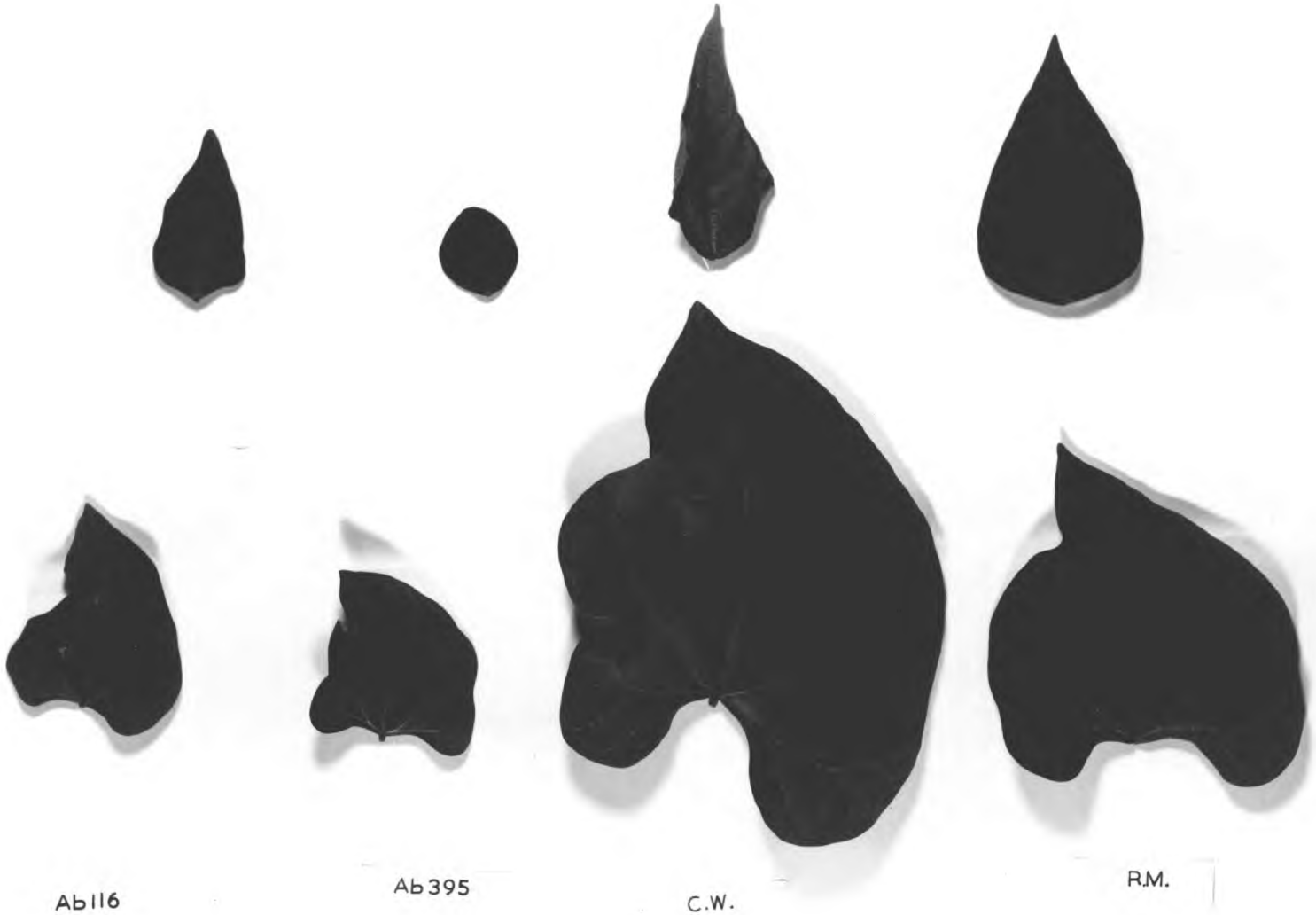


Figure 14.

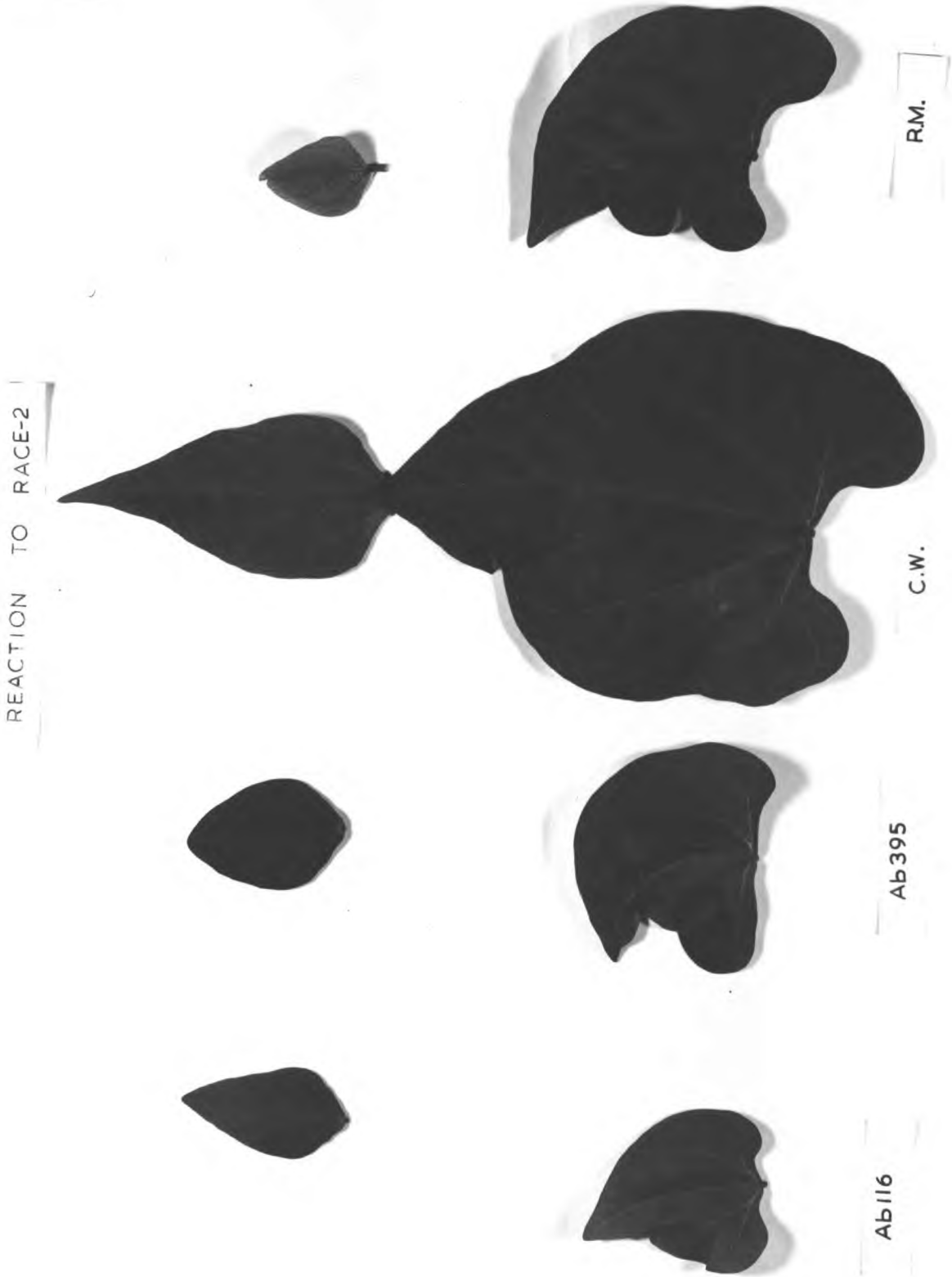
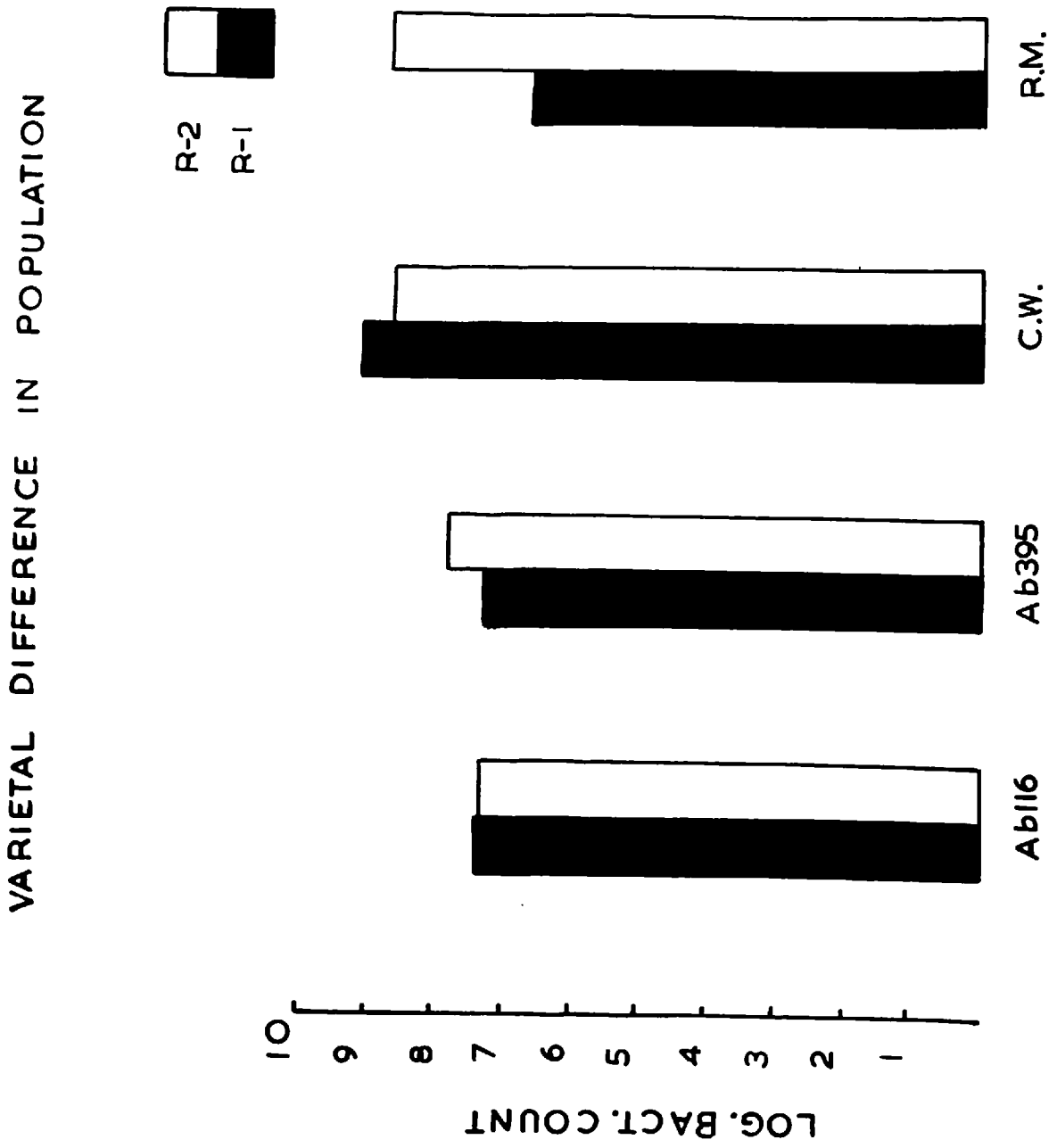


Figure 15.


VARIETAL DIFFERENCE IN POPULATION
R-2
R-1
R.M.
C.W.
Ab395
Ab116
LOG. BACT. COUNT
10
9
8
7
6
5
4
3
2
1

PART IV EFFECT OF LEAF AGE ON SUSCEPTIBILITY

A. - Number of lesions formed

Preliminary observations indicated that disease symptoms are more pronounced in inoculated young leaves, whereas old leaves are more tolerant.

Two isolates representing the two races were used to study the effect of leaf age. The four upper most trifoliates of susceptible variety were sprayed with a fine mist of inoculum until the whole undersurface was uniformly covered, inoculated plants were transferred to conditions of high humidity for 24 hours before being returned to greenhouse bench.

Seven days after inoculation disease was estimated by counting the number of lesions within a circle made by inprint of metal cap on basal leaflet.

Results of the count according to leaf number, leaf 1 being the oldest are summarized in Table 3.

B. - Bacterial multiplication

From the above experiment it was apparent that there is an increase in resistance with age. To investigate the possibility of a mechanical barrier that hinders disease development in old leaves, multiplication of bacteria in the monofoliates of two age groups was compared.

Plants in the first group were used three days after emergence, when the monofoliates were 2-2.5 inches broad while the second group had reached the second trifoliate leaf stage when the monofoliates were inoculated.

Prescribed areas were inoculated by infiltration method and uniformity in inoculation was maintained as in previous experiments. Table 4 shows results of sampling over a period of 5 days.

Soon after inoculation multiplication rate in young leaves was consistently much higher than that in old leaves. After 5 days viable number per leaf disk was 8.5×10^8 whereas that in old leaves was only 13.8×10^6. This indicates that resistance of old leaves is not due to mechanical barrier, and that other factors in the leaf tissue are responsible for age resistance.

Table 3. Effect of leaf age on number of lesions

Isolate	Race	Mean number of lesions/unit area			
		Leaf number 1	2	3	4
604	1	0.3	1.1	24	38.7
1341	2	0.5	1.0	29.8	38

Average of 12 plants for each determination

Table 4. Effect of leaf age on bacterial multiplication

Inoculated leaf	Mean viable number/leaf disk x 10^2 Time after inoculation					
	0	1 day	2 days	3 days	4 days	5 days
Young	4.7	14.6	98	4625	385,000	8,500,000
Old	5.8	8.7	28	717	63,000	138,000

Figures are means of 4 replicates for each determination.

PART V EXPERIMENTS WITH LEAF EXTRACTS

(i) Buffering capacity of leaf extracts

Garber (1961) reported a correlation between the viable number of Ps. tabaci, and the buffering capacity of the medium in which they were grown. In poorly buffered media the increasing alkalinity was associated with less growth; well buffered media supported better growth. This was related to the fact that juice from leaves of susceptible hosts had a greater buffering capacity than juice from resistant host.

To examine whether extracts from Red Mexican and Canadian Wonder have different buffering capacities, prefrozen 30 g. of young leaves and fully expanded leaves of each of the two varieties were macerated in a mortar and the macerate was pressed through two layers of muslin. The collected filtrate was then centrifuged at 15,000 r.p.m. for 30 minutes at 5°C. the buffering capacity of the supernatant was determined by measuring the volume of 0.01 N NaOH needed to change 2 ml. extract to pH 8 and the results are given in Table 5.

There was no difference in pH values of the four extracts and the volume of 0.01 N NaOH needed to give pH 8 ranged from 4 ml. to 3.5 ml. These differences are unlikely to be significant.

(ii) Behaviour of bacteria in leaf extracts

A. Healthy susceptible and resistant leaf extracts

Garber (1956) in his nutrition - inhibition hypothesis of pathogenicity based on a study of several animal and plant bacterial pathogens, stated that resistance could depend either on the absence of a nutrient factor in the host which is essential to the pathogen or to an inhibitor in, or produced by the host. Chand and Walker (1964) found that Ps. lacrymans multiplied equally well in extracts of both resistant and susceptible cucumber. To see if Red Mexican contains inhibitory substances that would restrict the growth of Race-1, the multiplication of the bacterium in extracts of the two varieties was examined.

250 g. fresh weight of leaves from Red Mexican and Canadian Wonder were kept at - 20°C. for 24 hours then extracted as in previous experiment. The leaf extract was filter sterilized through an 'Oxoid' membrane filter before being transferred to sterile flasks in 50 ml. quantities. Each flask was inoculated with 0.05 ml. (approx.) of 24 hours old washed bacterial suspensions of Race-1. The number of viable bacteria per ml. of extract was determined immediately after inoculation and at intervals during the following 96 hours. Flasks were incubated at 25°C. Results of viable

counts are shown in Figure 16.

The pattern of growth in the two extracts was the same suggesting no inhibitor in resistant leaf extract.

B. Growth behaviour of Race-1 in aqueous extracts from healthy and infected resistant leaves

Young monofoliates of Red Mexican plants were heavily infiltrated with Race-1 bacterial suspension so that the whole leaf became water-soaked.

After 4 days, 30 g. of infected and healthy leaves of the same age were frozen at - 20°C. before being macerated in 0.1 M phosphate buffer (pH 6) then centrifuged as in the previous experiments. The volumes were then reduced to 30 ml. each in a rotary evaporator at 40°C. under reduced pressure, then the extracts were filter sterilized and inoculated with a dilute washed suspension of Race-1 from 24 hours culture. Still leaf extract cultures were incubated at 25°C. and viable numbers were determined immediately after inoculation and during the following 96 hours. The results are given in Table 6.

Viable counts during the period of sampling showed inconsistent variation. Determination after 96 hours did not give much difference in bacterial number in the two extracts.

MULTIPLICATION OF RACE-I IN SUSCEPTIBLE AND RESISTANT HEALTHY LEAF EXTRACTS

Figure 16.

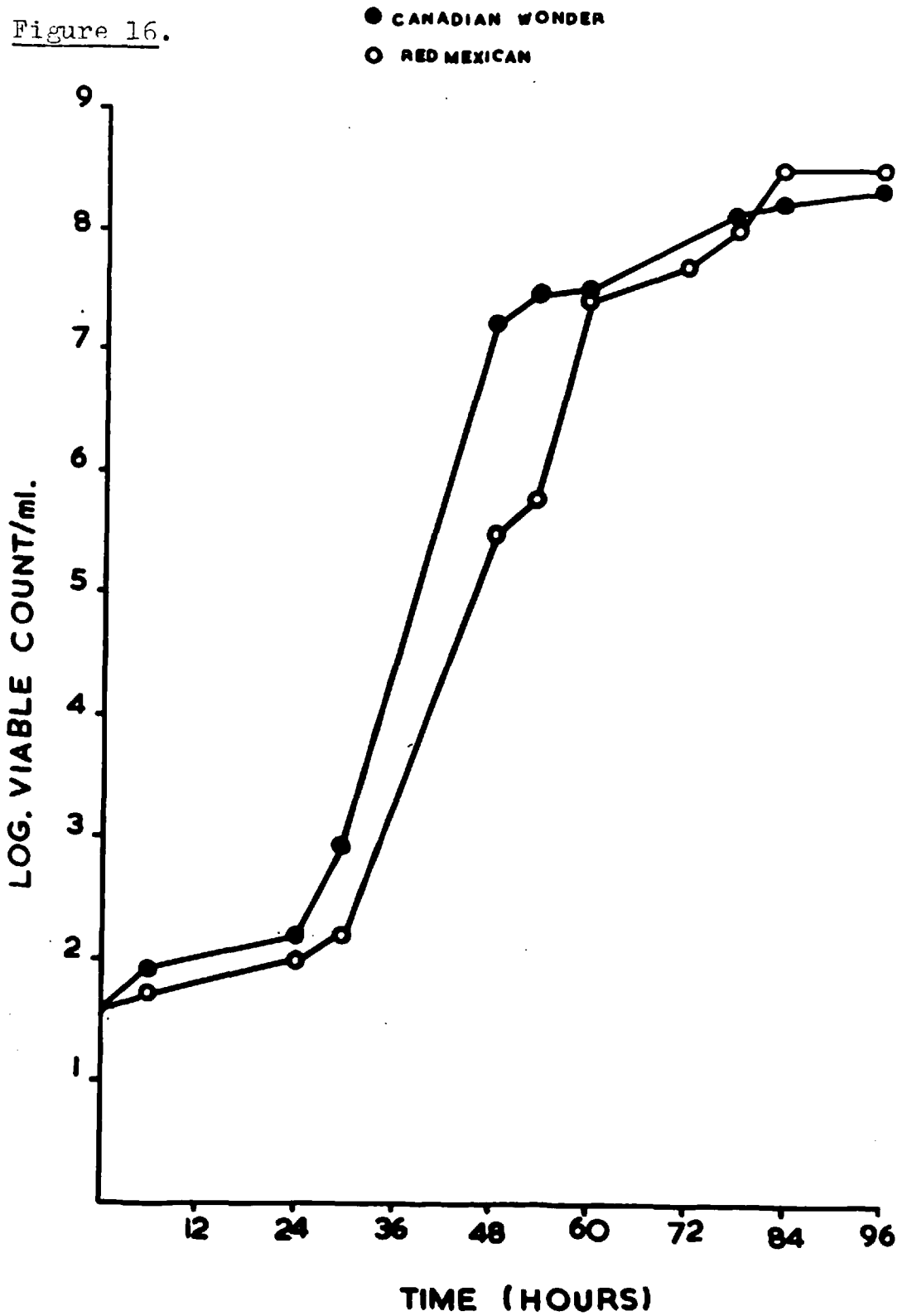


Table 5. pH and buffering capacity of leaf extracts (2 ml.)

	Canadian Wonder		Red Mexican	
	Young leaves	Expanded leaves	Young leaves	Expanded leaves
pH	5.9	6.1	6.1	6.1
0.01 NaOH Needed to give pH 8	3.6	4.0	3.8	3.5

Table 6. Growth of Race-1 in aqueous extracts from healthy and infected resistant leaves

Time (hours)	Mean viable count/ml.	
	Infected	Healthy
0	121	130
24	410	280
48	113×10^3	131×10^3
72	83×10^6	237×10^6
96	265×10^6	244×10^6

Average of 3 determinations.

PART VI EFFECT OF ADDITION OF NUTRIENTS TO THE INOCULUM ON BACTERIAL MULTIPLICATION IN VIVO

The effect of easily utilizable carbon and nitrogen sources was studied separately and in combination.

a. Effect of glucose

Prescribed areas of susceptible and resistant monofoliates were inoculated with suspensions of equal number of bacteria/ml. containing 0.0, 0.5 and 1 per cent glucose. Inoculated leaves were sampled after 2 days and 4 days. Results of viable counts are shown in Table 7.

Two days after inoculation disks receiving 0.0, 0.5 and 1 per cent glucose gave 98 x 10^2, 82 x 10^2 and 92 x 10^2 viable cells in susceptible, corresponding numbers in resistant variety were 81 x 10^2, 86 x 10^2 and 84 x 10^2. From these figures it appears that in neither of the two varieties there seems to be any stimulation of multiplication. Lack of stimulation was also observed in samples taken four days after inoculation. Not much difference between population in the two varieties was noted in samples taken 2 days after inoculation and the expected increase in susceptible variety only became much more apparent after 4 days.

b. Effect of caseinhydrolysate

A similar experiment was done with caseinhydrolysate instead of glucose and results are summarized in Table 8.

In susceptible variety, two days after infiltration an average of 16 x 10^3 viable cells was recorded when no caseinhydrolysate was added to the inoculum, 26 x 10^3 and 37 x 10^3 for 0.5 and 1 per cent levels. Figures for the same period in Red Mexican were 17 x 10^3, 28 x 10^3 and 31 x 10^3 for 0.0, 0.5 and 1 per cent caseinhydrolysate. Unlike the case of glucose there seems to be a slight increase in viable number when caseinhydrolysate was added, however this was only the case of two day samples, viable counts taken after 4 days often fell below that of control and results were inconsistent.

c. Supplementing glucose and caseinhydrolysate

Bacterial suspensions for inoculation were prepared in solutions containing:-

(1) 0.5 per cent glucose and 0.5 per cent caesin-hydrolysate.

(2) 1 per cent glucose and 1 per cent caesinhydrolysate.

(3) Control suspension was in sterile distilled water.

All suspensions contained the same number of bacteria/ml. Table 9 gives bacterial numbers in the two varieties.

Table 7. Effect of addition of glucose to the inoculum on bacterial multiplication in vivo

Days	Variety	Mean viable count/disk		
		No glucose	0.5% glucose	1% glucose
2	C.W.	9830	8200	9230
	R.M.	8140	8670	8440
4	C.W.	252×10^5	276×10^5	190×10^5
	R.M.	813×10^2	760×10^2	963×10^2

Average of 3 disks for each treatment.
C.W. = Canadian Wonder R.M. = Red Mexican

Table 8. Effect of addition of caseinhydrolysate to the inoculum on bacterial multiplication in vivo

Days	Variety	Mean viable count/disk		
		No casein-hydrolysate	0.5% casein-hydrolysate	1% casein-hydrolysate
2	C.W.	16100	26200	37200
	R.M.	17700	28800	31800
4	C.W.	520×10^5	453×10^5	596×10^5
	R.M.	943×10^2	763×10^2	893×10^2

Average of 3 disks for each treatment.
C.W. = Canadian Wonder R.M. = Red Mexican

Table 9. Effect of addition of glucose and caseinhydrolysate to the inoculum on bacterial multiplication in vivo

Days	Variety	Mean viable count/disk		
		Water control	0.5% glucose + 0.5% caseinhydrolysate	1% glucose + 1% caseinhydrolysate
2	C.W.	223×10^2	320×10^2	300×10^2
	R.M.	65.6×10^2	100×10^2	108×10^2
4	C.W.	147×10^6	156×10^6	183×10^6
	R.M.	220×10^3	212×10^3	227×10^3

Mean of 3 determinations for each treatment.
C.W. = Canadian Wonder.
R.M. = Red Mexican

The trend was very much the same as in above mentioned experiment, a slight stimulation after two days and not much difference in samples taken four days after inoculation.

The lack of response of bacterial multiplication to added nutrients may be due to translocation of nutrients away from infection site early before bacteria could make full use of them.

PART VII MIXED INOCULA

(1) Effect of preinoculation with Race-1 on the number of water-soaked lesions caused by Race-2 in Red Mexican

From previous experiments it was apparent that one difference between the two races is the inability of Race-1 to multiply as much as Race-2 in Red Mexican. The capacity of Race-2 to multiply at a faster rate is associated with water-soaking while infection with Race-1 at all concentrations which were used failed to show this symptom. In the following experiment the effect of preinoculation with Race-1 on the production of water-soaked lesions by Race-2 was studied.

Half unifoliate leaves of Red Mexican variety were inoculated with washed suspensions of Race-1 at the following concentrations; 10^4, 10^5, 10^6 and 10^7 cells/ml. The other half was prewater-soaked with sterile distilled water and was shielded when suspensions of Race-1 were being infiltrated. Excess bacteria were then washed off with sterile distilled water. Two hours later when the water-soaking effect had disappeared, both half leaves were again uniformly infiltrated with dilute suspension of Race-2, 5×10^3 cells/ml. After 5 days, small, discrete microscopic water-soaked lesions about1 mm. diameter appeared on both halves. Five disks 15 mm. diameter, taken at random, were

cut out from each half leaf and the number of water-soaked lesions was counted under a binocular. This was repeated and the results of the two experiments are given in Table 10.

Necrosis of the tissues was the predominant symptom at 10^7 and 10^6 Race-1 levels. At 10^7 cells/ml. an average of 1.0 water-soaked lesion in the first experiment and 1.4 in the second experiment was recorded for disks which received mixed inocula. Corresponding control figures were 10.3 and 18.5. Reduction in number of lesions in leaf halves preinoculated with Race-1 was also observed at 10^6 level where the average number of water-soaked lesions was 1.1 in first experiment and 0.8 in second. When Race-1 was reduced to 10^5 cells/ml. more lesions were formed in half leaves receiving mixed inocula than at higher levels of Race-1 yet the number per unit area was still much less than control halves preinfiltrated with water only. There was no reduction in number of lesions when the non-virulent race was 10^4 cells/ml.

These results indicate an inverse relation between concentration of Race-1 preinoculated and number of lesions formed as a result of inoculating the virulent race in the same place.

Table 10. Effect of different concentrations of Race-1 on number of water-soaked lesions caused by Race-2

Expt. No.	Mean number of water-soaked lesions/15 mm. disk							
	10^4	C	10^5	C	10^6	C	10^7	C
1	10.6	10	8	15.2	1.1	14.0	1.0	10.3
2	15.8	15.9	14.4	17.7	0.8	20.2	1.4	18.5

15 disks/treatment.

(2) Interaction between the two races in vivo

Klement and Lovrekovich (1961) demonstrated inhibition of Xanthomonas juglandis, pathogenic to walnut by Pseudomonas syringae, (lilac strain) when bean pods were injected with increasing numbers of the latter mixed with a constant number of the former. The two organisms in culture are not antagonistic to one another. Averre and Kelman (1964) reported that severity of bacterial wilt in tobacco, tomato and eggplant is reduced markedly if a high proportion of an avirulent strain is mixed with a virulent race of Pseudomonas solanacearum. This also happens when cells of an isolate of Race-1 pathogenic to tobacco are mixed with cells of Race-2 pathogenic to banana.

To study the interaction between Race-1 and 2 of Ps. phaseolicola in vivo, washed suspensions from 48 hour cultures of the two races in sterile distilled water were

standardized to equal turbidity then mixed together in the following proportion of R-2 to R-1, 100:0, 100:25, 100:50, 100:75 and 100:100. The concentration of Race-2 in all suspensions was thus kept constant. Control plants were inoculated with R-1 only, after the inoculum was adjusted to the correct number/ml. by adding sterile water. Prescribed areas of Red Mexican monofoliates were infiltrated with each of these bacterial mixtures in the usual manner and viable counts per leaf disk were determined immediately after inoculation and 4 days later. The results are summarized in Table 11.

Race-1 at concentrations used in this experiment did not induce much reduction in the final populations and total counts from mixed inocula were either slightly higher or less than that of R-2 only. The same experiment was repeated this time R-1 was constant and R-2 varied to give 100:0, 100:25, 100:50, 100:75 and 100:100 R-1/R-2. The results are given in Table 12 and Figure 17.

One limitation here is that multiplication in the mixture could only be compared with that of control, as there is no cultural method of separating the two races the final viable counts of mixed inocula are the total contributions of both races. When R-1/R-2 was 100:25 multiplication of mixture was almost ten fold less that of R-2 control. Increasing the ratio of R-2 to 50 per cent that

of original concentration also resulted in less multiplication and counts from corresponding R-2 control were nearly three times higher. Race-2 multiplication at 75 and 100 per cent levels was slightly higher than that of corresponding mixed inocula. This means that the avirulent race did affect multiplication of the virulent race at lower concentrations of the latter.

Table 11. Effect of mixing different concentrations of Race-1 with constant number of Race-2 on bacterial multiplication in Red Mexican

Time of sampling	Mean bacterial number/leaf disk								
	R-2 Only	Percentage R-1 added to constant R-2							
		25		50		75		100	
		M	C	M	C	M	C	M	C
Immediately after inoculation	77×10^2	88.5×10^2	16.5×10^2	89×10^2	31×10^2	112×10^2	46×10^2	121.5×10^2	50×10^2
4 days	157×10^6	199×10^6	4.7×10^6	182×10^6	6.5×10^6	101×10^6	6.7×10^6	119×10^6	4.0×10^6

Average of two replicates

M = R-1 + R-2 (Mixed inocula)

C = R-1 Only (Control)

Table 12. Effect of mixing different concentrations of Race-2 with constant number of Race-1 on bacterial multiplication in Red Mexican

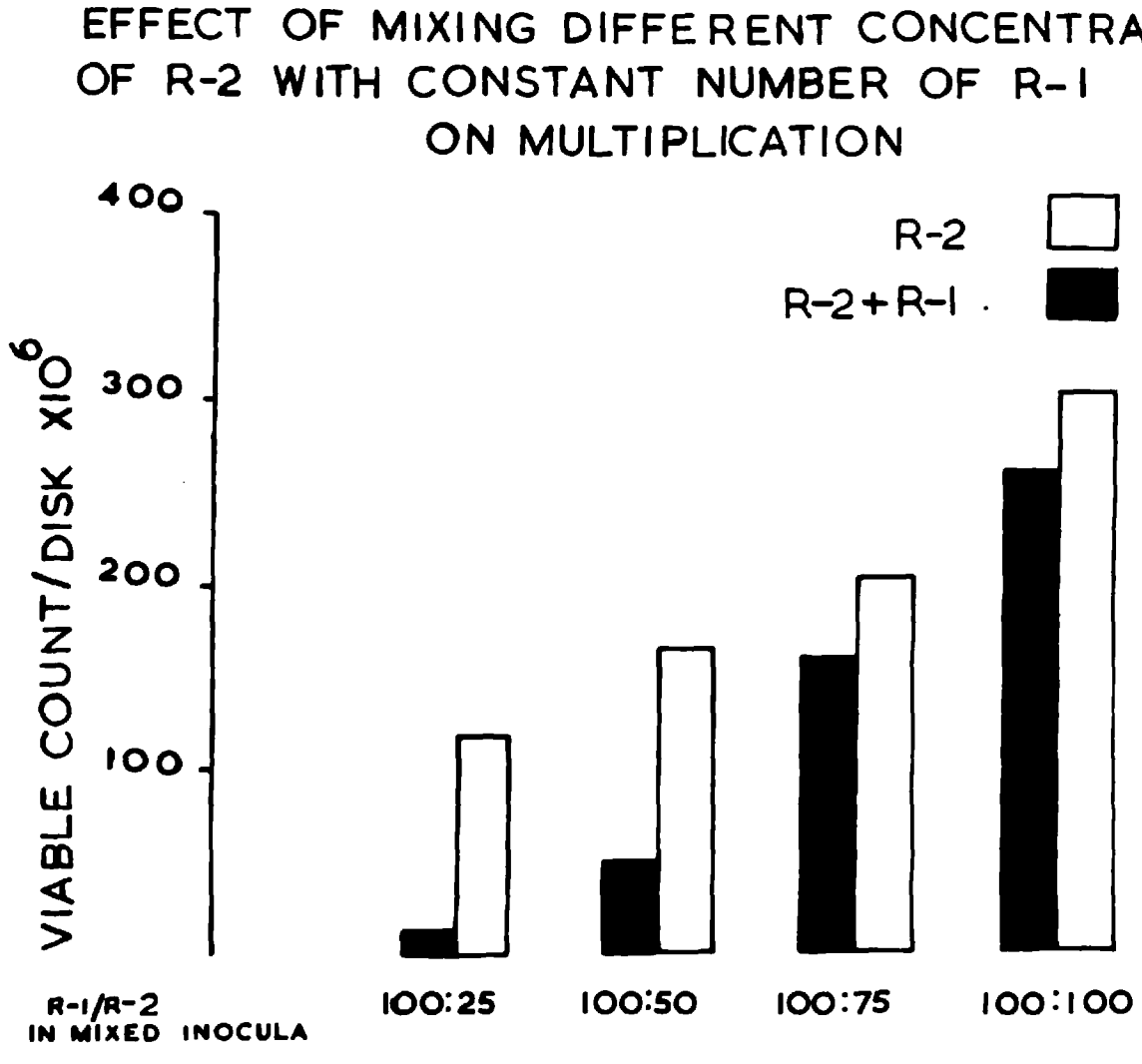
Time of sampling	Mean bacterial number/leaf disk								
	R-1 Only	Percentage R-2 added to constant R-1							
		25		50		75		100	
		M	C	M	C	M	C	M	C
immediately after inoculation	$22x10^2$	$28x10^2$	$9.9x10^2$	$46x10^2$	$12.6x10^2$	$54.8x10^2$	$15.3x10^2$	$56.5x10^2$	$16.3x10^2$
4 days	$84x10^5$	$115x10^5$	$118x10^6$	$49x10^6$	$167x10^6$	$163x10^6$	$206x10^6$	$265x10^6$	$306x10^6$

Average of two replicates

M = R-1 + R-2 (Mixed inocula)

C = R-2 only (Control)

Figure 17.


EFFECT OF MIXING DIFFERENT CONCENTRATIONS OF R-2 WITH CONSTANT NUMBER OF R-1 ON MULTIPLICATION
400
300
200
100
VIABLE COUNT/DISK X10^6
R-2
R-2+R-1
R-1/R-2 IN MIXED INOCULA
100:25
100:50
100:75
100:100

(3) Effect of preinoculation with Race-1 on multiplication of Race-2 in Red Mexican

Washed bacterial suspensions of Race-1 and Race- 2 from 48 hour cultures were standardized to give equal turbidities. Half unifoliates of Red Mexican were infiltrated with Race-1 while shielding the other half and any excess bacteria were washed off with sterile water. Race-2 infiltration on either side of the mid-rib between two main veins was done;

1. Immediately after the water-soaking had disappeared.
2. Six hours after infiltrating Race-1.
3. Twenty four hours after infiltrating Race-1.

Samples for viable count were taken 5 days from the start of the experiment. The results are given in Table 13.

In all cases there was a great reduction in viable number of R-2 caused by preinoculation with R-1. Differences in population of mixed inocula due to time lag were inconsistent.

Table 13. Effect of preinoculation with Race-1 on multiplication of Race-2 in Red Mexican

Mean bacterial number/leaf disk x 10^5 after 5 days						
Race-1	Time after inoculation of Race-1 (hrs)					
	0		6		24	
	M	C	M	C	M	C
58	43.1×10^2	92.4×10^2	21.8×10^2	103×10^2	38.2×10^2	84.2×10^2

Average of 3 replicates

M = R-1 + R-2 (Mixed inocula)

C = R-2 Only

PART VIII TESTING FOR PECTIC AND CELLULOLYTIC ENZYMES

1. Pectic enzymes

Lesions formed by Ps. phaseolicola are restricted in size; very young leaves produce slightly bigger lesions than those which have expanded at the time of inoculation. Incubating inoculated plants under humid conditions did not favour spread of lesions and their size remained more or less the same as controls kept on the greenhouse bench. Addition of nutrients as glucose and caseinhydrdysate did not stimulate an increase of viable numbers of bacteria and there was no spread beyond the area of inoculation. These observations suggested that a look into the organisms' ability to produce pectolytic and cellulolytic enzymes might be profitable.

Sabet and Dowson (1951) using a pectate gel medium reported that the organism causes liquefaction. Oxford (1944) on the other hand could not detect any cell wall degrading enzymes. Smith (1958) found that out of 3 isolates tested one isolate showed neither pectin glucosidase (PG) nor methylesterase activity while two isolates had pectin glucosidase but no methylesterase activity.

In a preliminary experiment the organism was grown on the medium used by Sabet and Dowson (1951). It was prepared

by adding 5 g. sodium polypectate to 500 ml. water at 70°C. on a magnetic stirrer. This was followed by 0.5 g. ammonium dihydrogen phosphate and 1.0 g. sodium asparaginate and the mixture was adjusted to pH7 with 0.1 N NaOH. The medium was then dispensed in test tubes before sterilization and after sterilization allowed to stand in water at room temperature; when set, gels were stabbed with the eight isolates available and tubes were incubated at 16°C. All isolates gave very poor growth and no liquefaction was apparent in any of the inoculated tubes.

Growing bacteria in liquid media supplemented with pectic substrates

The basic liquid medium was 0.4 per cent caseinhydrohysate, KH_2PO4, $MgSO4.7\ H_2O$ and 0.1 per cent glucose. This was supplemented with 0.9 per cent pectin or sodium polypectate. The latter was dissolved separately then added to the other components. The medium was adjusted to pH7 before autoclaving at 10lb/Sq.in. After four days growth in shake culture, filtrates from cultures of 4 isolates were tested for macerating activity. Potato disks 0.5 mm. thick were injected with water under reduced pressure then flooded with culture filtrates (pH 7.2) and the coherence of tissues was compared with that of disks in sterile medium.

None of the isolates caused maceration over the period of testing (24 hours). Vital staining (Tribie, 1951) of disks pretreated with culture filtrate for 6 hours then transferred to a mixture of KNO_3, 0.5 ml.; 0.1 per cent neutral red chloride, 1.0 ml; and buffer (pH7.6), a few drops, showed no difference from control disks deposited in sterile medium for the same period of time then stained in the same way. This showed that the culture filtrate did not kill potato cells over this period.

Testing the effect of filtrates on viscosity of pectin and sodium polypectate solutions

The ability of culture filtrate to reduce the viscosity of pectic solutions has been used by many workers to estimate pectingalacturonase activity.

Six Cannon-Fenske viscometers were set in a constant water bath controlled at 25°C. by a mercury - toluene thermostat and stirred mechanically. Flow time for 10 ml. phosphate buffer solutions were determined. The effect of culture filtrate on viscosity of substrate was measured by the flow time at intervals of 1, 30, 60 minutes and 3 hours after adding the test solution. Substrate reaction mixtures consisted of 5 ml. of substrate (Pectin or sodium polypectate, 1 per cent w/v solution adjusted to pH 6.5 with 0.1 N NaOH), 2 ml. of 0.2M phosphate buffer pH 6.5, 2 ml

distilled water and 1 ml. test filtrate delivered in each viscometer. Tables 14 and 15 give specific viscosity at 1, 30, 60 minutes and after 3 hours.

Specific viscosity calculated from

$$n_{sp} = \frac{n - n_o}{n_o}$$

where n_{sp} = specific viscosity.

n_o = viscosity of solvent (buffer solution).

n = viscosity of solution.

None of the four tested filtrates reduced the viscosity of tested substrate under the set conditions of the experiment. This showed that none had any polygalacturonase activity. This is a surprising result because most species of Pseudomonas are active in this way.

2. Cellulolytic enzymes

Substituting pectic substances with carboxymethyl cellulose in the medium and then testing culture filtrate for cellulase activity using the viscosity method mentioned above also showed no activity. The results are given in Table 16 None of the filtrates gave any reduction of viscosity of solution under test and flow time remained more or less constant suggesting a complete lack of cellulolytic activity. This too was a rather surprising result because other pathogenic species e.g. Ps. solanacearum secrete cellulase freely under the conditions used in this experiment.

Table 14. Testing effect of culture filtrate on viscosity of pectin solution

Isolate tested	Specific viscosity			
	Time after addition of culture filtrate (minutes)			
	1	30	60	180
380	5.96	5.79	5.79	5.79
604	5.31	5.34	5.34	5.34
1341	5.81	5.76	5.76	5.75
1098	5.80	5.78	5.77	5.77

Table 15. Testing effect of culture filtrate on viscosity of sodium polypectate solution

Isolate tested	Specific viscosity			
	Time after addition of culture filtrate (minutes)			
	1	30	60	180
380	5.82	5.93	5.93	5.88
604	5.81	5.73	5.75	5.73
1341	5.87	5.83	5.87	5.87
1098	4.93	5.04	4.98	4.99

Table 16. Testing culture filtrate for cellulase (Cx) activity

Isolate tested	Specific viscosity			
	Time after addition of culture filtrate (minutes)			
	1	30	60	180
380	4.99	4.99	4.96	4.95
604	5.16	5.13	5.13	5.11
1341	5.17	5.12	5.13	5.14
1098	7.97	7.81	7.54	7.58

PART IX EXPERIMENTS ON PHYTOTOXIN

Isolates of the same toxigenic bacteria may differ in their ability to produce the toxic metabolite. The only reported difference between Ps. tabaci and Ps. angulata is the toxigenicity of the former but not of the latter species. These species could not be separated on the basis of morphological, physiological, cultural or serological characteristic (Braun, 1937). Furthermore, non-toxigenic strains of Ps. tabaci obtained in vitro could not be distinguished from Ps. angulata. Fulton (1950) was not able to identify the species using bacteriophages isolated from each species. While some investigators maintained that these species are identical, others consider one to be a variant of the other, that is toxigenicity or its absence being taxonomically a trivial character.

Jensen and Livingston (1944) reported the failure of some isolates of Ps. phaseolicola to produce chlorosis on beans in conditions otherwise optimum for production of symptoms. Preliminary observations indicated that the halo and chlorosis associated with disease are very much affected by the stage of maturity of leaves when inoculated as well as by the concentration of inoculum applied. The younger the leaves the more pronounced and more spreading was the

chlorosis. When old leaves were inoculated hardly any halo was observed. Moreover, massive infiltration of hypersensitive Red Mexican with Race-1, when the leaves are still young, produced systemic chlorosis of developing trifoliates. These, however, regained their normal green colour after few days.

Since lesions on young leaves sustain a much higher population of bacteria this suggests that chlorosis is a function of bacterial multiplication within the tissues. It is also possible that mature leaves are much more tolerant of the action of the metabolite.

To compare the capacity of the different isolates to produce toxic metabolites in vivo the following experiment was conducted. Bacterial suspensions from seven isolates, standardized in a nepholometer to give equal number of cells, were point infiltrated into monofoliates at a stage of growth when leaves were most susceptible. Four plants were inoculated with each isolate. After 12 days the halo surrounding each infected region was measured along two diameters. The degree of chlorosis of first and second trifoliates was assessed as percentage of chlorotic leaflets. The results are summarized in Table 17.

So far as the size of halo was concerned, the isolates tested did not produce much difference because the diameter

of the zone varied in size only between 18 and 22 mm. Chlorosis of developing trifoliates was highest with 1098 (R-1) and 1341 (R-2).

Table 17. Size of halo and percentage chlorosis of first and second trifoliates induced by isolates of Ps. phaseolicola

Isolate Number	Zone of chlorosis (mm.)	Percentage chlorosis of trifoliates
1098	21	78
1057	22	62
1103	18	39
604	21	46
605	18	66
1341	20	69
380	21	64

Average of 4 plants for each treatment.

Production of symptoms by culture filtrate

In a preliminary experiment, 100 ml. of dextrose-peptone medium in flasks was inoculated with Race-1 and incubated at 22°C. Culture filtrates were collected by centrifugation and filtration through a membrane filter after 3, 7, 10 and 15 day's growth and the filtrates were tested for activity in four different ways.

(1) 0.05 ml. drops of filtrates were spotted on the upper surface of undetached Canadian Wonder leaves then pricked with sterile needle. Treated plants were immediately transferred to conditions of high humidity under artificial light.

(2) Water-soaking prescribed areas of the undersurface of leaves with culture filtrate.

(3) Water-soaking half unifoliates with culture filtrate.

(4) Placing cut ends of shoots in culture filtrate for 24 hours in a growth cabinet before transferring to Long Ashton nutrient solution.

To assess phytotoxicity due to other components of the medium that might produce effects similar to those of toxin, each of these tests was provided with two control sets of plants, one treated with autoclaved medium and the second with sterile water. Monofoliates for tests 1, 2 and 3

were 4-5 cm. broad (not fully expanded) when used, while cut shoots were in the fully expanded monofoliate stage.

Plants treated with 3 day old culture filtrates did not become chlorotic. Although chlorosis of monofoliates was apparent when leaves were treated with 7 day old filtrates, cut shoots treated with 7 day filtrates failed to show any symptoms whereas 10 and 15 day old filtrates gave the typical chlorosis of developing trifoliates. On cut shoots, chlorosis of the developing trifoliates was the only symptom of a phytotoxin, and mature leaves were completely unaffected. Chlorotic leaves, however, recovered and became green as they expanded. Apart from a slight puckering of infiltrated leaves, the dextrose-peptone medium alone had little toxicity. Cut shoots suffered from flaccidity of the leaves and a slight wilting but soon recovered when transferred to nutrient solution.

Symptoms of chlorosis on infiltrated leaves started to appear after two days and reached a maximum expression four days from the time of infiltration. When half leaves were infiltrated quite frequently symptoms spread to other half of the leaf (Figure 18).

Testing infected leaf extract

Monofoliates of a large number of susceptible plants were mass infiltrated at a number of points with 24 hour old



Figure 18. Chlorosis caused by culture filtrate

washed bacterial suspension. Seven days later when the lesions had developed, 30 g. of infected tissues were extracted in 60 ml. of water in a mortar. The extracts were centrifuged twice at 15,000 r.p.m. and the supernatant was reduced to 30 ml. in a rotary evaporator at 40°C. The extract was then filter sterilized by passing through an 'Oxoid' membrane filter. An equal weight of healthy leaves was also extracted, adjusted to same volume and sterilized in the same way.

No evidence of a phytotoxin was obtained when the extract from diseased tissues was infiltrated into bean leaves and extracts from healthy leaves produced much the same effect. Cut shoots allowed to take up the extract before they were transferred to nutrients also failed to develop chlorosis of developing trifoliates. This lack of activity may be due to the fact that the toxin is produced only in small amounts and is continuously translocated from infected tissue. Another factor and one which may be more important is that the toxic principle may combine with other substances in the plant making it inactive when crude extracts are tested. It is also conceivable that toxin is not highly potent and because healthy extracts show some phytotoxicity when infiltrated into bean leaves, activity of the small amount of toxin in the extract may be masked by that of other substances.

Comparison of activity of culture filtrates from seven isolates

The seven isolates previously compared for production of halo symptoms and chlorosis of developing trifoliates were tested for ability to produce the chlorosis inducing principle *in vitro*. Twelve day old culture filtrates of cultures on dextrose-peptone medium were tested by the infiltration technique. Results are summarized in Table 18.

Apart from isolate 1103 and 605 which did not give uniform chlorosis, all other filtrates were active and chlorosis spread to uninoculated half leaves.

Table 18. Testing culture filtrate obtained from seven different isolates

Isolate	Chlorosis
1098	++
1057	++
1103	+
604	++
605	+
1341	++
380	++

++ Uniform chlorosis + Slight chlorosis

Comparison of different liquid media for production of phytotoxin

The object of this experiment was to compare different liquid media for production of phytotoxin. Both chemically defined media and media containing organic sources of nitrogen were tried. The basic chemically defined medium was that of Vogel and Bonner (1956) used by Garber (1959) for growing the organism. This medium consists of the following:-

	$Mg\ SO_4.7H_2O$	0.02 per cent.
	Citric acid H_2O	0.20 per cent.
	$K_2H\ PO_4$ (anhydrous)	1.00 per cent.
	$NaNH_4\ PO_4$ (anhydrous)	0.35 per cent.
and	Glucose	1.00 per cent.

This chemically defined medium was used alone and supplemented with 0.4 per cent L-asparagine (monohydrate) or glutamine other media were dextrose-peptone, glucose-caesaminoacids, and nutrient broth (see Materials and Methods).

Bacteria grew best in the glucose-caesaminoacid and dextrose-peptone media causing distinct turbidity with formation of pellicle and substantial sediment. A slight turbidity was noted in nutrient broth while the chemically defined media gave very poor growth. When the culture filtrates were tested by the infiltration method, only

glucose-caseinhydrolysate and dextrose-peptone filtrates gave definite symptoms. Less clear chlorosis was obtained from nutrient broth and filtrates from chemically defined media.

In all subsequent experiments glucose-caesinhydrolysate medium and isolate 1098 of Race-1 and 1341 of Race-2 were used for obtaining active culture filtrates.

Assessing activity of culture filtrate

1. Chlorophyll estimation -

One of the direct effects of the phytotoxin on infected or culture filtrate injected plants is the reduction in chlorophyll content. Total chlorophyll content of infiltrated leaves when expression of symptoms was maximum normally four days after infiltration, has been found to be a good measure for assessing activity of filtrates.

Equal number of disks were cut with a cork borer from different areas of infiltrated half leaves. The fresh weight was determined and samples were frozen at - 20°C. The method for total chlorophyll extraction and determination was that given under Materials and Methods (P. 30).

2. Another effect of culture filtrates was the retardation of expansion of the infiltrated leaf. Table 19 gives total chlorophyll content per g. fresh weight of leaves

infiltrated with culture filtrate, medium or water. Whereas sterile medium did not interfere with chlorophyll synthesis and had little if any effect on expansion of infiltrated not fully expanded leaves, the culture filtrates reduced chlorophyll content of infiltrated leaf halves by over 30 per cent that of water treatment, moreover, there was a pronounced retardation of leaf expansion.

Relation of incubation temperature to activity of culture filtrate

Goss (1940), and Patel and Walker (1963) found that halo production is favoured by low temperature and that there is a definite reduction in the size of chlorotic areas surrounding infection point at temperature of 24°C. and above.

To study the effect of temperature on toxin production in liquid medium, still cultures were incubated at 12, 18, 22, 25 and 30°C. The rate of growth at these temperatures was measured in a nephelometer and after 12 days incubation culture filtrates were tested for activity using infiltration method. Four days after infiltration samples were taken from half leaves for chlorophyll estimation. These were compared with medium infiltrated leaves. Figure 19 gives growth curves at these temperatures and total chlorophyll estimations are given in Table 20.

Filtrates from cultures incubated at 12, 18 and 22°C. reduced chlorophyll by over 30 per cent whereas incubation at 25°C. caused an average reduction of only 17 per cent. Filtrates from cultures kept at 30°C. were inactive and only 4.1 per cent reduction was observed.
Bacterial growth rate was highest at 22°C. and least at 30°C.

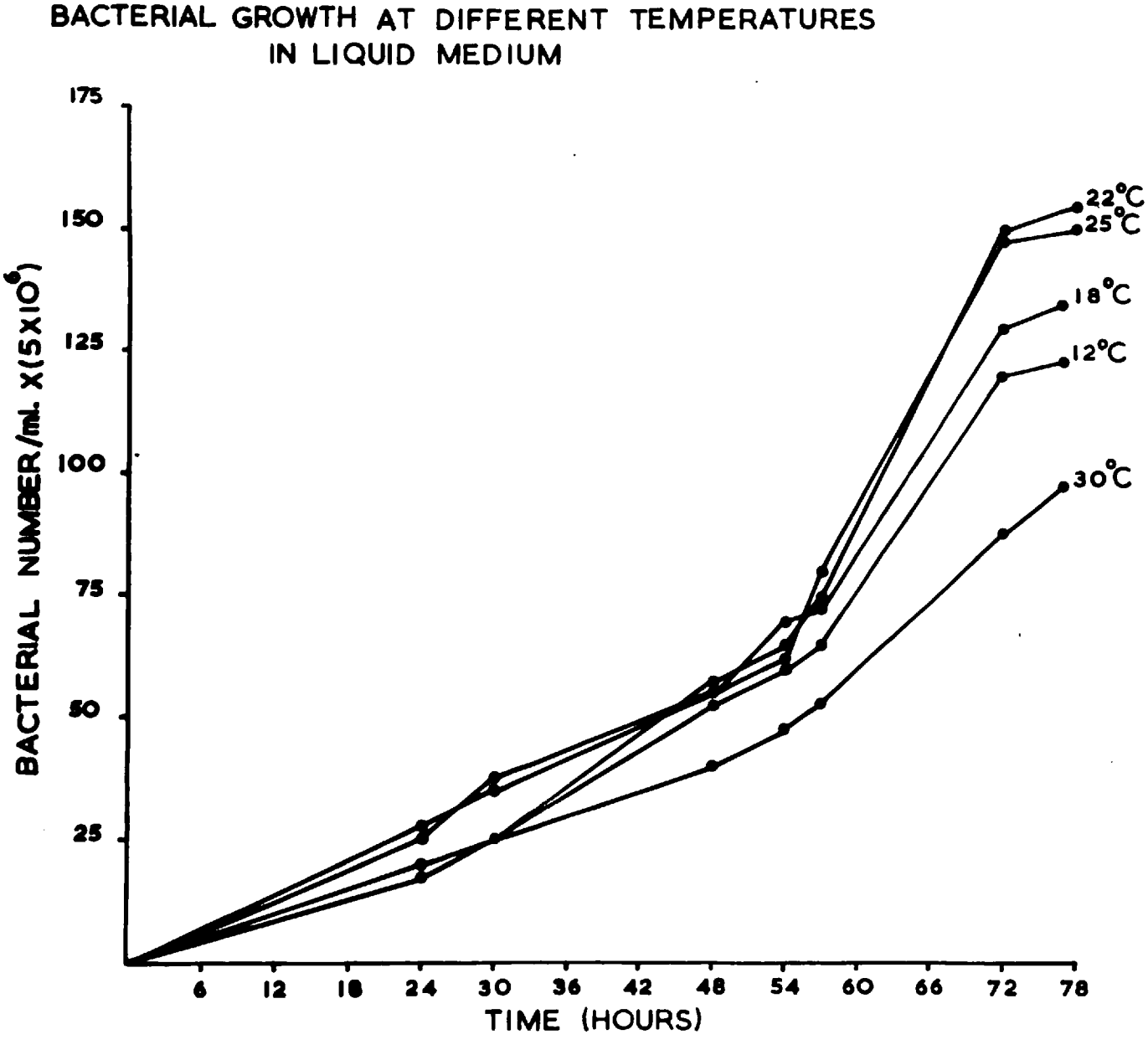

BACTERIAL GROWTH AT DIFFERENT TEMPERATURES
IN LIQUID MEDIUM
BACTERIAL NUMBER/ml. X(5X10⁶)
175
150
125
100
75
50
25
6
12
18
24
30
36
42
48
54
60
66
72
78
TIME (HOURS)
22°C
25°C
18°C
12°C
30°C

Figure 19.

This experiment indicates that temperatures between 12 and 22°C. are favourable for production of toxic principle responsible for chlorosis, a range which is much the same as that optimum for production of symptoms in inoculated plants.

Table 19. Effect of culture filtrate on total chlorophyll and area of infiltrated half leaf

	culture filtrate treated		Medium treated		water treated	
	Tc (mg./g.f.wt.)	Area (inch)	Tc (mg./g.f.wt.)	Area (inch)	Tc (mg./g.f.wt.)	Area (inch)
1	1.05	3.55	1.53	4.08	1.60	4.12
2	1.07	3.82	1.60	4.18	1.55	4.22

Average of 3 replicates.

Table 20. Effect of incubation temperature on activity of culture filtrates measured as reduction in total chlorophyll of infiltrated leaves

	Incubation temperature					
	12°C.	18°C.	22°C.	25°C.	30°C.	Medium
mg. total chlorophyll/g. fresh wt.	1.08	1.16	1.15	1.41	1.63	1.70
Reduction (%)	36.4	31.7	32.3	17	4.1	-

Average of 3 replicates.

Non-specificity of the toxin

Some plant toxins are host specific affecting only the susceptible host of the pathogen. A classical example is victorin from Helminthosporium victoriae (Meehan and Murphy, 1947). To determine whether the toxin plays any active role in pathogenicity of Race-2 and failure of Race-1 to parasitize the Red Mexican variety, culture filtrates from the two races were infiltrated into half unifoliates of Canadian Wonder and Red Mexican plants. Four days when the expression of symptoms was optimum, leaf samples from culture filtrate treated plants and control as media - infiltrated leaves were taken for estimation of total chlorophyll content. The results (Table 21) represent the average for three samples of total chlorophyll per g. fresh weight.

They show ~~that~~ as percentages a reduction of total chlorophyll. Leaves infiltrates with culture filtrates from Race-1 caused 28 per cent reduction in susceptible Canadian Wonder and 31 per cent reduction in resistant Red Mexican leaves. The corresponding figures for Race-2 culture filtrates are 34 per cent reduction in Canadian Wonder and 27 per cent in Red Mexican. These figures show clearly that the toxin is non-specific. In this respect it resembles the wildfire toxin.

Effect of culture filtrate on root elongation

It has been observed that culture filtrates, beside causing yellowing, check the expansion of infiltrated leaves. It has also been established that the effect of culture filtrate is non-specific. It was now of interest to test the effect of filtrates on root elongation. Cress, Lepidium sativum, tap-roots were preferred to bean roots on account of their fast and uniform elongation. Seeds for this purpose were surface-sterilized in sodium hypochlorite solution containing 2 per cent available chlorine to which a drop of Tween 80 was added and then washed in several changes of sterile water. Seeds were then transferred to sterile Petri dishes and flooded with 3 ml. culture filtrate. Two batches of seeds were used as controls, one soaked in sterile water and the second in autoclaved medium. The average root lengths after 24, 48 and 72 hours are given in Table 22.

The culture filtrate did not check root elongation; the average length of roots was much the same as that of controls.

Effect of dilution on culture filtrate activity

To assess the potency of the crude culture filtrate the effect of dilution on the capacity to induce chlorosis was studied.

Table 21. Effect of filtrates from cultures of the two races on total chlorophyll content

	Variety infiltrated					
	Canadian Wonder			Red Mexican		
	culture filtrate from		Medium alone	culture filtrate from		Medium alone
	R-1	R-2		R-1	R-2	
Total chlorophyll (mg./g. fresh wt)	1.11	1.01	1.55	1.09	1.16	1.59
Reduction (%)	28	34	-	31	27	-

Table 22. Effect of culture filtrate on cress root elongation

	Average length (mm.)		
	Time (hours)		
	24	48	72
Culture filtrate	5.5	9.5	14.3
Medium	6.0	9.0	14.7
Water	6.9	9.2	14.9

Filtrates from glucose-casaminoacid cultures were diluted with water 5, 10, 50 and 100 times then tested for ability to cause chlorosis. Observations are recorded in Table 23.

At a dilution of 1 in 5 the culture filtrate was as active as the undiluted filtrate. Ten fold dilution reduced the activity and chlorosis was not uniform through the infiltrated half leaf. No sign of chlorosis was observed at fifty times dilution and above.

Table 23. Effect of dilution on activity of culture filtrate

Dilution	Chlorosis
Undiluted culture filtrate	++
Culture filtrate diluted 1 in 5	++
Culture filtrate diluted 1 in 10	+
Culture filtrate diluted 1 in 50	-
Culture filtrate diluted 1 in 100	-

++ Marked chlorosis.
+ Slight chlorosis.
- No symptoms.

Effect of heating on culture filtrate

Filter sterilized filtrate from 12 day old cultures was subjected to:

(1) Heating at 60°C. for ten minutes in a hot water bath.

or (2) Autoclaved at 120°C. for 15 minutes.

Unheated and heat treated culture filtrates were tested by the infiltration technique. Control plants were injected with autoclaved medium. Three samples of equal weight were collected from each group of plants 4 days after infiltration for total chlorophyll estimation. Table 24 gives mean total chlorophyll per g. fresh weight and the percentage reduction compared with the controls.

Unheated filtrate caused 34 per cent reduction in total chlorophyll, the average reduction caused by culture filtrate heated at 60°C. for 10 minutes was 32 per cent whereas autoclaved culture filtrate caused 27 per cent reduction. From these figures it is clear that high temperature has little if any effect on activity of the toxin.

Table 24. Effect of heat on activity of culture filtrate

	Unheated culture filtrate	Heated C.F. (60°C. for 10 minutes)	Auto.C.F. (120°C. for 15 minutes)	Medium
Mean mg. Tc/g. fresh wt.	1.02	1.06	1.14	1.56
Per cent reduction	34	32	27	-

pH range of culture filtrate activity

The pH of culture filtrate originally 6.4 was adjusted to values of 4, 5, 7 and 9 by adding appropriate volume of 0.1 N HCl or 0.1 N NaOH before infiltrating into bean leaves.

Symptoms as chlorotic as those obtained from unadjusted culture filtrate were given by all filtrates, so the effect is not pH dependent unless the leaf is able to nullify the reaction of the solutions.

Effect of dialysis on culture filtrate

Culture filtrate was dialysed against water (600 ml. approx.) at 6°C. for 24 and 48 hours. The water was changed 4 times every day. When dialysed filtrates were tested activity was very much reduced after 24 hours and had completely disappeared in 48 hours.

Effect of solvents

(a) Diethyl ether - 100 ml. culture filtrates were concentrated to half volume in a rotary evaporator at 40°C. under reduced pressure then an equal volume of diethyl ether was added shaken for a few minutes and the solutions were allowed to separate in a separating funnel. The ether phase was collected and more ether was added and separated in the same way. Residue collected from combined ether fractions was inactive when tested.

(b) Absolute alcohol - 50 ml. culture filtrate were evaporated to dryness in the rotary evaporator and the residue was shaken in 50 ml. ethyl alcohol. When water solution of alcohol soluble fraction was tested in bean plants no chlorosis was detected.

Ion exchange resins

When culture filtrate was passed through ion exchange resin the active principle was neither absorbed in anion exchange resin, De-acidite (SRA 69), nor cation exchange resin Zeo-Karb 225 (SR 13).

Precipitation with acetone

Addition of 3-5 volumes of acetone to one volume of culture filtrate at a temperature of 6°C. resulted in separation of anabundant white flocculent precipitate. This was separated by centrifugation at 3,000 r.p.m. and traces of acetone removed from precipitate under a jet of air. The precipitate was soluble in water and contained the active part responsible for chlorosis, It was insoluble in ethanol, ether or chloroform. Time did not permit the development of further studies with the active principle.

Protein estimation

It has been reported that samples from the halo region and chlorotic leaves from infected plants have a lower soluble protein content than healthy leaves (Rudolph and Stahmann, 1964).

In this series of experiments soluble proteins were estimated: (a) Histochemically, and (b) quantitatively from the following samples.

1. Halo region surrounding infection point.
2. Systemically chlorotic leaves.
3. Healthy leaves.
4. Culture filtrate treated leaves.

Samples from the halo region, chlorotic leaves and healthy leaves were taken for estimation 12 days after inoculation. Culture filtrate and medium treated leaves were used 4 days after infiltration.

(a) Histochemical reaction

Leaf disks were extracted in hot ethanol to remove chlorophyll and free amino acids. When completely decolourized histochemical reaction of bound tryptophane was carried according to Doty *et al.*, (1946). Tissues were treated with *p*-dimethylamino-benzaldehyde reagent (0.5 ml. 0.15 per cent sodium nitrate solution, 1.0 ml. of 2.5 per cent *p* dimethylamino-benzaldehyde, and 2.5 ml. concentrated hydrochloric acid).

Blue colour formation was taken as an indication of protein as bound tryptophane. The colour reactions of culture filtrate and medium treated disks are represented in Figure 20.

A lower intensity of blue colour formation was noted in disks treated with culture filtrate 5 hours after addition of p-dimethylamino-benzaldehyde reagent. A similar reduction in intensity of the blue colour was observed in disks taken from the halo region and from chlorotic leaves.



Figure 20 Histochemical reaction - After treating with p dimethylamino benzaldehyde reagent.
Dark blue disk from culture filtrate treated leaf
Light blue disk from medium treated leaf

(b) Quantitative estimation of soluble proteins

Total soluble proteins were determined according to Lowery et al., (1951).

Reagents used:

1. 10 per cent trichloroacetic acid.
2. Tris buffer pH 7.4 [2.5 ml. 0.2 M 2-amino-2-(hydroxy-methyl) - propane - 1:3 diol, 40 ml. 0.1 N HCl and 35 ml. H_2O]
3. Crystalline bovine albumin serum (obtained from Koch-Light Laboratory).
4. IN NaOH.
5. Alkaline Copper reagent consisting of:
   A. 20 g. $NaNO_3$, 0.2 g. NaK tartarate diluted to 1 litre.
   B. 5 g./litre $CuSO_4.5H_2O$.

50 ml. of A mixed with 1 ml. of B for use on the same day.

6. Folin and Ciocalteu's phenol reagent (B.D.H) - This was diluted with water (1:1 V/V).

Extraction and estimation

Fresh samples of known weight were extracted thoroughly in Tris buffer (1 g. fresh weight in 20 ml.). Insoluble plant debri was removed by repeated centrifugation at 18,000 r.p.m. at 5°C. and protein in the supernatant was

precipitated by adding 10 per cent trichloroacetic acid (1:1 V/V) followed by centrifugation at 1,000 r.p.m. The sediment was washed and resedimented with 10 per cent trichloroacetic acid. Precipitated protein was dissolved in 2 ml. N NaOH by placing the samples in hot water for 10 minutes.

A standard curve was prepared from crystalline bovine albumin. To 1 ml. of solutions containing 10-100 ug. of standard protein 5 ml. of alkaline copper reagent were added and the mixture allowed to stand for 10 minutes. Then 0.5 ml. of diluted phenol reagent was rapidly added and immediately mixed in. The optical density of the solution was read 30 minutes later at 750 mu in a spectrophotometer. The quantity of protein in 1 g. leaf fresh weight was obtained by diluting the concentrated protein solution following the same procedure and then determining the protein equivalent from the standard graph.

Table 25 gives soluble protein in terms of protein equivalents per unit fresh weight from halo zones and chlorotic trifoliates. The estimates were consistently less than those from healthy control leaves. Reduction in soluble protein content was also induced when culture filtrate was injected into healthy leaves (Table 26).

Table 25. Estimation of total soluble protein in samples from halo, chlorotic and healthy leaves

Expt. number	mg. protein equivalents/g. fresh weight			
	Monofoliates		Trifoliates	
	Halo region	healthy leaves	Chlorotic	Healthy
1	16.3	17.8	15.5	28.0
2	13.0	22.3	12.6	21.3
3	20.0	25.5	18.8	23.5

Figure shown are means of 3 determinations

Table 26. Effect of culture filtrate on total soluble protein content

Expt. number	mg. protein equivalents/g. fresh weight	
	Culture filtrate	Medium
1	15.1	25.5
2	17.5	24.8
3	18.3	18.8

Figure shown are means of 3 determinations

Estimation of free amino acid nitrogen

Patel and Walker (1963) have shown that leaf samples from halo and chlorotic leaves of infected plants contain considerably more free amino acids than healthy leaves.

The total free amino acid nitrogen was estimated from the following samples:

1. Halo region and corresponding healthy leaves.
2. Chlorotic leaves and corresponding healthy leaves.
3. Leaves treated with culture filtrate and controls infiltrated with sterile medium.

The tissues were extracted in 80 per cent ethanol (5 ml./g. fresh weight), and extracts were separated and collected by centrifugation at 3,000 r.p.m. for 10 minutes at $0^{\circ}C$.

Determination of free amino acid nitrogen was carried according to a modification of Pope and Steven's (1939) method based on the formation of a copper complex of amino acids followed by iodimetric determination of copper (Belozerkij and Proskurjakow, 1956).

Reagents used

1. $CuCl_2$, 6.81 g. in 250 ml. H_2O.
2. Na_2HPO_4 16.1 g. in 125 ml. H_2O.
3. Borate buffer 28.6 g. borax, 750 ml.H_2O and 50 ml. N HCl made up to a litre.
4. 0.001 N sodium thiosulphate prepared freshly from 0.01 N stock solution.

5. Acetic acid.

6. Potassium iodide solution 4 g./20 ml. H_2O.

7. 1 per cent soluble starch.

The copper suspension was prepared by adding 50 ml. of reagent 1 to 100 ml. of reagent 2. The resulting precipitate was centrifuged and then resuspended in 150 ml. borate buffer. After adding the copper suspension to the plant extracts, 3 ml. distilled water were added and the mixture was shaken for three minutes. The solution was then filtered and 10 ml. of the filtrate, 0.5 ml. acetic acid, 1 ml. potassium iodide solution and 2-3 drops of starch solution were added and the resulting free iodine was titrated against standard freshly prepared 0.001 N sodium thiosulphate; free amino nitrogen (mg.) = ml. 0.01N thiosulphate X0.28.

Samples from halo leaves away from lesions were taken 7, 10 and 12 days after inoculation of prescribed areas. Table 27 gives the total free amino nitrogen from chlorotic leaves and corresponding healthy leaves and Table 28 gives the total free amino nitrogen from halo samples and healthy leaves. For leaves treated with culture filtrate the free amino acid nitrogen was determined immediately after infiltration and for each of the following four days. The results of the experiments are shown in Table 29.

Whereas the free amino acid nitrogen content in healthy trifoliates ranged between 78-82 ug. per g. fresh weight that in chlorotic leaves was considerably higher and often exceeded double this value. Similarly, samples from halo surrounding infection areas had much more free amino acids than corresponding samples from healthy leaves. In all samples taken from healthy leaves after 7, 10 and 12 days the free amino acid content remained more or less the same whereas that from halo region was highest after 12 days. When the medium and culture filtrate were injected into healthy leaves the expected amino acid increase in medium infiltrated leaves was apparent in first sample taken immediately after infiltration in the first experiment and was still higher than that of culture filtrate treated leaves one day after infiltration when the experiment was repeated, yet in later samples a much greater increase was estimated from culture filtrate treated leaves.

Table 27. Determination of free amino acid nitrogen in chlorotic leaves and corresponding healthy leaves

Expt. number	Mean ug. free amino acid nitrogen/g. fresh wt.		
	Chlorotic leaves from infected plants	Corresponding healthy leaves	Per cent increase
1	169	82	106.0
2	205	78	162.8
3	169	80	111.3

Figures are means of 3 determinations.

Table 28. Determination of free amino acid nitrogen in samples taken from halo region and Healthy control

Days	Mean ug. free amino acid nitrogen/g. fresh wt.		
	Halo samples	Healthy samples	Per cent increase
7	140	74	89.2
10	169	77	119.5
12	180	75	140.0

Figures are means of 3 determinations.

Table 29. Effect of culture filtrate on free amino acid content of the leaves

Days after infiltration	Mean ug. amino acid nitrogen/g. fresh wt.					
	Experiment (1)			Experiment (2)		
	Medium	Culture filtrate	Per cent increase	Medium	Culture filtrate	Per cent increase
0	153	148	-	194	119	-
1	147	241	63.9	165	117	-
2	144	253	75.7	141	238	68.7
3	132	280	112.1	150	233	55.3
4	174	275	58.0	157	236	50.3

Figure are means of 3 determinations

PART X ATTEMPTS TO COUNTERACT CHLOROSIS

It has been established from previous experiments that chlorosis whether induced by infection or by injecting sterile culture filtrates into leaves is associated with reduction in soluble protein and an increase in free amino acids. This is also characteristic of senescent leaves.

In this series of experiments the effect of the following chemicals on chlorosis was studied.

a. Benzimidazole.

b. Kinetin.

c. Zeatin.

and d. the three above mentioned in combination with Indoleacetic acid (IAA)

Stock solutions were made up by dissolving the chemicals in a few drops of alcohol which was then made up to volume in distilled water. Solutions prepared in this way remained clear without precipitation.

a. Experiments with benzimidazole

Wang et al., (1959, 1960) have shown that benzimidazole a synthetic analogue of adenine, maintains the normal rate of chlorophyll synthesis and prevents the destruction of the green pigments in light and in darkness. It has also been reported that benzimidazole maintains the soluble

nitrogen content of detached wheat leaves at a level higher than in water controls (Person et al., 1957).

In the following experiments the effect of different concentrations of benzimidazole on development of chlorosis of infected leaves and leaves treated with culture filtrate was studied.

(i) Infected plants

Plants for this experiment were inoculated in the standard manner. Each monofoliate was infected at four points with washed bacterial suspension then three days later inoculated leaves were sprayed with benzimidazole solutions at 25, 50 and 100 p.p.m. on both surfaces to run off. The growing points of the plants were protected during the spray which was repeated once daily for 5 days. Plants during this period were kept in humid chamber and were only taken out for spraying. Ten days after inoculation the effect of the chemical on spread of haloes, chlorosis based on total chlorophyll estimation, chlorosis of developing trifoliates and other effects were assessed. Table 30 gives the average spread of haloes in mm. and the number of chlorotic trifoliates out of 8 plants for each treatment.

There was reduction of halo size with increase in benzimidazole concentration. The diameter of the halo surrounding infection area was 15 mm. at 25 p.p.m., 10 mm. at 50 p.p.m. and 8 mm. at 100 p.p.m. Control plants sprayed

with water had an average halo diameter of 22 mm. All plants sprayed with 100 p.p.m. had green trifoliates, two plants sprayed with 50 p.p.m. and 25 p.p.m. had chlorotic trifoliates and seven plants sprayed with water had chlorotic trifoliates. Samples from the halo zones had total chlorophyll contents of 1.57, 1.59, 1.33 and 1.12 mg. for benzimidazole concentrations of 100, 50, 25 and 0.0 p.p.m. Total chlorophyll content in healthy leaves of the same age was 1.85 mg. (Figure 21A). Concentration of 100 p.p.m. was somewhat phytotoxic; this was confined to edges of leaves causing marginal necrosis.

(ii) Culture filtrate treated plants

Sterile filtrates were injected into half unifoliates and when the effect of water-soaking disappeared, half an hour to two hours later benzimidazole solutions were sprayed twice during the same day and once on each of the following three days. Here again, plants for the four days following injection of filtrates were kept under humid chambers. Similar concentrations of benzimidazole were applied as in above experiment and samples from each treatment were taken for chlorophyll assessment. The results are given in Figure 21 B.

Averages for chlorophyll estimates from benzimidazole sprayed leaves were slightly higher than leaves sprayed with

water. However, in no case was it comparable to green control leaves. Chlorophyll estimates from diseased plants sprayed with benzimidazole were consistently higher than corresponding estimates from culture filtrate treated leaves. This suggests that benzimidazole could be affecting spread and depth of chlorosis in infected plants indirectly by inhibiting bacterial multiplication and hence reducing toxin production. As a sequel to this the effect of the chemical on bacterial multiplication was examined.

(i) In vivo effect of benzimidazole

Much the same experiment described in page117 was repeated and viable counts were estimated after 10 days. Table 31 gives the results.

Control plants sprayed with water gave an average viable number of 73×10^7 per leaf disk. Estimates from 25 p.p.m. were close to this number but at higher concentrations of 50 p.p.m. and 100 p.p.m. there was a substantial reduction in viable numbers.

Table 30. Effect of benzimidazole on chlorosis of infected leaves

	Concentration of benzimidazole (p.p.m.)			
	0.0	25	50	100
Mean halo diameter (mm.)	21 (± 0.7)	15 (± 0.6)	11 (± 0.6)	8 (± 0.2)
Number of chlorotic 1st trifoliates	7	2	2	0
Number of chlorotic 2nd trifoliates	4	2	1	0
Necrosis of leaf edge	0	0	1	8

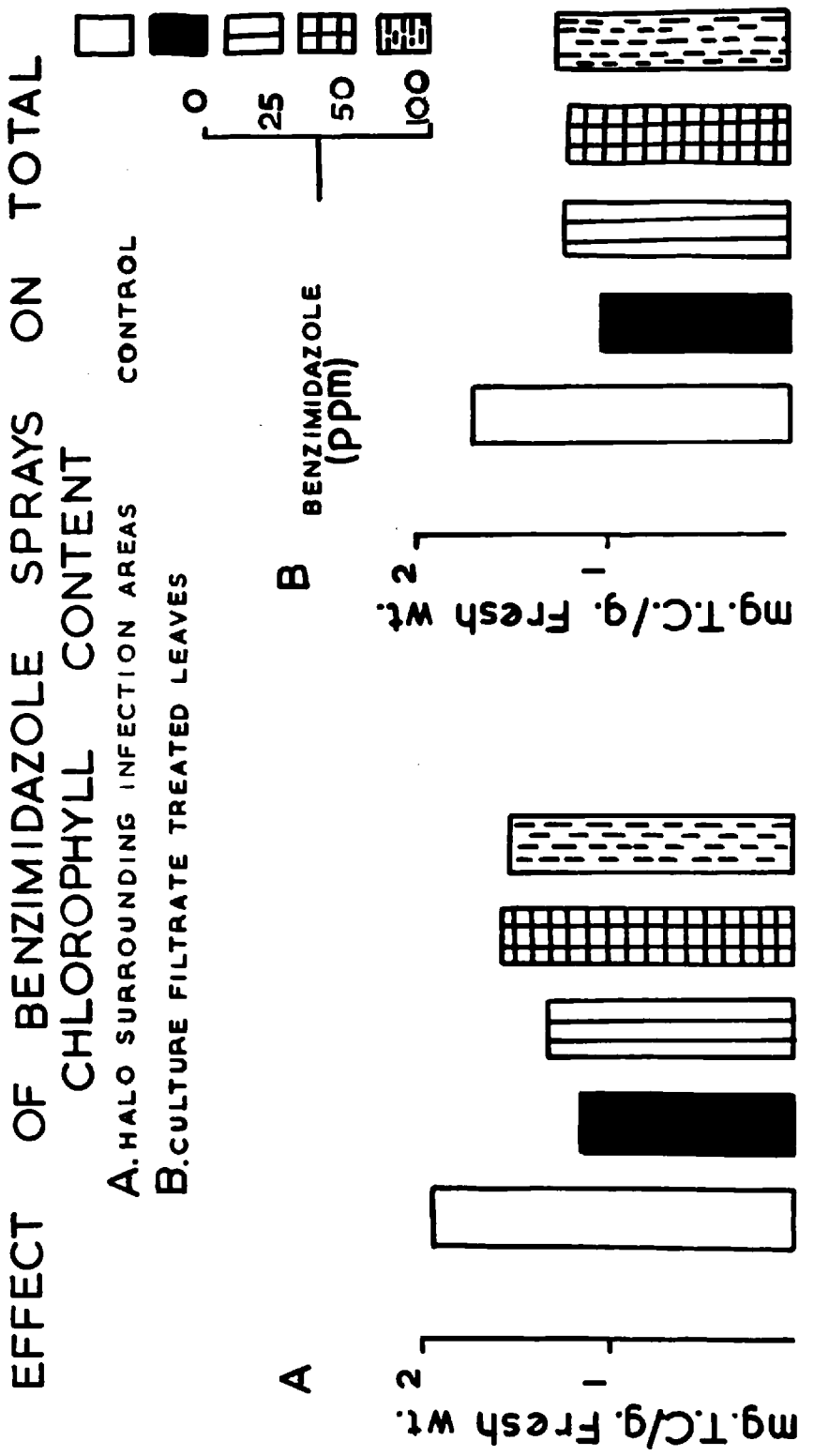
Total of 8 plants/treatment
Figures between brackets are standard errors of the means

Table 31. Effect of benzimidazole on bacterial multiplication in vivo

	Benzimidazole concentration (p.p.m.)			
	0.0	25	50	100
Viable count x 10^6	730	692	133	42

Average of 3 disks

Figure 21.


EFFECT OF BENZIMIDAZOLE SPRAYS ON TOTAL CHLOROPHYLL CONTENT
A. HALO SURROUNDING INFECTION AREAS
B. CULTURE FILTRATE TREATED LEAVES
CONTROL
0
25
50
100
BENZIMIDAZOLE (ppm)
A
B
mg. T.C./g. Fresh wt.
2
1

(2) In vitro effect of benzimidazole

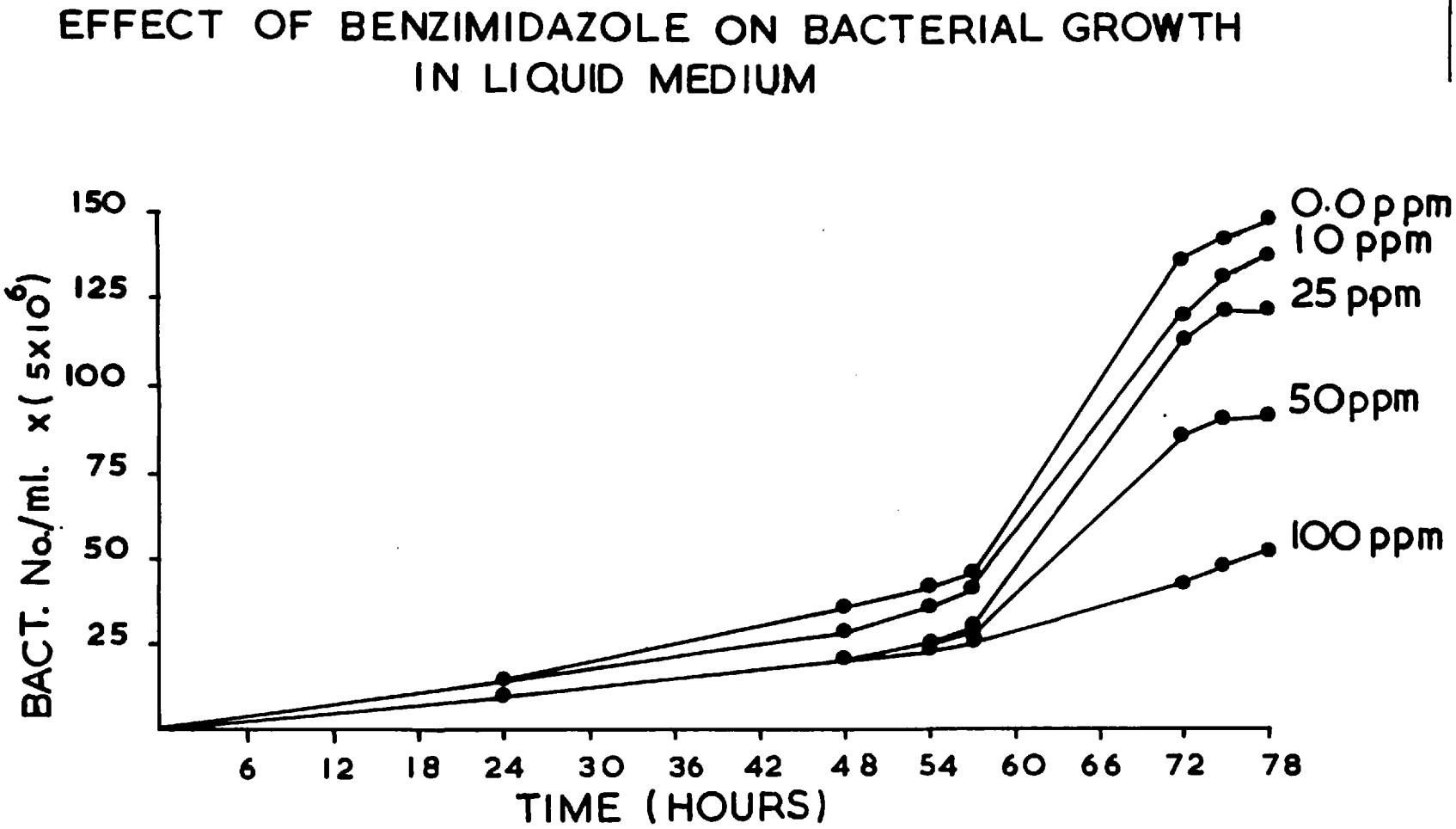
A benzimidazole solution was sterilized by filtration through an 'Oxoid' membrane filter then added to tubes of sterile glucose-casein hydrolysate liquid medium at 0.0, 10, 25, 50 and 100 p.p.m. Tubes were inoculated with 0.1 ml. of dilute bacterial suspension and incubated at 22°C. The growth was determined by turbidity measurements in a nephelometer at various intervals. The results are given in Figure 22.

The general pattern of growth was more or less the same for the first 58 hours; wider differences became apparent later on and there was a consistent reduction in turbidity with increasing concentration indicating a definite bacteriostatic effect of benzimidazole.

b. Kinetin (6-furfurylamino-purine)

Amongst the properties of this substance is the ability to retard senescence of leaves (Richmond and Lang, 1957; Osborne, 1962; Suguira, Umemura and Oata, 1962). Farkas et al., (1963) suppressed the chlorosis inducible in tobacco leaves by culture filtrates of Ps. tabaci by spraying kinetin at concentration of 10^{-5}M. Since symptoms caused by Ps.phaseolicola resemble those of wildfire toxin it seemed profitable to find out if chlorosis could be influenced by kinetin treatment.

Figure 22.


EFFECT OF BENZIMIDAZOLE ON BACTERIAL GROWTH
IN LIQUID MEDIUM
BACT. No./ml. x(5x10^6)
150
125
100
75
50
25
6
12
18
24
30
36
42
48
54
60
66
72
78
TIME (HOURS)
0.0 ppm
10 ppm
25 ppm
50ppm
100 ppm

(i) Infected plants

These were sprayed with 5, 10, and 20 p.p.m. kinetin solutions once every day for 5 days starting on the third day after inoculation. Control plants were sprayed with distilled water and plants during this period were kept in a humid chamber. The effect on chlorosis was assessed 10 days after the start of the experiment and the results are given in Table 32. The effect on depth of chlorosis of halo region in terms of total chlorophyll per g. fresh weight is given in Figure 23A.

The average halo diameter at 5 p.p.m. was slightly less than in the water control. The former was 16 mm. and the latter 18 mm. Leaves sprayed with 10 and 20 p.p.m. had an average halo zone of 13 mm. in both cases. There was no consistent difference between the number of chlorotic trifoliates on kinetin treated and non-treated plants. Kinetin at concentrations used in this experiment had no effect on depth of chlorosis and in no case there was much increase in total chlorphyll content above that of controls.

(ii) Culture filtrate treated leaves

Here again there was a lack of effect on depth of chlorosis when the same concentrations of kinetin, used in the above experiment, were sprayed on culture filtrate treated leaves (Figure 23B).

c. Zeatin

This is a growth promoting substance related to kinetin and claimed to be more potent in some of its physiological activities. Shaw (1965) stated that zeatin is active at a concentration as low as 0.1 ug./L. No mention was made about the bioassay.

The effect of this growth substance on chlorosis of infected plants and culture filtrate treated leaves was studied in the same way as in the benzimidazole and kinetin experiments, with concentrations of 5, 10 and 20 p.p.m. The results of the effect on halo spread and number of chlorotic trifoliates of infected leaves are given in Table 33. Degree of chlorosis of the halo in terms of total chlorophyll per unit fresh weight is given in Figure 24.

Zeatin at these concentrations had no effect on spread of chlorosis and the average halo diameters were nearly the same as those of the controls. The intensity of chlorosis was also unaffected by zeatin spray.

Table 32. Effect of Kinetin on chlorosis of infected plants

	Concentration of Kinetin (p.p.m)			
	0.0	25	50	100
Mean halo diameter (mm.)	18 (± 0.7)	16 (± 1.0)	13 (± 0.7)	13 (± 0.6)
Number of chlorotic 1st trifoliates	5	6	4	5
Number of chlorotic 2nd trifoliates	3	3	2	3

Total of 8 plants/treatment.

Figures between brackets are standard errors of the means

Table 33. Effect of zeatin on chlorosis of infected plants

	Concentration of zeatin (p.p.m.)			
	0	5	10	20
Mean halo diameter (mm.)	19 (± 0.7)	19 (± 0.7)	18 (± 0.5)	18 (± 0.6)
Number of chlorotic 1st. trifoliates	5	5	6	6
Number of chlorotic 2nd. trifoliates	3	3	3	5

Total of 8 plants/treatment.

Figures between brackets are standard errors of the means

Figure 23.

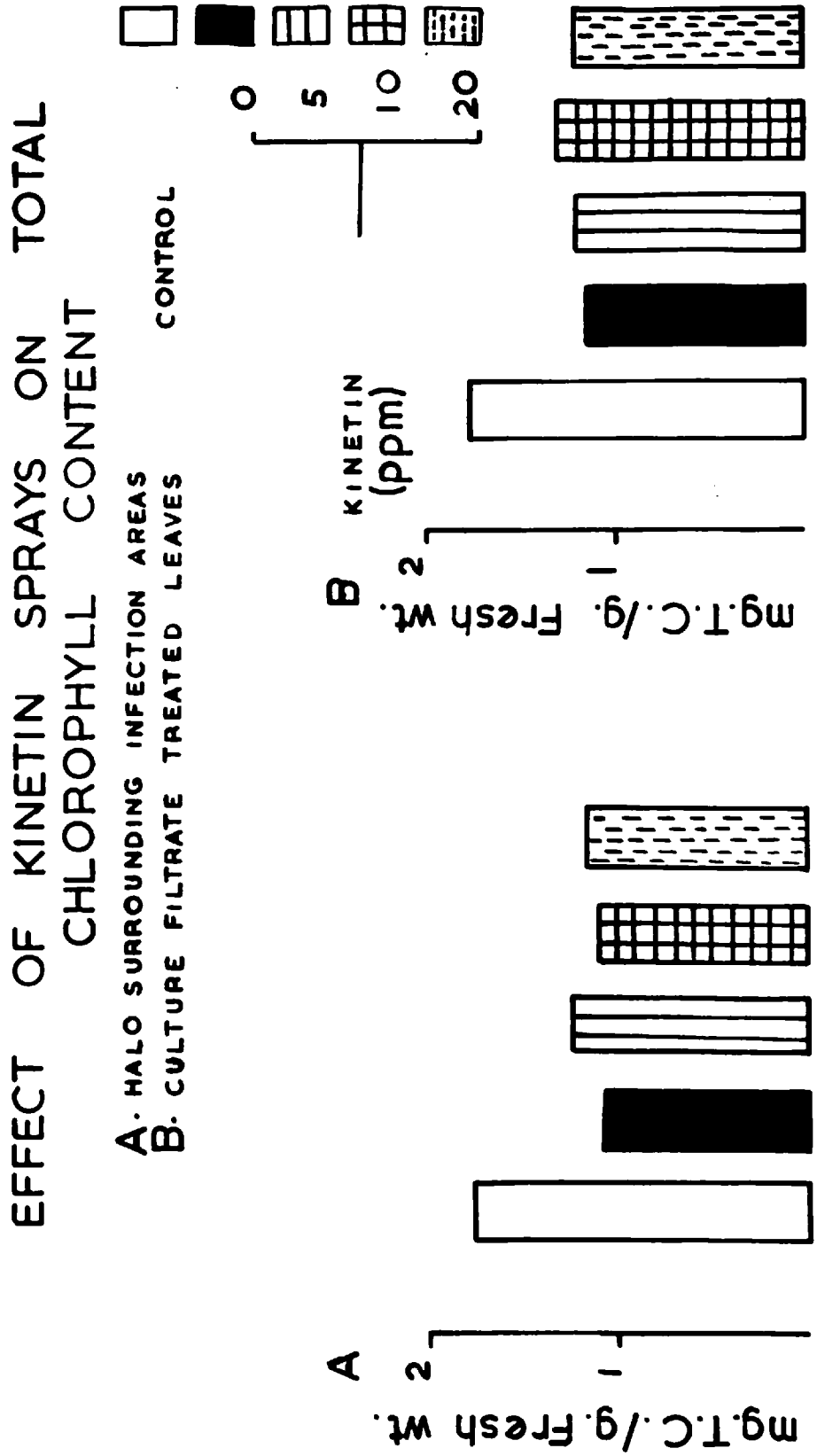
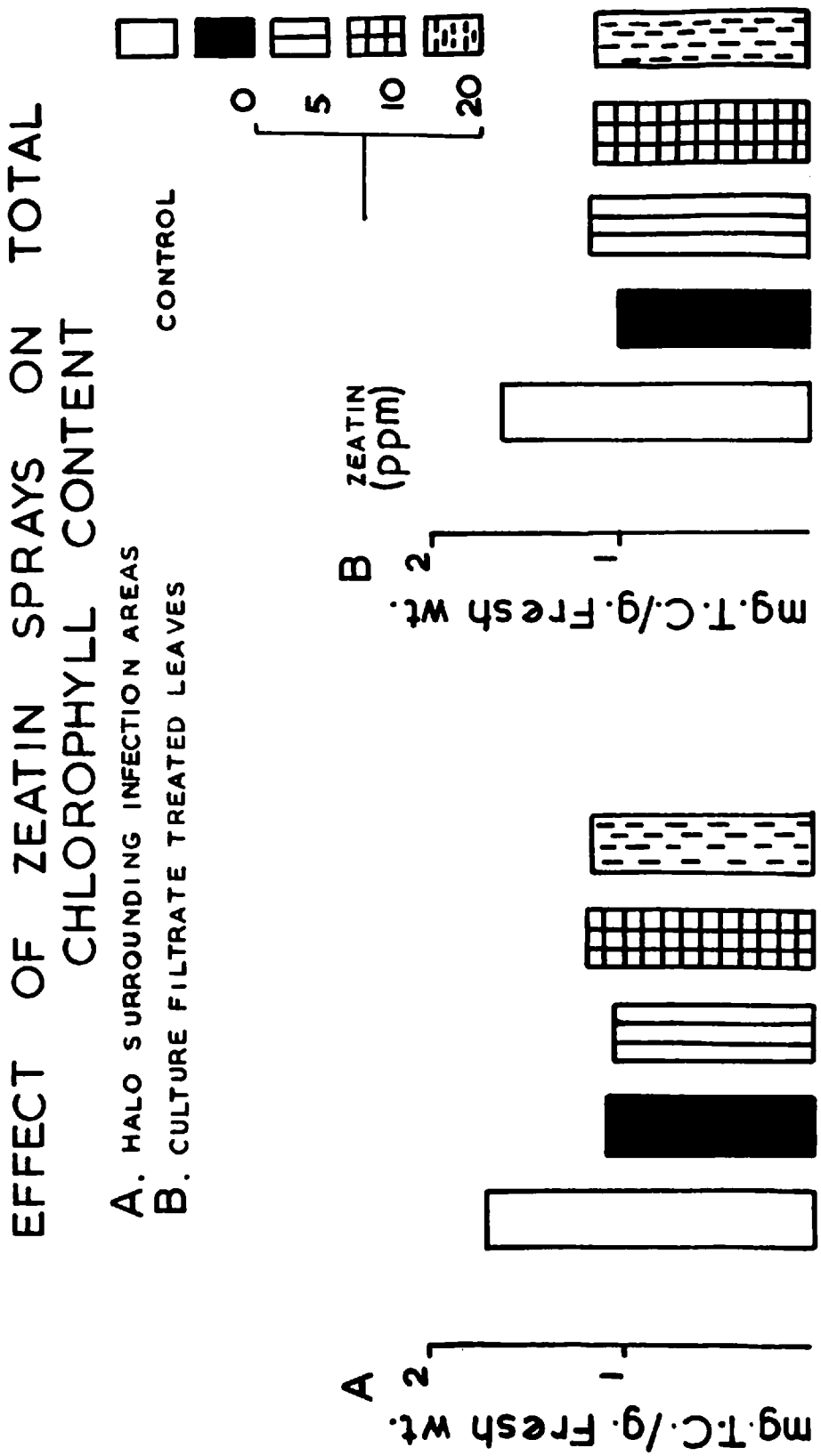

EFFECT OF KINETIN SPRAYS ON TOTAL CHLOROPHYLL CONTENT
A. HALO SURROUNDING INFECTION AREAS
B. CULTURE FILTRATE TREATED LEAVES
CONTROL
0
5
10
20
KINETIN (ppm)
A
B
mg.T.C./g. Fresh wt.
2
1

Figure 24.


EFFECT OF ZEATIN SPRAYS ON TOTAL CHLOROPHYLL CONTENT
A. HALO SURROUNDING INFECTION AREAS
B. CULTURE FILTRATE TREATED LEAVES
CONTROL
0
5
10
20
ZEATIN (ppm)
A
B
mg.T.C./g. Fresh wt.
2
1

d. Effect of adding indoleacetic acid to benzimidazole kinetin and zeatin solutions

Indoleacetic acid sprayed alone at concentration of more than 10 p.p.m. caused etiolation of the stem and flaccidity of the monofoliates. In this experiment the effect of two levels of IAA mixed with constant concentrations of each of the three chemicals tested before was studied. IAA concentrations were 0.0, 5 and 10 p.p.m. That of benzimidazole was 50 p.p.m. and kinetin and zeatin were 10 p.p.m. each. Solutions were tested on culture filtrate injected leaves and spraying was done as before, twice on the same day after infiltration and once every day for the following 3 days. Degree of chlorosis was estimated 4 days after the start of the experiment and results are given in Table 34. The effect on infiltrated half leaf expansion was also measured and average half leaf areas are given in Table 35.

None of these treatments suppressed chlorosis. Apart from treatments containing zeatin half leaf areas were close to those sprayed with water only. When zeatin was incorporated the average leaf areas were slightly highter than controls.

Table 34. Effect of IAA benzimidazole kinetin and zeatin on total clorophyll of chlorotic half leaves

	Mean mg. total chlorophyll/g. fresh wt.			
	IAA concentration (p.p.m.)			Medium (Control)
	0.0	5	10	
Benzimidazole (50 p.p.m.)	1.22	1.14	1.20	
Kinetin (10 p.p.m.)	1.19	1.26	1.29	
Zeatin (10 p.p.m.)	1.16	1.04	1.11	
Water (Control)	1.02	1.11	1.09	1.71

Average of 3 replicates/treatment.

Table 35. Effect of IAA benzimidazole kinetin and zeatin on infiltrated half leaf area

	Average half leaf area (sq.in.)			
	IAA concentration (p.p.m.)			Medium (Control)
	0.0	5	10	
Benzimidazole (50 p.p.m.)	3.81	4.02	3.92	
Kinetin (10 p.p.m.)	4.12	4.08	4.21	
Zeatin (10 p.p.m)	4.32	4.36	4.28	
Water (Control)	4.11	3.96	4.06	4.52

DISCUSSION

The purpose of the work involved in this thesis is to inquire into some aspects of the disease caused by the two races of Pseudomonas phaseolicola with special emphasis on the behaviour of the pathogen in vivo, its translocation, multiplication and some of the factors affecting multiplication as well as its toxin production.

Experiments on translocation indicated that once the pathogen reaches the xylem elements it gets translocated in the xylem fluids to regions above and mainly remains confined to vascular tissue causing little if any invasion of mesophyll. The movement of bacteria in susceptible plant is less restricted than in resistant variety suggesting that xylem resistance is correlated with that of mesophyll. When inoculated at cotyledonary nodes chlorosis of developing trifoliates was observed in susceptible plants but not in resistant. During the course of infection only young leaves became chlorotic, mature leaves apart from flaccidity which occurred later during infection were to all appearances unaffected. When circumscribed areas of undetached leaves were inoculated away from the main veins halo regions surrounding infection areas contained very few bacteria, a lesser number of bacteria was found in chlorotic leaves above inoculated leaves. These results substantiate those of earlier workers (Zaumeyer, 1932 and Waitz and Schwatz, 1956).

The cell number of Race-1 in susceptible leaves increased in a pattern much similar to that expected *in vitro*; characterized by a logarithmic phase entering into a stationary phase in a matter of few days after inoculation. Viable counts from Red Mexican indicated that though the plant is resistant the bacterium is capable of substantial multiplication. The entry to the stationary phase in both susceptible and resistant varieties coincides with symptom expression, i.e. water-soaking in susceptible and browning of tissues in the resistant. The water-soaking symptom might be the result of activity of the pathogen in the host tissues while the browning evoked by the damaged tissues themselves. Browning of infected tissue in susceptible variety occurs later when the cells are completely damaged. The intensity of the reaction, water-soaking in the susceptible and browning of resistant tissue, depends on inoculum concentration and also the maturity of the leaf. The higher the injected inoculum and the younger the leaves the more intense is the reaction. Weak symptoms in resistant variety remain constant and do not become stronger.

It is relevant to mention here that Dye (1958) from a number of inoculations and reisolations of *Xanthomonas spp.* on beans came to the conclusion that some phytopathogenic bacteria have no specific host range, and that bacterial

plant pathogens could be adapted to infect plants not naturally susceptible to them. Sharen (1959) stated that when large numbers of bacteria are introduced in resistant leaves the pathogen appeared capable of overcoming the resistance of the host, proceeding to multiply and ultimately destroying the tissues. Relatively smaller initial numbers of cells does not seem to be able to surmount the resistant reaction and the relationship is turned in favour of the host. Results obtained during the course of this work are in disagreement with those of Sharen (1959). Increasing inoculum concentration in Red Mexican failed to give the typical susceptible reaction and always there was a lack of water-soaking phase. At any given concentration the population maximum in resistant leaf tissue was always far less than that in susceptible variety and that only in the susceptible leaf tissue bacteria appeared capable of starting progressive infection. Race-2 in differential variety, Red Mexican, behaved exactly like Race-1 in the susceptible. pH values of healthy leaf extracts from differential variety, Red Mexican and susceptible variety, Canadian Wonder, were nearly the same. Increasing alkalinity ascribed by Garber (1961) as the possible explanation for resistance to Ps. tabaci among Nicotiana spp. is not likely to be the cause of resistance of Red Mexican to Race-1 since it was not possible to relate buffering capacity of healthy

resistant and susceptible leaf extracts with resistance or susceptibility.

Antibacterial activity has been recorded from many plant species, Little and Grubough (1946) found their extracts to be active against a number of phytopathogenic bacteria and obtained indications that in the case of corn, resistance to bacterial wilt due to Bacterium stewarti (Xanthomonas stewartii) may be due to the presence of antibacterial substances as these were found in the juice of resistant varieties. Dadd (1961) found that among Nicotiana spp. Undiluted extracts from resistant species inhibited growth of Ps. tabaci whereas extracts from susceptible did not.

Race-1 bacteria multiplied in both filter sterilized resistant and susceptible healthy leaf extracts to the same extent, moreover, there was no consistent difference between growth in diseased and healthy resistant leaf extracts. An important point to be mentioned here is that the main site of bacterial multiplication is the intercellular spaces where the pathogen initially depends on the intercellular fluids for its metabolism and multiplication. The juice expressed from leaves cannot reflect either the composition or properties of the intercellular fluid. During the extraction and centrifugation any possible preformed inhibitor might have been inactivated in the process.

There is also the possibility that the nutrient released during the extraction process might have masked any inhibitory substance preformed in resistant plant or stimulated as a result of incompatible reaction.

Apart from varietal resistance the susceptible plants exhibit age resistance. This has been reported to be the case in many bacterial diseases, in cucumber against the angular leaf spot pathogen, Pseudomonas lachrymans (Van Gundy and Walker, 1964) and in tomato against X. vesicatoria (Nayudu and Walker, 1960). In beans this is due to internal factors rather than a superficial host barrier since the rate of bacterial multiplication was much higher in young leaves than old mature ones. The results obtained in this connection are in agreement with those of Goss (1940) and Patel and Walker (1963), the latter workers found no correlation between susceptibility of young leaves, tolerance of old leaves and amino acid pattern.

When relatively high concentration of Race-1, 10^5 cells/ml. or more were infiltrated into resistant Red Mexican followed by a dilute bacterial suspension of Race-2, 5×10^3/ml. a remarkable reduction in number of water-soaked lesions was observed. Again mixing Race-2 with relatively high proportion of Race-1 reduced increase in viable count to a value much below that expected from Race-2 alone.

A ratio of 25/100 Race-2/Race-1 resulted in a final population 10 fold less that of the virulent race. The assumption being that the metabolic activity of the non-virulent race responsible for atypical reaction changes the microenvironment (site of inoculation) and this change retards further multiplication. The results obtained in this connection show great similarity to those of Müller and Börder (1940) who demonstrated that inoculation of potato variety with non-virulent race of Phytophthora infestans confers protection when inoculum of the virulent race is placed in regions that had previously been inoculated with non-virulent race or if a mixture of the two races was used. The reactions in beans are very much comparable with hypersensitive reaction associated by Müller (1959) with the development of a phytoalexin in the case of fungus pathogens. Relatively few workers have investigated the role of phytoalexin like substances in bacterial diseases. Cruickshank and Perrin (1963) are of the opinion that these substances are stimulated by fungi, germination fluids, certain chemicals but not bacteria. Klement and Lovrekovich (1961) on the other hand support the concept that phytoalexin - like reaction may result when phytopathogenic bacteria are injected into tissues of a non-susceptible host. Comparison of effects of inocula

containing different proportions of cells of Ps. syringae which evokes a rapid host defence reaction in bean pods and X. juglandis which elicits a slow mild response showed that the increase in cell number of X. juglandis was inversely proportional to the initial number of Ps. syringae. Inhibition of the virulent race of Ps. phaseolicola was not increased by establishing the non-virulent race and delaying time when the virulent race was infiltrated.

Attempts to induce production of pectolytic enzymes in liquid media failed to reveal any macerating or pectingalacturonase activity. Again there was a complete lack of cellulolytic activity when carboxymethyl cellulose was used.

It is accepted that systemic chlorosis and halo symptoms are presumably caused by an exotoxin produced by the pathogen. It was readily produced in liquid media indicating that its formation is not dependent on an interaction between the pathogen and substances in the infected leaves as was previously assumed by Waitz and Schwatz (1956). When seven different isolates were inoculated into susceptible leaves they all gave comparable chlorosis, similarly all the isolates were capable of producing the toxin in liquid cultures. The effect of culture filtrates was, as in the case of infected plants, more pronounced in young leaves

than mature expanded ones. Trifoliates produced on cut shoots after they had been allowed to take up filtrate, were chlorotic whereas the mature leaves were unaffected. Culture filtrate interfered with chlorophyll retention as well as expansion of infiltrated region which was greatly reduced. The toxin is non-specific and culture filtrates from Race-1 interfered with chlorophyll synthesis of resistant leaf in much the same way as the susceptible indicating that it does not play an active role in specificity of the pathogen. When infected leaf extract was tested no activity was detected, this is possibly due to the fact that the toxin is produced in small quantity at a time and presumably continuously translocated away from infection point. It is conceivable that the little amount extracted gets masked or inactivated during the process. The fact that the activity of culture filtrate is greatly reduced by 10 fold or more dilutions also suggests that the toxin is not a highly potent one. When cultures were incubated at different temperatures activity of filtrates was between 12°C. to 22°C., a range very much similar to that of in vivo production reported by Goss (1940) and Patel and Walker (1963). The active principle was stable over a wide range of pH and did not loose activity by heating at 60°C. or even autoclaving at 15 lb./sq. in. for 15 minutes. It is dialysable but was

neither absorbed to anion exchange resin De-acidite (SRA 69) nor cation exchange resin, Zeo-Karb 225 (SR 13). It was not soluble in organic solvents.

Soluble protein estimates from haloes surrounding infection areas and chlorotic leaves were consistently less than healthy leaves. The reduction in soluble protein associated with chlorosis was also observed when leaves were injected with cell free culture filtrates. Reduction in soluble protein was accompanied with increase in free amino acids which was in many cases twice as much as in healthy leaves.

Accumulation of amino acids has been correlated with certain disease symptoms. Steinberg (1947) suggested that release of excessive quantities of free amino acids in the tissues a probable factor in production of morphological abnormalities and interveinal chlorosis in tobacco frenching disease, moreover a specific toxicity response to dl-isoleucine simulates most of the symptoms of the disease. Patel and Walker (1963) who detected accumulation of ornithine and B alanine along with some other amino acids suggest that the increase in amino acids might be responsible for chlorosis in beans. The increase in free amino acids may be a direct result of the toxin or conversely it could be a result of proteolysis. The organism produces proteolytic

enzymes (Clara, 1934). In this case the main site of greatest accumulation would be the infection area and high levels of free amino acids in chlorotic trifoliates may be brought about by translocation. Garber (1959) assumes that an unusual biosynthesis pathway for an amino acid in the halo forming Pseudomonads is somehow related to the production of an analogue that is excreted to diffuse into the host cells of the leaf where it behaves as an antimetabolite for the specific amino acid. It is also possible that an exotoxin may occur in several species as an antimetabolite for a specific amino acid but occur as different analogues of that amino acid.

Experiments with benzimidazole indicated that the chemical reduces the size of halo and chlorosis of trifoliates by checking the growth of bacteria in vivo. The viable counts decreased with increasing concentration and the result was confirmed when the effect of the chemical was examined in liquid medium. The inhibitory effect of benzimidazole on the growth of microorganisms was provided by Woolley (1944) and Klotz and Mellody (1948). Benzimidazole could be inhibiting the growth of the bacterium in the same way as that of Escherichia coli reported by Woolley (1944) who suggested that the action of benzimidazole involves an interference with purine metabolism of the

microorganism by acting as an antimetabolite antagonist of adenine.

Benzimidazole spray on culture filtrate treated leaves failed to prevent chlorosis and the total chlorophyll in benzimidazole sprayed leaves was much the same as that in water sprayed controls. The chemical has been suggested to be more selective in retarding senescence of monfoliates and have no effect on the retention of the green pigment in leaf tissue of bean, sunflower, tobacco or Xanthium leaves (Wang et al., 1961 and Osborne and McCalla 1961).

Kinetin reduced halo size but had no effect on depth of chlorosis of infected leaves or culture filtrate treated. A similar result was obtained by Hoitink et al., (1964) who found no correlation between depth of chlorosis and kinetin spray. Zeatin neither caused reduction of halo diameter nor reduced degree of chlorosis. The effect of the three chemicals on chlorosis was not improved by the addition of IAA.

SUMMARY

1. Sixteen varieties of beans were tested for their reactions to the two races of Pseudomonas phaseolicola. Except Red Mexican, all the other varieties were susceptible to Race-1. These varieties and Red Mexican were susceptible to Race-2. When two new lines were tested their reactions to the two races were intermediate.

2. Translocation of bacteria in susceptible plants inoculated at cotyledonary nodes was less restricted than in the resistant.

3. Very few bacteria were isolated from halo and chlorotic leaves away from necrotic lesions.

4. Multiplication of bacteria in susceptible variety was at higher rate than in the resistant, and the peak reached varied with initial inoculum concentration.

5. The susceptible reaction was characterized by a water-soaking phase followed by necrosis while in the resistant variety no water-soaking was observed.

6. Resistant reaction was neither affected by increasing bacterial number nor addition of nutrients to the inoculum.

7. There was no difference in pH between extracts from susceptible and resistant leaves and host reaction was unrelated to the buffering capacity of leaf extract.

8. Growth rate of bacteria in resistant leaf extract was nearly as high as that in susceptible leaf extract and therefore suggesting absence of any inhibitor in resistant leaf extract.

9. Preinoculation of high number of Race-1 into resistant leaves reduced the number of water-soaked lesions formed as a result of inoculating a dilute suspension of Race-2 in differential variety, Red Mexican.

10. A relatively high proportion of Race-1 mixed with small number of Race-2 reduced the multiplication in differential variety to a value much below that expected from Race-2 alone.

11. The organism was neither capable of utilizing pectic substances (pectin or sodium polypectate) nor carboxymethyl cellulose.

12. The halo symptoms and chlorosis of developing trifoliates were produced by all isolates when inoculated into susceptible plants. The intensity and spread of chlorosis were affected by maturity of inoculated leaves and inoculum concentration, the higher the inoculum and the younger the leaves the more pronounced was the chlorosis. Moreover, all isolates produced the chlorosis inducing principle when grown in liquid media.

13. When culture filtrate from Race-1 was injected into leaves of differential variety it interfered with chlorophyll retention in much the same way as the case in susceptible

and hence toxin is non-specific.

14. Attempts to produce chlorosis by injecting healthy leaves with infected leaf extract failed, and it was suspected that this was because of deactivation during extraction or more likely translocation of the small quantity produced at a time.

15. Whereas the optimum temperature for bacterial growth in liquid medium was 18-25°C. production of toxic principle was favoured by low temperature (12-22°C.) and was greatly reduced at 25-30°C.

16. The pH of the culture filtrate, within wide limits, had no discernible effect on activity.

17. Heat treatment did not reduce the capacity of culture filtrate to induce chlorosis.

18. The toxic principle was dialysable, non-soluble in organic solvents and was neither adsorbed to anion exchange resin De acidite (SRA 69) nor cation exchange resin Zeo karb 225 (SR 13). When acetone was added to culture filtrate the water soluble precipitate induced typical chlorosis.

19. The halo surrounding infection area and chlorotic leaves produced above infected leaves contained less soluble proteins and more free amino acids than healthy leaves of the same age. Soluble protein reduction and increase in free amino acid content was reproduced when sterile culture filtrates were injected into healthy leaves.

20. Attempts to counteract chlorosis by spraying benzimidazole indicated that though the chemical reduced chlorosis by checking bacterial growth it had notmuch effect on chlorophyll retention of culture filtrate treated leaves. Kinetin reduced halo size but had no effect on depth of chlorosis. Zeatin and IAA also failed to prevent destruction of the green pigments.

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