UNIVERSITY OF EDINBURGH

A study of the genus Pseudomonas with special reference to the species Pathogenic to plants

by

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General Introduction

Pathological changes in plants caused by bacteria interests both the plant pathologists and the bacteriologists. The importance of the subject is emphasized by the great loss of yield of some very important crops both growing in the field and in storage. Plant diseases also prevent the use of potentially valuable crop plants in some parts of the world. Many years ago the plant pathologists were interested in the study of the isolation and identification of the organisms which cause such diseases, and in the study of the symptoms of disease, and the infection cycles of the pathogens. Control of the diseases also received attention. A great deal of information has accumulated about most of these questions.

The control of plant diseases, which is obviously the most important aspect of all could be achieved when we understand the pathogenic properties of the organism concerned, and the relationship between these pathogenic organisms and the plant.

The bacteria giving rise to plant diseases are well documented. Bergey's Manual (1957) cites about 183 species of phytopathogenic bacteria which belong to five Genera.

Pseudomonas	90 species
Xanthomonas	60 species
Erwinia	17 species
Corynebacterium	11 species
Agrobacterium	5 species

There is some agreement amongst plant pathologists on the generic

level of these bacteria, but there is, however, much controversy over identification of the species, mainly because the species have been named according to the host plant from which the organism was isolated. Few attempts were made to compare the isolated organism with other known species. Comparative studies, however, have considerably reduced the number of these species.

Wormald (1932) who first described Pseudomonas mors-prunorum, compared that organism with Pseudomonas prunicola (Pseudomonas syringae), both of which are responsible for the bacterial canker of stone-fruit trees and found certain cultural differences between the two species. Rosen & Bleeker (1933) on the basis of serological and pathological studies found that Ps. prunicola, Ps. cerasi and an organism they isolated from infected pear, were probably the same as Ps. syringae. Erikson (1945) studied the biochemical characteristics of Ps. mors-prunorum and other phytopathogenic species including Ps. prunicola and the Ps. fluorescens and Ps. pyocyaneus. He concluded that there was no justification in raising these species to specific rank. Friedman (1953) studied the serology of phytopathogenic pseudomonads and included in his study Ps. fluorescens. His results indicated the presence of common antigenic substances among the species and no difference between the phytopathogenic species and Ps. fluorescens. A close relationship between Ps. mors-prunorum and Ps. syringae was also found by Paton (1956) who stated that these species could not be distinguished by the laboratory methods

he applied in his study. He included in his study pseudomonads isolated from different sources and found that strains isolated from plants, including the plant pathogens, could be separated from strains isolated from soil by a number of tests, such as sucrose utilization with or without acid production, the formation of levan from sucrose, the lack of copper reducing substances produced from gluconate.

Starr (1959) in his review stated that there is no definite correlation between nomenspecies and serotype. Rhodes (1959) studied 169 strains of fluorescent pseudomonads including different nomen species using 45 characters and concluded that several of the named phytopathogens showed a close relationship or apparent identity with soil and water isolates. Perlasca (1960) also came to the conclusion that pathogenicity of 4 isolates of Ps. syringae, which showed a range of variability in pathogenic, antigenic and phage relationships, was not associated with any particular antigenic structure. However Lovrekovich & Klement (1961) reported a thermostable antigen which is specific to Ps. tabaci, and therefore useful in distinguishing Ps. tabaci, Ps. angulata from Ps. mors-prunorum, Ps. lachrymans and Ps. mori. They reported that the aspecific reaction between these organisms is due to a thermo-labile antigen.

Stolp (1961) found that fluorescent pseudomonads from soil were similar to the plant pathogenic pseudomonads in phage sensitivity. However Crosse & Garrett (1961) used phage strains isolated from soil and found two lysotypes of Ps. mors-prunorum.

As both types are specifically associated with either cherry or plum tree isolates, they suggested that the specific phage sensitivity is correlated with specific virulent factor, they also stated that all Ps. mors-prunorum isolates were physiologically homogeneous and quite distinct from Ps. syringae.

Stolp (1961) found that leaf necrosis of pelargonium plants is caused not only by Xanthomonas pelargonii but also by X. campestris, Ps. delphinii, Ps. lapsa and Ps. mors-prunorum. Dye (1958) also found that many xanthomonads isolated from different hosts could infect a common host and claimed that host specificity does not exist among the xanthomonads, at least to the extent previously claimed. The similarity between different xanthomonas species was also found by DeLey & Van Muylem (1963) who examined the DNA composition of 10 different xanthomonas species and found them to have a Guanine + cytosine content ranging from 66.0% to 68.2% a value close to that of pseudomonads (60-68%). However Colwell & Liston (1961) concluded from their taxonomic study that Xanthomonas spp. Pseudomonas spp. and Ps. solanacearum were clearly distinct at the genus level. Lovrekovich & Klement (1960) studied the tolerance of phytopathogenic bacteria to triphenyl tetrazolium chloride, and found that 22 different species of xanthomonads, except X. stewerti and X. uredovora, were more sensitive to T.T.C. than pseudomonad species. Stolp, Starr & Baigent (1965) also stated that the fluorescent pseudomonads, xanthomonads and Ps. solanacearum form separate and distinct clusters within the phytopathogenic pseudomonadaceae and suggested

that Ps. solanacearum should be placed in a separate genus on this account.

DeLey, Park, Tijtgat and Van Ermengem (1966) studied the DNA homology of xanthomonads and pseudomonads, and they took the opposite view to that expressed above, finding no reason to keep the xanthomonads in a separate genus and proposed placing them in a single genetic species Pseudomonas campestris on the basis of similarity in DNA composition of pseudomonads and xanthomonads. Crosse & Garrett (1963) found that Ps. mors-prunorum differed from Ps. syringae strains on the ground that Ps. mors-prunorum failed to liquify gelatin and to hydrolyse aesculin and arbutin but they were tyrosinase positive and gave white growth in 5% sucrose broth. They indicated that there are some intermediate forms between the two species, and concluded that the species differences is a matter of definition and that the species names may be retained as a label with the actual meaning of an ecotype. The same authors studied 23 phages isolated with Ps. mors-prunorum and Ps. syringae against 200 pseudomonads, including named plant pathogens, and a variety of saprophytes and found that the majority of the phages did not distinguish between plant pathogens and saprophytes. Billing (1963) found that phage sensitivity tests can play a useful part in the identification of phytopathogenic bacteria if used in conjunction with other tests. Paton (1960) discussed the invasive ability of the pathogenic pseudomonads and stated that the pathogenicity remains to a very great extent an unsolved mystery, the virulent pathogen should be able to invade

tissue at a greater rate than the host can control it. Toxins produced by some plant pathogenic pseudomonads e.g. Ps. tabaci do not account alone for the usual rapid spread of the disease, although it will probably assist in the establishment of the organism within plant tissue. He also suggested 4 characters required by plant pathogens to make them aggressive towards plant tissue.

1. They must live actively on the nutrients present in the host tissue. 2. They must live actively in the specific pH and eH environment existing in the normal tissue or adapt that environment to one more suitable to themselves. 3. They produce a toxic substance such as is formed by the tobacco pathogen. 4. They must cause the pathogenic effects to spread through the tissue beyond the area of actual infection, although there is no direct evidence that such spreading is related to any specific factor produced by the bacteria.

Klement (1963) found that all the plant pathogenic pseudomonads he tested caused the death of the tobacco leaf tissue when injected into leaf, while the non-pathogenic pseudomonads caused no pathological symptoms. Klement, Farkas & Lovrekovich (1964) also found that all the phytopathogenic pseudomonads species they tested multiply in tobacco leaf tissue, when injected whereas the saprophytic pseudomonads were unable to do so.

From the literature it is clear that confusion exists about the identification of the phytopathogenic Pseudomonas species, as well as how close is the relationship between the plant pathogenic

and the saprophytic pseudomonads.

Little work has yet been done to explain the mechanism of pathogenicity of these organisms to plants.

The aim of this study is to examine the differences, between some of the plant pathogenic Pseudomonas species, and to attempt to find differences between plant pathogenic and the saprophytic pseudomonads from soil and other sources in the hope of discovering why pathogens become established in the plant tissue and why the non-pathogenic pseudomonads are unable to do so.

Materials and General Methods

The organisms tested

Reference Number	Name of the organism	Source	Origin
NZ1	Pseudomonas syringae	peach	N.Z.
NZ3	" "	cowpea	S.A.
NZ4	" "	apricot	S.A.
NZ6	" "	apricot	N.Z.
NZ7	" "	cherry	N.Z.
NZ8	" "	almond	N.Z.
NZ9	" "	citronella	N.Z.
NZ10	" "	lilac	N.Z.
NZ12	" "	apricot	N.Z.
NZ13	" "	willow	N.Z.
NZ14	" "	arbutus	N.Z.
NZ16	" "	grapefruit	N.Z.
NZ18	" "	pear	N.Z.
NZ20	" "	passion	N.Z.
NZ21	" "	buttercup	N.Z.
NZ24	" "	apple	N.Z.
P1	" "		Dowson U.K.
P37	" "	lilac	" "
P42	" "		" "
S5	" "	pear	Crosse "
S8	" "	"	" "

S20	Pseudomonas syringae	pear	Crosse U.K.
S36	" "	"	" "
W1	" "	"	" "
W183	" "	"	" "
G2	" "	citrus	" "
P38	Ps. syringae f. sp. populea	poplar	Dowson U.K.
P39	" "	"	" "
P40	" "	"	" "
P6	Pseudomonas mors-prunorum		Dowson U.K.
P47	? " "	cherry	Paton "
D1	" "	plum	Crosse "
D2	" "	"	" "
D5	" "	"	" "
D16	" "	"	" "
N5	" "	cherry	" "
C9	" "	"	" "
C22	" "	"	" "
C46	" "	"	" "
P12	Pseudomonas phaseolicola	bean	Crosse U.K.
P13	" "	"	" "
P14	" "	NCPPB 52	
P16	" "	NCPPB 607	
P18	" "	bean	Crosse U.K.
NZ41	" "	"	" N.Z.
P44	Pseudomonas medicaginis	"	Department culture

M44	Pseudomonas tabaci	Department culture
M45	" "	" "
P3	Pseudomonas marginalis	Dowson U.K.
P5.P3	" "	Graham U.K.
667	" "	NCPPB
1558	" "	NCPPB
1559	" "	NCPPB
1604	" "	NCPPB
Bt1	Pseudomonas spp.	From the rhizosphere of beet
Bt6	" "	" "
Bt7	" "	" "
Cb1	" "	the rhizosphere of cabbage
Cb2	" "	" "
Cb4	" "	" "
Lk1	" "	" leek
Lk2	" "	" "
Lk7	" "	" "
Bn3	" "	" bean
F300	" "	plum
F307	" "	pear
F309	" "	pear
P18	" "	apple Paton U.K.
P19	" "	plum " "
P46	" "	cherry " "
Pt2	" "	the rhizosphere of potato

5A8	Pseudomonas spp.		soil	
6A1	"	"	"	
7A9	"	"	"	
4A3	"	"	"	
5A2	"	"	"	
1A1	"	"	"	
CS4	"	"	"	
F9	"	"	"	
F11	"	"	"	
S60	"	"		Crosse U.K.
6A12	"	"	"	
3D13	"	"	"	
Ps.73	"	"	"	Graham U.K.
R1	"	"	egg	Board U.K.
R2	"	"	"	" "
R3	"	"	"	" "
R4	"	"	"	" "
R5	"	"	"	" "
R6	"	"	"	" "
R7	"	"	"	" "
R8	"	"	"	" "
	Pseudomonas aeruginosa			Sutherland U.K.

The Pseudomonas spp. from soil some of them were isolated by the author and some were received from Dr. A.J. Holding

X1	Xanthomonas phaseoli		Graham U.K.
FXI	"	"	U.K.

X2	*Xanthomonas phaseoli* var. *fuscans*		Graham U.K.
4	*Agrobacterium tumefaciens*	NCPPB	
5	*A. rhizogenes*	"	
223	*A. tumefaciens*	"	
396	" "	"	
397	" "	"	
398	" "	"	
794	" "	"	
925	" "	"	
930	" "	"	
1001	" "	"	
8149	*A. radiobacter*	NCIB	
8150	*A. tumefaciens*	NCIB	
LK11	*Agrobacterium spp.*	soil	
ZM1	" "	"	
76V	*Erwinia atroseptica*		Graham U.K.
G126	" "		" "
G112	" "		" "
HT/1	" "		" "
276	" "		" "
SR4/1	" "		" "
QB4	" "		" "
G110	" "		" "
1172	*E. carotovora*		" "
G147	" "		" "

E32	*E. carotovora*		Graham	U.K.
J6	" "		"	"
340	" "		"	"
312	" "		"	"
G117	" "		"	"
1640	*E. aroideae*		"	"
ENA10	" "		"	"
A8IV	" "		"	"
74V	" "		"	"
J2	" "		"	"
140V	" "		"	"
911V	" "		"	"
119V	" "		"	"
H2	" "		"	"
EP3	*E. chrysanthemi*		"	"
402	" "		"	"
8154	*Alcaligenes viscosus*	NCIB		
8596	" "	"		
8156	*A. faecalis*	"		
8551	*A. tolerans*	"		
8734	*A. metalcaligenes*	"		
9018	" "	"		
9021	" "	"		
C58	*Alcaligenes spp.*	Soil	Holding	U.K.
C511	" "	"	"	"

N.Z. = New Zealand
U.K. = United Kingdom
S.A. = South Africa
NCPPB = National Collection of plant pathogenic bacteria U.K.
NCIB = National Collection of industrial bacteria U.K.

Maintenance of cultures:-

Maintenance of stock cultures was at 4°C on sloped media in 1 oz., screw-capped vials of nutrient agar + 2% (v/v) glycerol.

Nutrient broth:-

The nutrient broth used had the following composition - Peptone (Evans) 0.25 gm; peptone (Bacto) 0.25 gm; Lab lemco (Oxoid) 0.5 gm; NaCl 0.25 gm; distilled water 100 ml. To each litre of medium 5 ml. 1 $\bar{N}$ NaOH was added to give final pH value of 7.0.

Nutrient agar:-

Nutrient broth was solidified with 1.2% (w/v) agar (Oxoid No.3). Additional carbon sources were added on a (w/v) or (v/v) basis.

Buffers:-

Buffers were as described in Mackie & McCartney's Handbook of Bacteriology (Cruickshank, 1960).

PART I

Nutritional Study of the Organisms.

Nutritional Study of the Organisms

The activity of organisms on different carbon compounds

Introduction:-

There are many reports on acid production from sugars by different Pseudomonas species, Sears & Gourlay (1928) found that Ps. aeruginosa produced an acid reaction with glucose as the carbon source only when the nitrogen content of the medium was at low concentration. At high nitrogen concentration, the products of nitrogen metabolism neutralized the acids. With other sugars no acid reaction was observed, even in medium with low nitrogen content, although the sugars had been utilized by the organism. Lacey (1932) in his study of pseudomonads isolated from different plants, found that 90% of the cultures examined produced acid from glucose, 68% from sucrose and none from lactose. Clara (1934) claimed that acid production from glucose and sucrose were important characteristics of plant pathogenic pseudomonads (quoted from Paton, 1956). Wormald (1932) reported that Ps. mors-prunorum produced an alkaline reaction followed by an acid reaction from lactose and in that property they differed from Ps. syringae. However in 1956, Paton found that there were no differences between Ps. syringae, Ps. mors-prunorum in acid production from glucose, sucrose and lactose. However he found that pseudomonads isolated from plant sources produced an acid from sucrose and were different from pseudomonads isolated from sources other than plants which failed to produce an acid reaction from sucrose in peptone medium. His results are in

agreement with Clara (1934), Burkholder & Starr (1948) who also found that acid production from sucrose was a useful test. Klinge (1960) found that none of the pseudomonads he examined produced acid from starch, salicin, lactose or maltose, and 70% of the Ps. fluorescens and 83% of the Ps. putida strains produced acid from sorbitol. All the pseudomonads examined by him (no plant pathogenic pseudomonads were included) produced acid from mannose, glucose, galactose, fructose, arabinose, xylose and glycerol. Most of the work in the study of the carbohydrate metabolism of pseudomonads is concentrated on Ps. fluorescens and Ps. aeruginosa and has been reviewed by DeLey (1960). Little work however has been carried out on the plant pathogenic pseudomonads, although it seems that phytopathogenic pseudomonads degrade glucose in the same manner as does Ps. fluorescens. All the enzymes of the Entner-Doudoroff pathway were found in Ps. fluorescens, Wood & Schwerdt (1953). Their presence has also been established in Ps. angulata, Ps. coronofaciens, Ps. tabaci and Ps. syringae (Katznelson, 1958).

It was of interest to study the activity of organisms on various carbohydrates in an attempt to discover differences, if any, between the plant pathogenic pseudomonads and the non-pathogenic pseudomonads.

Experiment 1. The acid production from different carbohydrates by pseudomonads

It is generally known that pseudomonads produce small amounts of acid from sugars, the fact that acid production could be detected from a carbohydrate in one basal medium and not in another was emphasized by Paton (1956) who found that acid production from the sugars he tested depends on the nature and the concentration of the nitrogen source of the medium.

This experiment was conducted in medium which has the following composition:-

1% (w/v) of the carbon source to be examined

0.008% (w/v) K_2HPO_4

0.002% (w/v) KH_2PO_4

0.02% (w/v) $MgSO_4$

0.1% (w/v) yeast extract (Difco)

0.3% (w/v) agar

Bromothymol blue was used as the indicator, the pH of the basal medium was adjusted to pH7 before sterilization at 121° for 15 min. The carbohydrates were added aseptically from Seitz-filter sterilized solutions to give a final concentration of 1% (w/v) in 5 mls. amounts of the medium in ½" test tubes. Preliminary work showed that yeast extract at the above concentration satisfied the nitrogen requirement of the organisms and that the alkaline reaction occurring in the basal medium was not strong as it was in a medium containing peptone.

The following compounds were used as representatives of monosaccharides, disaccharides, sugar alcohols and glucosides, glucose, xylase, sucrose, maltose, lactose, glycerol, sorbitol and ∝-methyl-D-glucoside. Preliminary work also indicated that neither growth nor acid was produced anaerobically when glucose was tested therefore all the other compounds were tested in unsealed tubes. The tubes were inoculated by stab from 24 hours old cultured grown on nutrient agar + 2% (v/v) glycerol. The tubes were incubated at 27°C and examined at different intervals for the acid production up to 20 days.

Results:- (Table 1)

There was no significant differences between the plant pathogenic and the non-pathogenic pseudomonads in the acid production from these carbohydrates. Ps. phaseolicola was the only species among the plant pathogenic which did not produce an acid from sorbitol which may be useful in the separation of that organism from other plant pathogens.

Discussion:-

Results obtained suggested that acid production from the different carbohydrates examined could not differentiate between the plant pathogenic pseudomonads and the non-pathogenic strains. Ps. phaseolicola was the only species of the plant pathogenic pseudomonads which did not produce acid from sorbitol. Maltose and ∝-methyl-D-glucoside were not utilized by any of the pseudomonads tested with the exception of three strains. The

Table I. Acid production from different carbohydrates. Results after 7 days.

Organism tested	glucose	xylose	sucrose	maltose	lactose*	glycerol	sorbitol	∝-methyl D-glucoside
Ps. syringae								
S5	+	+	+	-	+	+	+	-
S8	+	+	+	-	-	+	+	-
S20	+	+	+	-	-	+	+	-
S36	+	+	+	-	+	+	+	-
W1	+	+	+	-	+	+	+	-
W183	+	+	+	-	-	+	+	-
G2	+	+	+	-	-	+	+	-
Ps. mors-prunorum								
C9	+	+	+	-	+	+	+	-
C22	+	+	+	-	+	+	+	-
C46	+	+	+	-	+	+	+	-
N5	+	+	+	-	+	+	+	-
D1	+	+	+	-	-	+	+	-
D2	+	+	+	-	-	+	+	-
D5	+	+	+	-	+	+	+	-
D16	+	+	+	-	+	+	+	-

Table 1 (Contd.)

Organism tested	glucose	xylose	sucrose	maltose	lactose*	glycerol	sorbitol	∝-methyl D-glucoside
Ps. phaseolicola								
P12	+	+	+	-	-	+	-	-
P13	+	+	+	-	-	+	-	-
P14	+	+	+	-	-	+	-	-
P16	+	+	+	-	-	+	-	-
P18	+	+	+	-	-	+	-	-
NZ41	+	+	+	-	-	+	-	-
Ps. marginalis								
1604	+	+	+	-	-	+	+	-
1559	+	+	+	-	-	+	+	-
1558	+	+	+	-	-	+	+	-
667	+	+	+	-	+	+	+	-
Ps. tabaci								
M44	+	+	+	-	-	+	+	-
M45	+	+	+	-	+	+	+	-
Non-pathogenic strains								
Bn3	(+)	-	-	+	-	-	+	+
Cb1	+	+	+	-	-	+	-	-
Cb4	+	+	+	-	+	+	-	-
Bt6	+	+	+	-	+	+	+	-

Table 1 (Contd.)

Organism tested	glucose	xylose	sucrose	maltose	lactose*	glycerol	sorbitol	∝-methyl D-glucoside
Non-pathogenic strains								
Lk2	+	+	+	-	-	+	-	-
Lk7	+	+	+	-	-	+	+	-
Pt2	(+)	-	-	+	-	-	-	+
6A12	+	+	-	-	-	-	-	-
5A8	+	+	-	-	-	-	-	-
1A1	+	+	+	-	-	+	-	-
CS4	+	+	+	-	-	+	+	-
4A3	+	+	-	-	-	+	+	-
7A9	(+)	-	-	+	-	-	-	+
Ps73	+	+	+	-	-	+	-	-
F300	+	+	+	-	-	+	-	-
F307	+	+	+	-	+	+	+	-
F309	+	+	+	-	-	+	-	-
F9	+	+	-	-	-	-	+	-

+ = acid reaction

- = alcali reaction

* in the case of lactose results were after 14 days from the incubation.

results from maltose were in agreement with those reported by Klinge (1960) and with those reported for the fluorescent pseudomonads by Stainier, Palleroni & Doudoroff (1966). The three exceptions, mentioned above, were non-pathogenic Pt2-Bn3 and 7A9. These strains, however, were not fluorescent and were the only strains examined which could not utilize xylose. Results obtained from lactose indicated that lactose was not readily utilized by pseudomonads since an acid reaction occurred only after a long time of incubation (between 10-20 days). The results were in agreement with those reported by Paton (1956) and also no differences were found between Ps. mors-prunorum and Ps. syringae as claimed before, Wormald (1932).

All the plant pathogenic pseudomonads produced an acid from sucrose. This was in agreement with that reported by Paton (1956), Billing (1963). It was also found that a proportion of the non-pathogenic pseudomonads were able to produce an acid from sucrose, these strains were mainly isolated from the rhizospheres of different plants and isolated from plant leaves, and agrees the results of Paton (1956).

Experiment 2. The production of levan

Introduction:-

It is well known that some pseudomonads synthesize a polysaccharide from sucrose. This ability was used by Wormald (1932) in isolation of Ps. prunicola on nutrient agar with 5% sucrose; Crosse (1953) also used this character for the identification of Ps. mors-prunorum. Erikson (1945) found that both Ps. mors-prunorum and Ps. prunicola produced levan from sucrose while Ps. marginalis, Ps. crasi and the saprophytes Ps. fluorescens and Ps. pyocyaneus did not. Paton (1956) examined pseudomonads isolated from different sources for their ability to synthesize levan from sucrose and found that the majority of levan producing strains were those isolated from plant sources. A high percentage of these were found to be the pathogens. Fuchs (1956) reported that some pseudomonads which did not originate from diseased plants produced levan from sucrose, therefore levan production is not a specific character of plant pathogenic pseudomonads. This was investigated in the following experiment.

Plant pathogenic pseudomonads (31 strains) belonging to different species, and non-pathogenic pseudomonads (20 strains) isolated from various sources were grown on nutrient agar containing 5% (w/v) sucrose, The channel plate technique method described by Paton (1960) was also used.

Results:- (Table 2)

Levan was produced by all the plant pathogenic pseudomonads and by many of the non-pathogenic strains most of which were

isolated from plant sources.

Table 2. The production of levan by different pseudomonads

Organisms tested	Number of strains tested	Number produced levan
Ps. syringae	10	10
Ps. mors-prunorum	9	9
Ps. phaseolicola	6	6
Ps. marginalis	4	4
Ps. tabaci	2	2
Non-pathogenic pseudomonads		
pseudomonads from soil	10	2
pseudomonads associated with plants	10	8

Experiment 3.

In a few cases it was difficult to decide whether or not levan had been formed (a strain of Ps. syringae and of Ps. phaseolicola, and 2 saprophytic strains). Therefore the following experiment was conducted to examine, chemically, whether levan was formed. Levan producing strains were used as controls. Identification of polysaccharide component was by paper chromatography.

The method:-

The organisms were grown on nutrient agar plus 5% (w/v) sucrose for three days at 27°C in Roux bottles. The cells and

the polysaccharide were harvested and were shaken vigorously for about 10 mins. After centrifugation the cloudy supernatent liquid was tested for polysaccharides.

The isolation of the polysaccharide:-

To the cloudy solution, ethanol 95% (v/v) was added, 3 volumes to 1 volume of the supernatent, mixed thoroughly and left at room temperature for about 20 mins. Within that time the polysaccharide precipitated and the ethanol was decanted. The precipitated polysaccharide was redissolved in water and the procedure was repeated two times more to wash the polysaccharide free of the sucrose of the medium and other compounds.

The hydrolysis of the polysaccharide:-

A proportion of the polysaccharide, about 0.3 gm. wet weight, and 5 ml. of 0.05$\bar{N}$ H_2SO_4 were heated at 100°C in a water bath for about 10 mins. A saturated solution of barium hydroxide was then added to neutralize the acid. Finally the hydrolysed polysaccharide was filtered from the precipitated barium sulphate and spotted onto Whatman No.4 paper for chromatographic study. Two solvents were used n-Butanol-pyridine-water (3:1:1) used by Paton (1956) and isopropanol-water (160:40) Smith (1960). A descending chromatogram was run for 6 hours at room temperature. The sugars spots were located with two different reagents.

1. Phloroglucinol, and
2. Aniline diphenylamine reagent, both as described by Smith (1960).

Results:-

All the organisms tested were found to produce a polysaccharide from sucrose, which, when hydrolysed was found to contain only fructose.

The above method although laborious, gave unequivocal results in the cases normally difficult to judge by visual means. There proved to be no differences between the different Pseudomonas species in this respect.

Experiment 4. The quantitative determination of the levan produced by pathogenic and non-pathogenic Pseudomonads

Corey & Starr (1957) found a direct correlation between the amount of polysaccharide produced and the pathogenicity of 4 colony types in Xanthomonas phaseoli, therefore it was of interest to discover whether there were any differences between the plant pathogenic and the non-pathogenic pseudomonads in the amount of levan produced from sucrose.

Representative strains were grown on nutrient broth for 24 hours and 1 ml. of the growing cultures was added to 50 mls. of nutrient broth + 5% (w/v) sucrose in 300 mls. flasks. Flasks were incubated at 27°C in shaker for 48 hours.

The determination of the amount of polysaccharide produced:-

Modification of Corey & Starr (1957) method was used. After 48 hours the cultures were centrifuged and the cells resuspended

in 50 mls. of water and shaken for 1 hour to dissolve the polysaccharide. Then the cells and the polysaccharide mixture was centrifuged. The polysaccharide was washed free of cells, and the polysaccharide dissolved in the growing medium was precipitated with 95% (v/v) ethanol and redissolved in water and reprecipitated. The polysaccharide was dried at 105°C to constant weight.

Results:- (Table 3)

These results indicate no significant differences between either the different plant pathogenic Pseudomonas species or between non-pathogenic and pathogenic species.

Table 3. The amount of levan produced from sucrose by different Pseudomonas species in 50 mls. of medium

Ps. syringae		Ps. marginalis	
S5	322 mg.	1559	392 mg.
S8	410 mg.	667	311 mg.
Ps. mors-prunorum		Ps. tabaci	
D1	378 mg.	M45	320 mg.
D2	325 mg.		
Ps. phaseolicola		Non pathogenic Pseudomonads	
P12	257 mg.	Cb4	396 mg.
P14	279 mg.	F300	230 mg.

The production of copper reducing compounds from sucrose by the organisms

Paton (1956) found that a large number of pseudomonads including pathogens isolated from plants were capable of producing a substance which reduced Benedict's solution at 100°C when grown on sucrose medium, whereas a large number of the pseudomonads isolated from sources other than plants and similarly grown did not produce a reducing substance.

Experiment 5. The effect of sucrose concentrations and time of incubation on the copper reduction test

Representative strains (11) were grown on nutrient broth containing the following concentrations of sucrose, 0.5%-1%-2% and 4% (w/v). The tubes were incubated at 27°C, the cultures examined at different intervals for their ability to reduce Benedict's solution at 100°C for 10 min. over a 21-day incubation period.

Results:- (Table 4) indicated that reducing activity was very strong after 2 days in most cases, especially at the concentrations of 2 and 4% (w/v). These cultures were able also to reduce Benedict's solution after 21 days of incubation. At 0.5% (w/v), however, it was found that most of the cultures were unable to reduce Benedict's after 14 days of the incubation. This suggested that the reducing compounds produced were either utilized or present in amounts undetectable by this method. Ps. aeruginosa and the non-pathogenic strain 5A8 failed to form a reducing compound.

Table 4. The effect of sucrose concentration and time of incubation on copper reduction test

Organism tested	0.5% sucrose				1% sucrose			2% sucrose		4% sucrose	
	Days of incubation										
	2	5	10	14	5	10	14	2	21	2	21
Ps. syringae											
S5	+	++	++	-	++	++	(+)	++	++	++	++
S8	+	++	+	-	++	++	-	++	++	++	++
Ps. phaseolicola											
P12	+	++	++	-	++	++	-	++	++	++	++
P14	+	++	++	-	++	++	(+)	++	++	++	++
Ps. mors-prunorum											
D5	+	++	+	-	++	++	(+)	++	++	++	++
N5	+	++	++	-	++	++	(+)	++	++	++	++
Ps. marginalis											
1559	+	++	++	-	++	++	(+)	++	++	++	++
1558	+	++	++	(+)	++	++	(+)	++	++	++	++
Non-pathogenic pseudomonads											
5A8	-	-	-	-	-	-	-	-	-	-	-
Cb4	+	++	++	-	++	++	(+)	++	++	++	-
Ps. aeruginosa	-	-	-	-	-	-	-	-	-	-	-

++ = strong reaction
+ = moderate reaction
(+) = weak reaction
- = No reduction of the Benedict's solution.

Experiment 6. The production of copper reducing compounds from sucrose and gluconate

The organisms under test were inoculated into nutrient broth + 2% (w/v) sucrose and into Na gluconate 4% (w/v) medium as

described by Haynes (1951). The organisms were tested for their ability to reduce Benedict's solution at 100°C for 10 mins. at different intervals up to 7 days of incubation at 27°C.

Results:- (Table 5) indicated that plant pathogenic pseudomonas species tested reduced Benedict's solution when grown on sucrose, the reaction was detected after 2 or 3 days and was very strong. In the case of gluconate none of the plant pathogenic pseudomonas species tested, with the exception of Ps. marginalis, produced a reducing compound from gluconate. Some of the non-pathogenic pseudomonads produced a reducing substance from sucrose. These strains were mainly of the levan producing group and were isolated from the rhizosphere and the plant surfaces. In the case of gluconate, it was found that the strains which produced a reducing compound from gluconate were mainly of the non-pathogenic type which included Ps. aeruginosa and Ps. marginalis strains.

The ability to produce a reducing compound from sucrose has no correlation with the ability to produce one from gluconate. The inability of Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola and Ps. tabaci to produce a copper reducing compound from gluconate was confirmed again when 2 representative strains from each species were grown on Na gluconate medium in shaking cultures, (to test the effect of cultural conditions) as Haynes (1951) recommended the use of aerated culture.

Table 5. The production of reducing substances from gluconate and from sucrose by Pseudomonads

Organism tested	sucrose	gluconate	Organism tested	sucrose	gluconate
Ps. syringae			*Ps. tabaci*		
S5	+	-	M44	+	-
S8	+	-	M45	+	-
S20	+	-	Non-pathogenic strains		
S36	+	-	Bn3	-	-
W1	+	-	Cb1	+	-
W183	+	-	Cb4	+	-
G2	+	-	Bt6	+	-
Ps. mors-prunorum			Lk2	+	+
C9	+	-	Lk7	+	-
C22	+	-	Pt2	-	-
C46	+	-	5A8	-	-
N5	+	-	1A1	+	+
D1	+	-	Cs4	+	-
D2	+	-	4A3	-	-
D5	+	-	7A9	-	-
D16	+	-	Ps.73	+	+
Ps. phaseolicola			F300	+	+
P12	+	-	F307	+	-
P13	+	-	F309	+	+
P14	+	-	F9	-	-
P16	+	-	*Ps. aeruginosa*	-	+
P18	+	-			
NZ41	+	-			
Ps. marginalis					
1604	+	+			
1559	+	+			
1558	+	+			
667	+	+			
P3	+	+			

Experiment 7. The relationship between acid production from sucrose, levan formation and the copper reduction test among the plant pathogenic Pseudomonads

The results obtained indicated that, in the case of all the plant pathogenic pseudomonads there was a positive correlation between acid production, levan production and the ability of organisms to produce a copper reducing compound from sucrose.

Experiment 8. The identification of the reducing substances produced from sucrose by the organisms

Representative strains (11) of the plant pathogenic pseudomonads as well as non-pathogenic Pseudomonas species were inoculated into a nutrient broth containing 2% (w/v) sucrose. The cultures were chromatogramed every day for 7 days, then examined at 10 and 14 days, for the identification of the reducing compounds produced from sucrose. Preliminary work indicated that the following solvent was the most suitable for the separation of the compounds on Whatman No.1 paper, isopropanol: water (160:40) a descending chromatogram was run for 6 hours at room temperature. The chromatograms were dried and stained with triphenyltetrazolium chloride according to Trevelyan, Procter & Harrison (1950). This reagent was found by Paton (1956) to react with glucose, fructose and 2-ketogluconate. Aniline diphenylamine reagent was also tested.

Results:- (Table 6) indicated that after 2-3 days incubation all the organisms which were able to produce a copper reducing compound from sucrose, produced 2-ketogluconate, which was recognised by its R_f value when P3 strain (which is Ps. marginalis) was used as a control. This strain was found by Paton (1956) to produce 2-ketogluconate from sucrose. Fructose and glucose were the other compounds detected in these cultures. Results also indicated that 2-ketogluconate was present in these cultures even after 14 days of incubation. No sucrose was detected in these cultures after 4-6 days of incubation.

Table 6. The identification of reducing compounds produced by pseudomonads grown on sucrose.

Results after 48 hours.

Organism tested	The presence of			
	sucrose	fructose	glucose	2-ketogluconate
Ps. syringae				
S5	+	+	+	+
S8	+	+	+	+
Ps. phaseolicola				
P12	+	+	+	+
P14	+	+	+	+
Ps. mors-prunorum				
D5	+	+	+	+
N5	+	+	+	+
Ps. marginalis				
P3	+	+	+	+
1559	+	+	+	+
667	+	+	+	+
non-pathogenic pseudomonads				
Cb4	+	+	+	+
5A8	+	-	-	-

Experiment 9. The accumulation of 2-ketogluconate from sucrose by the plant pathogenic pseudomonads

Further strains (6 Ps. syringae, 6 Ps. mors-prunorum, 4 Ps. phaseolicola, 2 Ps. marginalis as well as 2 Ps. tabaci) were grown on nutrient broth sucrose medium, and tested as before. All accumulated 2-ketogluconate from sucrose, indicating a positive relationship between the copper reduction test and the presence of 2-ketogluconate.

Discussion:-

The results described above indicated that the plant pathogenic pseudomonads produced 2-ketogluconate from sucrose. This characteristic, as will be shown later, may be of some importance in the establishment of plant pathogenic pseudomonads in the plant tissue. These results, however, are in disagreement with those reported by Paton (1956) who found that the reducing substances obtained from sucrose by Ps. mors-prunorum and Ps. syringae were hexoses and not 2-ketogluconate. This disagreement could be due to the different medium and cultural conditions used as well as the chromatographic procedure. The reducing substance obtained from gluconate was found to be 2-ketogluconate by Koepsell (1950). The inability of the plant pathogenic pseudomonads except Ps. marginalis, to produce 2-ketogluconate from gluconate was unexpected. However, this result was in agreement with that reported by Paton (1956) who also found that Ps. mors-prunorum and Ps. syringae were not able to utilize 2-ketogluconate. He also found that Ps. viridiflava was able to

utilize 2-ketogluconate but did not produce a copper reducing compound from sucrose. Katznelson (1956) also found that none of the plant pathogenic pseudomonads he tested were capable of utilizing 2-ketogluconate. Since 2-ketogluconate is not apparently utilized by the plant pathogenic pseudomonads, and a correlation was found by Paton (1956) between the production of 2-ketogluconate from gluconate and the utilization of 2-ketogluconate, the inability of the plant pathogenic pseudomonads to accumulate 2-ketogluconate from gluconate could be explained on the basis that these organisms are not able to utilize the keto acid. The keto acid was only produced from gluconate by the organisms able to utilize 2-ketogluconate. However, Garrett, Panagopoulas & Crosse (1966) reported that Ps. syringae and Ps. mors-prunorum were able to oxidize gluconate. Their method of testing for gluconate oxidation depended on the precipitate formed 24 hours after boiling the growing medium with Benedict's reagent. The ability of the plant pathogenic pseudomonads tested to accumulate 2-ketogluconate from sucrose could be explained on the basis that when these organisms metabolise sucrose, glucose is released and metabolized with the production of 2-ketogluconate according to Lackwood, Tabenkin & Ward (1941) and Norris & Campbell (1949) who reported that 2-ketogluconate was produced from glucose by pseudomonads.

Experiment 10. The production of reducing compounds from other disaccharides

Results obtained from a study of organisms on sucrose medium indicated that the plant pathogenic pseudomonads and a proportion of the non-pathogenic strains are able to produce 2 ketogluconate. Therefore it was of interest to examine the effect of organisms on some other disaccharides. Lactose and maltose were used at 2% (w/v) in nutrient broth media. The same representative strains were used as in experiment (8). After 2, 4 and 6 days the cultures were chromatogramed as described before. The chromatograms were stained with triphenyltetrazalium chloride reagent.

Results:-

Lactose or maltose were the only sugars detected. No other reducing compounds were detected with any of the cultures examined.

Experiment 11. The production of copper reducing compounds from sucrose by other plant pathogenic bacteria belonging to Xanthomonas, Agrobacterium and Erwinia.

As suggested earlier, the production of 2-ketogluconate by the plant pathogenic pseudomonads could be of some importance in the establishment of these organisms within the plant tissue. Therefore it was of interest to examine some other plant pathogenic bacteria belonging to other genera for their effect on sucrose. The medium used in this study was nutrient broth containing 2% (w/v) sucrose.

The organisms were inoculated into sucrose medium, and were examined every day during 7 days incubation, for their ability to reduce Benedict's solution at 100°C.

Organisms tested

Xanthomonas phaseoli X_1 and FX_1

X. phaseoli var. fuscans X_2

Agrobacterium tumefaciens 223 - 794 - 1001 and 8150 strains

Soft rotting coliforms 26 strains

Results:-

The 3 Xanthomonas strains examined produced a copper reducing compound which was best detected after 4 days of incubation.

The 4 Agrobacterium tumefaciens strains all produced a copper reducing compound from sucrose. The reaction was weak up to the fifth day of incubation but strong after further incubation.

The results obtained from the examination of the 26 strains

of Erwinia, representative of 4 species, showed that only 7 strains, all E. atroseptica, reduced Benedict's solution at 100°C. The reaction was strong and occurred after 24-48 hours of the incubation. Only one strain of E. atroseptica (strain 276) failed to produce any reducing substance from sucrose. However, the classification of that strain was questioned by Dr. D. Graham (Personal communication) on other grounds.

Experiment 12.

Bernerts & DeLey (1963) found that Agrobacterium tumefaciens and A. radiobacter produced a copper reducing compound, identified as 3-ketoglucoside, from the corresponding disaccharides and bionic acids. They examined different Gram's-negative organisms but found that only A. tumefaciens and A. radiobacter carried out this reaction. As they apparently did not examine plant pathogens in detail, it was of interest to examine plant pathogenic pseudomonads and different Erwinia species in lactose and sucrose media.

The following representative strains were tested.

Pseudomonads S5 - S8 - P12 - P14 - D1 - C9 - 667 - 1559 - 4A3 - Cb4.

Agrobacterium tumefaciens 8150 - 1001 - 794 - 223.

Erwinia QB4 - G110 - E32 - 1172 - A8IV - 340.

They were grown on lactose and sucrose media, as described by Bernerts and DeLey (1963). After 2 and 4 days of incubation the plates were flooded with Benedict's solution.

Results:-

Lactose medium:- None of the pseudomonads or erwinias examined produced 3-ketolactose. Of the 4 agrobacterium strains only 8150 and 1001 strains produced 3-ketolactose.

Sucrose medium:- None of the Agrobacterium strains produced a reducing compound round the mass of growth. The plant pathogenic pseudomonads and one of the non-pathogenic strains produced a weak reaction as well as E. atroseptica strains.

Experiment 13. The identification of copper reducing compounds produced from sucrose by Xanthomonas, Agrobacterium and Erwinia atroseptica

The 3 xanthomonads strains, the 4 Agrobacterium tumefaciens strains and the 7 E. atroseptica strains were grown on nutrient broth + 2% (w/v) sucrose. The cultures were chromatogramed after 2, 3, 4, 6, 8 and 10 days, the chromatographic method used was the same as described before.

Results:-

Xanthomonads:- The sucrose was the only sugar detected in the cultures up to the 4th day of incubation and after 6, 8 and 10 days glucose as well as fructose were the only reducing compounds detected. No 2-ketogluconate or other reducing compound was detected. However, because the growth of Xanthomonas was not very good in such a medium, another medium,

containing 2% (w/v) sucrose in a mineral salt medium containing 0.2% (w/v) yeast extract was used. Cultures were aerated and the growth was very good. The only reducing substances detected, however, were glucose and fructose.

Agrobacterium:- The results obtained from the examination of agrobacterium strains were unexpected in that sucrose was the only compound detected up to the 4th day. After 6, 8 and 10 days, glucose and fructose were the only reducing compounds detected. No 3-ketosucrose could be detected and this explains the failure of Agrobacterium to produce a yellow ring round the growth when tested on Bernerts & DeLey's sucrose medium.

Erwinia atroseptica:- Strains of E. atroseptica produce a reducing compound which reacted with the triphenyltetrazolium chloride reagent and was detected after 2 days incubation, no other reducing compound was detected. All the 7 E. atroseptica strains produced the same reducing compound, as indicated by the similarity of the R_f value of the compound. The compound was not 2 ketogluconate as it possessed a different R_f value to 2 ketogluconate.

Discussion:-

Xanthomonas species have been shown to produce an acid reaction from sucrose Dye (1958), as have the plant pathogenic pseudomonads described in this study. The Xanthomonas examined differed from the plant pathogenic pseudomonads in that they did not accumulate 2-ketogluconate from sucrose.

The results obtained with agrobacterium strains showed that

not all the strains of A. tumefaciens accumulated 3-ketolactose, as claimed by Bernerts & DeLey (1963), (2 strains were unable to do so). The results obtained from lactose medium also showed that Pseudomonas and different species of Erwinia are not able to accumulate 3-ketolactose. Results obtained from sucrose medium however indicated that A. tumefaciens did not accumulate 3-ketosucrose and were able to cleave the glucoside linkage in the sucrose molecule since glucose and fructose were the only reducing compounds detected. Results also showed that the medium described by Bernerts & DeLey (1963) for the production of 3-ketoglucoside from disaccharide is not useful for the identification of agrobacteria if sucrose is used. Furthermore it has been found in this study that pseudomonads as well as Erwinia atroseptica to a lesser degree, could give the reaction described by Bernerts and DeLey when sucrose was used.

Results obtained from the examination of the soft rotting coliforms revealed that Erwinia atroseptica, the black leg organism was the only Erwinia species which accumulated a reducing compound from sucrose. Graham (1964) examined 57 strains of soft rot coliforms for acid production from sucrose and he found that all the organisms he tested produced an acid reaction. He also found a remarkable uniformity in the biochemical reactions of these organisms. At present it is not possible to distinguish the erwinias by physiological tests (Graham & Dowson,(1960), Graham (1964)). However, the results described above indicated that E. atroseptica could be distinguished from the other erwinias by their ability to accumulate a reducing compound when they were grown on sucrose medium.

Experiment 14. The utilization of some organic acids by the organisms

This experiment was conducted in an attempt to discover which organic acids were used by pseudomonads, whether or not the plant pathogens could be separated from the non-pathogens on their choice of carbon source.

The basal medium used in this study has the following composition (w/v):- NH_4 H_2PO_4 0.1%, $MgSO_4$ 0.02%, K_2HPO_4 0.008%, KH_2PO_4 0.002%, yeast extract (Difco) 0.05%, bromothymol blue used as indicator.

The following organic acids were tested:-
Na tartrate, Na fumarate, Na citrate, Na succinate, Na lactate, Na benzoate, Na malonate, Na propionate, DL-malic acid and Ca gluconate. The above organic acids were added to the basal medium to a final concentration 0.5%, the medium was adjusted to pH 6.8 and sterilized at 121° for 15 mins. To determine whether failure to grow with an organic acid was due to toxicity at the concentration used, or inability to use the acid, glucose (0.5% w/v) was added to a second batch of organic acid media for control purposes. Increase of growth in acid containing medium over growth occurring on basal medium was used as measure of ability to use the acid. Also used in conjunction with the growth increase was the increase of pH in the acid containing medium.

Results:- (Table 7) indicated that the following organic acids were utilized by all the organisms tested. Ca gluconate,

DL-malic acid, Na malonate, Na succinate, Na citrate and Na fumarate.

Na propionate and Na benzoate were toxic to most organisms as indicated by the failure to grow on these organic acids even when glucose was added. Results did not differentiate between the plant pathogenic and the non-pathogenic pseudomonads. However Ps. syringae strains were different from Ps. mors-prunorum in that Ps. syringae strains were able to utilize lactate but not tartrate, whilst Ps. mors-prunorum were able to utilize tartrate but not lactate, with the exception of P47 strain.

Table 7. The utilization of organic acids by the Pseudomonads

Strains tested and the number examined	The number of strains utilized the organic acid										
	Na tar-trate	Na fuma-rate	Na cit-rate	Na oxa-late	Na succ-inate	Na lac-tate	Na benz-oate	Na malo-nate	Na prop-ionate	DL malic acid	Ca gluc-onate
Ps. syringae 8	0	8	8	0	8	8	-	8	-	8	8
Ps. mors-prunorum 9	8	9	9	0	9	1	-	9	-	9	9
Ps. phaseo-licola 6	0	6	6	0	6	0	-	6	-	6	6
Ps. margin-alis 4	4	4	4	0	4	4	0	4	-	4	4
Ps. tabaci 2	2	2	2	0	2	2	-	2	-	2	2
Non-patho-genic Pseudomonads 14	12	14	14	0	14	14	-,0	14	-	14	14

- = The organic acid or its salt is toxic

0 = The organic acid was not utilize

The nitrogen requirements

Experiment 15.

The nitrogen requirements of the organisms were studied in an attempt to discover differences between the plant pathogenic and non-pathogenic pseudomonads. Holding (1960) studied the nitrogen requirement of Gram-negative soil bacteria, including pseudomonads, and he grouped them according to their nitrogen requirements and other characters. Preliminary work indicated that both the medium containing 10% (v/v) yeast autolysate and that containing 0.5% (w/v) vitamin free casamino acids was toxic to some organisms.

The following basal medium was used, it contains (w/v) 0.5% glucose, 0.008% K_2HPO_4, 0.002% KH_2PO_4, 0.02% $MgSO_4$, 1.2% agar, the nitrogen compound was added at 0.1% concentration, except KNO_2 which was tested at 0.01% (w/v).

The following compounds were tested:-
Potassium nitrate, ammonium phosphate, potassium nitrite, yeast extract (Difco), vitamine-free casamino acids (Difco) and ammonium lactate. The media were adjusted to pH7 and sterilized at 121°C for 15 mins. The organisms were streaked onto these different media and the plates incubated at 27°C.

Results:- (Table 8)

Results indicated that the yeast extract and the amino acid media supported good growth of all the organisms tested, all growing very well after 24 hours. The ammonium lactate medium was found to support the growth of all the Ps. syringae strains

but of only one strain out of 9 of the Ps. mors-prunorum. No differences were found between the plant pathogenic and non-pathogenic pseudomonads on any of the media tested. None of the plant pathogenic pseudomonads could grow on KNO_2 medium. However 3 out of 14 non-pathogenic grew on that medium. Of the mineral nitrogen sources examined $NH_4H_2PO_4$ was found to support good growth for all the plant pathogenic pseudomonads. Ps. marginalis grew well on KNO_3 medium but most of the other pathogens gave weak growth or did not grow.

Table 8. The nitrogen requirement of the organism results after 7 days incubation at 27°C

Organism tested	Potassium nitrate KNO_3 0.1%	Potassium nitrite KNO_2 0.01%	Ammonium phosphate $NH_4H_2PO_4$ 0.1%	Ammonium lactate 0.1%	Vitamine free casamino acid 0.1%	Yeast extract 0.1%
Ps. syringae						
S5	(+)	-	+	+	++	++
S8	(+)	-	+	+	++	++
S20	(+)	-	+	+	++	++
S36	(+)	-	+	+	++	++
W1	+	-	+	+	++	++
W183	-	-	+	+	++	++
G2	-	-	+	+	++	++
Ps. mors-prunorum						
D1	+	-	+	-	++	++
D2	(+)	-	+	-	++	++
D5	(+)	-	+	-	++	++
D16	(+)	-	+	-	++	++
C9	(+)	-	+	-	++	++

Table 8 (Contd.)

Organism tested	Potassium nitrate KNO_3 0.1%	Potassium nitrite KNO_2 0.01%	Ammonium phosphate $NH_4H_2PO_4$ 0.1%	Ammonium lactate 0.1%	Vitamine free casamino acid 0.1%	Yeast extract 0.1%
Ps. mors-prunorum						
N5	(+)	-	+	-	++	++
C22	(+)	-	+	-	++	++
C46	(+)	-	+	-	++	++
P47	(+)	-	+	+	++	++
Ps. phaseolicola						
P12	(+)	-	+	-	++	++
P13	-	-	+	-	++	++
P14	(+)	-	+	-	++	++
P16	-	-	+	-	++	++
P18	-	-	+	-	++	++
NZ41	-	-	+	-	++	++
Ps. marginalis						
1559	+	-	+	+	++	++
1558	+	-	+	+	++	++
1604	+	-	+	+	++	++
667	+	-	+	+	++	++
Ps. tabaci						
M44	(+)	-	+	-	++	++
M45	(+)	-	+	-	++	++
non-pathogenic Pseudomonads						
14	10	3	11	10	14	14

- = No growth
(+) = weak growth
+ = good growth
++ = very good growth

The numbers are the number of non pathogenic strains grown

Experiment 16. The utilization of ammonium lactate as carbon and nitrogen source

Results obtained from the utilization of organic acids indicated that lactate was utilized by all the Ps. syringae strains, whereas the Ps. mors-prunorum strains did not utilize lactate with the exception of one strain. The same result was obtained when ammonium lactate was examined as nitrogen source. It was of interest therefore to study the utilization of ammonium lactate as the only source of carbon and nitrogen by the organisms.

The medium used has the following composition:-

0.5% (v/v) ammonium lactate

0.002% (w/v) KH_2PO_4

0.008% (w/v) K_2HPO_4

0.02% (w/v) $MgSO_4$

1.2% (w/v) agar

The medium was adjusted to pH 7 and sterilized at 121° for 15 mins. The plates were streaked and incubated at 27°C.

Results:- (Table 9)

Results indicated that all the Ps. syringae strains are able to utilize ammonium lactate as the only source of carbon and nitrogen whereas Ps. mors-prunorum strains did not with the one exception mentioned above strain P47.

These results suggest that the utilization of ammonium lactate as carbon and nitrogen source could differentiate between Ps. syringae and Ps. mors-prunorum. However the majority of the

non-pathogenic pseudomonads could utilize that compound as well as Ps. marginalis strains.

Table 9. The utilization of ammonium lactate as carbon and nitrogen source

Number of strains tested		Number of strains utilized ammonium lactate
Ps. syringae	8	8
Ps. mors-prunorum	9	1
Ps. phaseolicola	6	0
Ps. marginalis	4	4
Ps. tabaci	2	2
non-pathogenic Pseudomonads	14	11

Experiment 17. Nitrate reduction

The ability of organisms to reduce nitrate to nitrite or to nitrogen gas was examined.

The media used in this study has the following composition:- 0.5% (w/v) glucose, 0.008% (w/v) K_2HPO_4, 0.002% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4$, 0.2% (w/v) yeast extract and 0.1% (w/v) KNO_3, the medium was adjusted to pH7 and sterilized at 121° for 15 mins. The medium was used as liquid medium which was tubed in 5 mls. amount in ½" test tubes and containing fermentation tubes to detect any gas produced.

The medium was also used as a soft agar (0.2% w/v agar) which was stab inoculated. The liquid medium was also tested under anaerobic condition when the tubes were sealed with liquid paraffin after inoculation. The three media were incubated at 27°C for 4 days and then examined for the presence of nitrite with sulphanilic acid and ∝-naphthylamine. All negative cultures were tested with zinc dust for the presence of nitrate, positive cultures were tested for nitrate in the presence of nitrite by adding sulphanilic acid and boiling to destroy nitrite, when cool nitrate was tested for by the addition of sulphanilic acid and ∝-naphthylamine and zinc dust.

Results:- (Table 10)

All the organisms tested gave good growth in the liquid medium, aerobically incubated, and the soft agar medium. There was no growth in the liquid medium which was sealed with paraffin, except with 4 organisms. These grew very well in all the 3 media

Table 10. The effect of organisms on KNO_3

Organism tested	The presence of NO_2	The presence of NO_3	Organism tested	The presence of NO_2	The presence of KNO_3
Ps. syringae			*Ps. phaseolicola*		
S5	(+)	+	P12	-	+
S8	(+)	+	P13	-	+
S20	(+)	+	P14	-	+
S36	(+)	+	P16	-	+
W1	-	"	P18	-	+
W183	-	+	NZ41	-	+
G2	-	+	*Ps. tabaci*		
Ps. mors-prunorum			M44	-	+
D1	(+)	+	M45	-	+
D2	(+)	+	*Ps. marginalis*		
D5	(+)	+	667	+	+
D16	(+)	+	1558	+	+
C9	-	+	1559	+	+
N5	-	+	*1604	-	-
C22	-	+	non-pathogenic pseudomonads		
C46	-	+	number tested		
			** 14	4	11

* = gas produced

** = 3 non-pathogenic Pseudomonads produced gas and no nitrate nor nitrite could be detected.

and produced N_2 gas, of these organisms, 3 were non-pathogenic and one was Ps. marginalis strain 1604. The results obtained when the cultures were tested for nitrite was the same in both the liquid medium and in the soft agar medium, and can be summarised as follows:-

1. Nitrate was still present in all the cultures with the exception of the 4 cultures which produced gas.

2. Ps. marginalis strains strongly reduced nitrate to nitrite as did some of the non-pathogenic organisms.

3. Ps. phaseolicola strains as well as Ps. tabaci did not reduce nitrate to nitrite.

4. Some of the Ps. syringae and Ps. mors-prunorum strains produced a weak nitrite reaction.

Experiment 18. The effect of haemin on the nitrate reduction

Fewson & Nicholas (1961) reported that both molybdenum and cytochromes participate in nitrate reduction. It was of interest therefore to study the effect of haemin on the nitrate reduction. Haemin was dissolved in a small amount of triethanolamine and was added to give a final concentration of 5 μg/ml of the soft agar nitrate medium described above. After 4 days of incubation, the cultures were examined for their ability to reduce nitrate.

Results:-

The addition of haemin has no effect on the nitrate reduction.

Experiment 19. Nitrate reduction by organisms previously grown on nitrate medium

Klinge (1960) reported that nitrate reduction by some strains of pseudomonads could be found only when the strains were passaged through a synthetic medium containing nitrate as the only source of nitrogen. Therefore representative strains were grown on the nitrate medium for 2 days and then inoculated into the nitrate soft agar medium as described before. The tubes were incubated for 4 days and then examined for nitrate reduction, the control treatment was with the same organisms inoculated directly from nutrient agar + 2% (v/v) glycerol into the nitrate soft agar medium.

Results:-

There were no differences between the nitrate passage treatment and the control treatment.

Experiment 20. The effect of organisms on nitrite (nitrite reduction)

The ability of organisms to reduce nitrite was studied in the same medium as was used for nitrate reduction. Nitrite was used of concentration 0.02% (w/v) KNO_2; the soft agar medium was used and incubated at 27°C for 7 days. Cultures which grow on that medium were examined for the presence or absence of nitrite.

Results:-

None of the Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola or Ps. tabaci strains were able to grow on that medium. When, however, the nitrite concentration was reduced to 0.01% (w/v) there was growth but nitrite was not reduced by these organisms. Nitrite was reduced only by the organisms which produced gas from nitrate.

Experiment 21. The utilization of amino acids as carbon and nitrogen source

Results obtained from experiment 15 indicated that the vitamin free casamino acids medium supported the growth of all the organisms tested. The following experiment was conducted, therefore, to see which amino acids could be utilized as the only source of carbon and nitrogen.

The basal medium used in this study had the following composition:- $MgSO_4$ 0.02% (w/v), K_2HPO_4 0.008% (w/v), KH_2PO_4 0.002% (w/v). The amino acids were added at concentration of 0.1% (w/v) and 0.05% (w/v) except for tryptophan which was tested at 0.1% (w/v) and 0.02% (w/v). The pH of the media were adjusted to pH7 and sterilized at 115° for 10 mins. The media were inoculated with one drop of slightly turbid bacterial suspension. The tubes were incubated for 7 days at 27°C. The following amino acids were tested:- glycine, β-alanine, DL-alanine, L-asparagine, D-asparagine, DL-aspartic acid, L-glutamic, L-leucine, L-lysine, DL-isoleucine, L-proline, DL-valine, DL-methionine, L-arginine monohydrochloride, DL-histidine, DL-serine, D-tryptophane, L-tryptophane, DL-tryptophane, L-tyrosine, DL-β-phenylalanine, creatine and creatinine.

Results:- (Table 11)

There were no differences in the results obtained from using the amino acids at 0.1% (w/v) or 0.05% (w/v).

Table 11. The utilization of amino acids as carbon and nitrogen sources by Pseudomonads

The organisms and number tested	The number of organisms used the amino acid					
	Glycine	β-alanine	DL-alanine	L-asparagine	D-asparagine	DL-aspartic acid
Ps. syringae (8)	0	0	8	8	0	8
Ps. mors-prunorum (9)	0	0	9	9	0	9
Ps. phaseolicola (6)	0	0	6	6	0	6
Ps. tabaci (2)	0	0	2	2	0	2
Ps. marginalis (4)	0	2	4	4	0	4
non-pathogenic Pseudomonads (14)	3	4	11	11	0	11

The organisms and number tested	The number of organisms used the amino acid					
	L-glutamic acid	L-leucine	DL-isoleucine	L-proline	DL-valine	DL-methionine
Ps. syringae (8)	8	8	0	8	0	0
Ps. mors-prunorum (9)	9	4	0	9	0	0
Ps. phaseolicola (6)	6	0	0	6	0	0
Ps. tabaci (2)	2	2	0	2	0	0
Ps. marginalis (4)	4	4	4	4	4	0
non-pathogenic Pseudomonads (14)	11	6	4	11	6	0

Table 11 (Contd.)

The organisms and number tested	The number of organisms used the amino acid					
	L-arginine Hcl	DL-histidine	DL-serine	L-tyrosine	L-tryptophan	D-tryptophan
Ps. syringae (8)	8	5	2	0	0	0
Ps. mors-prunorum (9)	5	4	2	2	0	0
Ps. phaseolicola (6)	0	0	0	0	0	0
Ps. tabaci (2)	0	0	0	0	0	0
Ps. marginalis (4)	4	4	1	4	4	0
Non-pathogenic Pseudomonads (14)	6	7	2	7	2	0

The organisms and number tested	The number of organisms used the amino acid				
	DL-tryptophan	DL-β-phenyl-alanine	creatine	creatinine	L-lysine
Ps. syringae (8)	0	0	0	0	0
Ps. mors-prunorum (9)	0	0	0	0	0
Ps. phaseolicola (6)	0	0	0	0	0
Ps. tabaci (2)	0	0	0	0	0
Ps. marginalis (4)	4	0	0	0	4
Non-pathogenic Pseudomonads (14)	2	3	0	0	5

The results could be summarised as follows:-

1. No clear cut difference was obtained between the plant pathogenic and the non-pathogenic pseudomonads on any of the amino acids tested.

2. Ps. marginalis strains seem to be closely related to some of non-pathogenic pseudomonads, as shown by the utilization of DL-isoleucine, DL-valine, L-tryptophane, and L-lysine. The results with the above amino acids showed they all are used by all the Ps. marginalis strains and some of the non-pathogenic strains, whereas none of the other plant pathogenic pseudomonads tested utilized these amino acids as carbon and nitrogen sources.

3. The following amino acids are utilized by all the organisms tested with the exception of the 3-non-pathogenic pseudomonads (7A9, Bn3 and Pt2 strains), L-asparagine, DL-aspartic acid, DL-alanine, L-glutamic and L-proline.

4. The following amino acids were not utilized by any of the organisms tested, D-asparagine, DL-methionine, D-tryptophane, creatine and creatinine.

It was also noticed that with all the amino acids tested, an alkali reaction developed when the organisms grew on them as indicated by bromo-thymal blue when added to the growing cultures.

Experiment 22. The production of the fluorescent pigment in the amino acid media

It was noticed from the above experiment that when the organisms grew on L-proline and L-glutamic acid media, most of the cultures were strongly fluorescent. These results suggest that these particular media may be useful for the detection of the fluorescent pigment produced by some pseudomonads.

Experiment 23. The arginine dihydrolase system

The anaerobic conversion of arginine to ornithine, ammonia and CO_2 through the action of arginine dihydrolase system was found by Thornley (1960) to be of great taxonomic value as general character to distinguish aerobic pseudomonads from other Gram-ve eubacterial rods. She found that of 156 pseudomonads examined, only one strain failed to give a positive result. She tested only two phytopathogenic pseudomonads and reported that they were negative. Stanier, Palleroni & Doudoroff (1966) found that every fluorescent strain examined was positive.

It was of interest therefore to study the arginine dihydrolase system of these organisms.

The method used was the same as described by Thornley (1960). The arginine medium was inoculated and sealed with sterile vaseline and incubated at 27°C and examined up to 21 days of incubation.

Results:- (Table 12) showed that of 30 non-pathogenic pseudomonads strains isolated from different sources, all except

3 strains (Pt2 - Bn3 and 7A9) gave positive results. The reaction was strong and occurred between 2 and 4 days of incubation. Of the 31 plant pathogenic pseudomonads tested only Ps. marginalis gave a positive reaction.

Table 12. The arginine dihydrolase system

	Number of the organisms tested	Number showed alkali reaction
Ps. syringae	10	0
Ps. mors-prunorum	9	0
Ps. phaseolicola	6	0
Ps. tabaci	2	0
Ps. marginalis	4	4
Non-pathogenic pseudomonads	30	27

Conclusions:-

The conclusions which could be made from the nutritional studies described above were as follows.

There were no clear cut differences between the plant pathogenic pseudomonads as one group and the non-pathogenic pseudomonads as the other group. The majority of pseudomonads, including the plant pathogens, could satisfy their carbon and energy requirements from a wide range of sugars and organic acids, as well as the amino acids. They were also able to satisfy their nitrogen requirements from inorganic and organic sources.

The differences between the plant pathogens and the saprophytes seemed most significant in the arginine dihydrolase test.

The results of the nutritional studies indicated the close relationship between Ps. marginalis and the non-pathogenic pseudomonads. There were differences between the different species of the plant pathogenic pseudomonads in their utilization of organic acids, and the utilization of ammonium lactate differentiated between Ps. mors-prunorum and Ps. syringae.

PART II

A study of the cytochrome oxidase activity of the organisms

A study of the cytochrome oxidase activity of the organisms

Introduction:-

The oxidase reaction, based upon the ability of certain bacteria to produce indophenol by the oxidation of dimethyl-P-phenylenediamine and α-naphthol, was introduced by Gordon & McLeod (1928) to aid identification of Gonococci. The use of a more sensitive and less toxic tetramethyl compound was advocated by Ellingworth, McLeod & Gordon (1929). Kovacs (1956) introduced a method for the identification of Pseudomonas aeruginosa; the test depends upon the detection of the cytochrome oxidase enzyme(s) and all 436 strains gave a positive reaction. The test was also valuable for the identification of non-pigmented strains of Ps. aeruginosa. Gaby & Hadley (1957) used another method for detecting the same enzyme(s) for the identification of Ps. aeruginosa and reported that other species of Pseudomonas will give a positive reaction but they were not certain if the presence of the cytochrome oxidase is characteristic of all members of the genus. Shewan, Hobbs & Hodgkiss (1960) proposed a scheme for the identification of certain genera of Gram-negative bacteria. They used the oxidase test as described by Kovacs (1956) and found that all the pseudomonads are oxidase positive, these authors did not examine plant pathogenic pseudomonads. However, Hodgkiss (1960) found in an attempt to differentiate the pseudomonads from the enterobacteriacea, all motile Gram's negative peritrichous rods give a negative oxidase

reaction whereas polarly flagellated Gram's negative rods, with the possible exception of certain plant pathogens, give a positive oxidase reaction. He did not give the identity of the species of plant pathogenic pseudomonads examined. Klinge (1960) found that the results of the oxidase test, as described by Gaby & Hadley (1957) depended on the strain of bacterium, on the broth medium and on the reagent. He also reported that the composition of the solid media does not influence the oxidase reaction when the reagent was dropped onto the growing colonies. Steel (1961) applied the test as described by Kovacs to 1660 strains of different genera and found that members of the pseudomonadaceae were generally oxidase positive, Billing (1963) reported that pseudomonads isolated from soil, water, and chicken meat were positive in the oxidase test, whereas many of the plant pathogenic pseudomonads are negative. Stanier et al.(1966) reported that the oxidase reaction was positive for every pseudomonas species they examined except Ps. maltophilia which is oxidase negative.

Experiment 24. The cytochrome oxidase activity

Two methods were used to study the cytochrome oxidase activity. One was the method described by Kovacs (1956) which is smearing bacterial growth on filter paper impregnated with 1% (w/v) aqueous tetramethyl P-phenylenediamine hydrochloride. The development of a dark purple colour indicated the presence of the enzyme(s). The other method used was that described by Gaby & Hadley (1957) in which the organisms were grown on nutrient broth for 24 hours at 27°C, and the appearance of blue colour on the addition of P-aminodimethylaniline oxalate, and an ethanolic solution of ∝-naphthol, indicated the presence of the enzyme(s).

Results:- (Table 13)

The same results were obtained when the above two methods were tested and were as follows:-

Of Ps. syringae strains (29) examined, 28 isolated from different hosts and different countries gave a negative reaction. Only one strain gave a positive reaction this was strain NZ21 which differs from the other Ps. syringae strains in many other characters. No attempts were made to determine the pathogenicity of that strain. The results also showed that Ps. mors-prunorum, Ps. phaseolicola, Ps. tabaci, and Ps. medicaginis were negative to that test which differentiates sharply the plant pathogenic group (apart from Ps. marginalis) from the non-pathogenic pseudomonads isolated from different sources, with the exception of one organism P19 received as a pseudomonas spp. isolated from plum.

The method described by Kovacs (1956) was very sensitive, the reaction occurred between 3 - 30 seconds with the non-pathogenic pseudomonads, Ps. marginalis and Ps. aeruginosa. Such a reaction did not occur with the other plant pathogenic pseudomonads studied, even after 10 mins. The method described by Gaby & Hadley (1957) was less sensitive and the reaction was slower than that of Kovacs. The blue colour developed with the non-pathogenic pseudomonads, Ps. marginalis and Ps. aeruginosa between 30 seconds and 2 mins. All the plant pathogenic pseudomonads which gave negative results by the Kovacs method, also gave negative results, since no blue colour developed within 10 minutes after the addition of the reagents. It was also noticed that after the addition of the reagents to the control medium a blue colour developed after 10 mins., and increased in intensity with time.

Table 13. The results of the cytochrome oxidase test

Organisms tested	Number of strains tested	Number gave positive reaction	Number gave negative reaction
Ps. syringae	29	1	28
Ps. mors-prunorum	10	0	10
Ps. phaseolicola	6	0	6
Ps. tabaci	2	0	2
Ps. medicaginis	1	0	1
Ps. marginalis	6	6	0
Ps. aeruginosa	1	1	0
Non-pathogenic Pseudomonads	37	36	1

Experiment 25. The effect of haemin on the cytochrome oxidase test

Jacobs & Conti (1965) found that Staphylococcus epidermidis when grown in medium supplemented with haemin had a high content of cytochromes. Therefore it was of interest to examine the effect of haemin on the cytochrome oxidase activity of the organisms. The medium used was nutrient agar to which haemin was added aseptically from a filter sterilized solution of haemin dissolved in triethanolamine to give a final concentration 5µg/ml. Representative strains of Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola, as well as non-pathogenic pseudomonads, were grown on the haemin medium and a control medium, which contained no added haemin. The cytochrome oxidase activity of the organisms was examined every day during 3 days of incubation at 27°C. The method of Kovacs was used.

Results:-

Results indicated that the addition of haemin had no effect on the plant pathogenic pseudomonads. None gave any positive reaction during this time of incubation. The addition of haemin did not hasten the reaction of the non-pathogenic pseudomonads.

Experiment 26. The effect of the composition of the medium on the cytochrome oxidase test

The composition of the growth medium was found to effect the results of the oxidase test. Klinge (1960), who used three different liquid media, used the method of Gaby & Hadley (1957)

and other reagents. He also reported that the composition of the solid medium has no effect.

The effect of the composition of the medium on the cytochrome oxidase test was studied. Nutrient agar medium supplemented with 2% of the following compounds was used, glucose, glycerol, sorbitol, Na succinate and L-glutamic acid. The media were adjusted to pH7 and autoclaved at 121° for 15 mins.

Representative strains (18) were tested, 10 non-pathogenic strains and 2 strains of the following pathogenic species, Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola and Ps. marginalis. The organisms were grown on the above media for 3 days and examined every day for the cytochrome oxidase activity as described by Kovacs (1956).

Results:-

All the organisms grew well onto the above media. The cytochrome oxidase reaction was very strong on Na succinate nutrient agar, compared with the reaction occur when the organisms grown on the control nutrient agar medium. A strong reaction was given by all the non-pathogenic pseudomonads as well as the Ps. marginalis strains. The plant pathogenic species gave a weak reaction compared with the non-pathogenic strains. In the case of the glucose nutrient agar medium, it was found that the non-pathogenic and Ps. marginalis strains gave either a negative reaction or a weak reaction. This finding was investigated further. The following medium was used and contained the following constituents:- 1% (w/v) glucose, 0.008%

(w/v) KH_2PO_4, 0.002% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4$, 0.1% (w/v) $NH_4H_2PO_4$, 0.2% (w/v) yeast extract, pH7, the medium autoclaved at 121° for 15 mins.

The same representative strains were tested as above and the results indicated that all the organisms tested were cytochrome oxidase negative.

None of the other compounds tested had significant effect on the cytochrome oxidase test.

Experiment 27a. The cytochrome oxidase reaction and its relation to the cytochrome system

Several respiratory components have been isolated from Ps. aeruginosa (Horio, 1958a). They are cytochrome.554 and cytochrome.551 of the c type, cytochrome.560 of the b type, blue protein and the pseudomonas cytochrome oxidase. The cytochrome oxidase preparation has complex absorption bands and some parts of the absorption spectrum are very similar to cytochrome-c and the blue protein. It apparently possesses two haem moities, a c type and a2 (DeLey, 1964) which are responsible for the organism's "oxidase" positive reactions.

The results obtained from experiment 24 indicated that the non-pathogenic pseudomonads as well as Ps. marginalis were cytochrome-oxidase positive whereas the other plant pathogenic pseudomonads were oxidase-negative. Therefore it was of interest to study the cytochrome systems of these organisms.

Methods:-

Representative strains (11) were studied three of them non-pathogenic and two strains from each of the following species. Ps. marginalis, Ps. syringae, Ps. mors-prunorum and Ps. phaseolicola. They were grown in Roux bottles containing nutrient agar + 2% (v/v) glycerol for 2 days. The cells were harvested and washed with distilled water and then resuspended in 0.05 M phosphate buffer pH7. Cell-free extracts was obtained by the exposure of the thick bacterial suspension to ultrasonic disintegrator for about 10 mins. They were then centrifuged to free the extract from

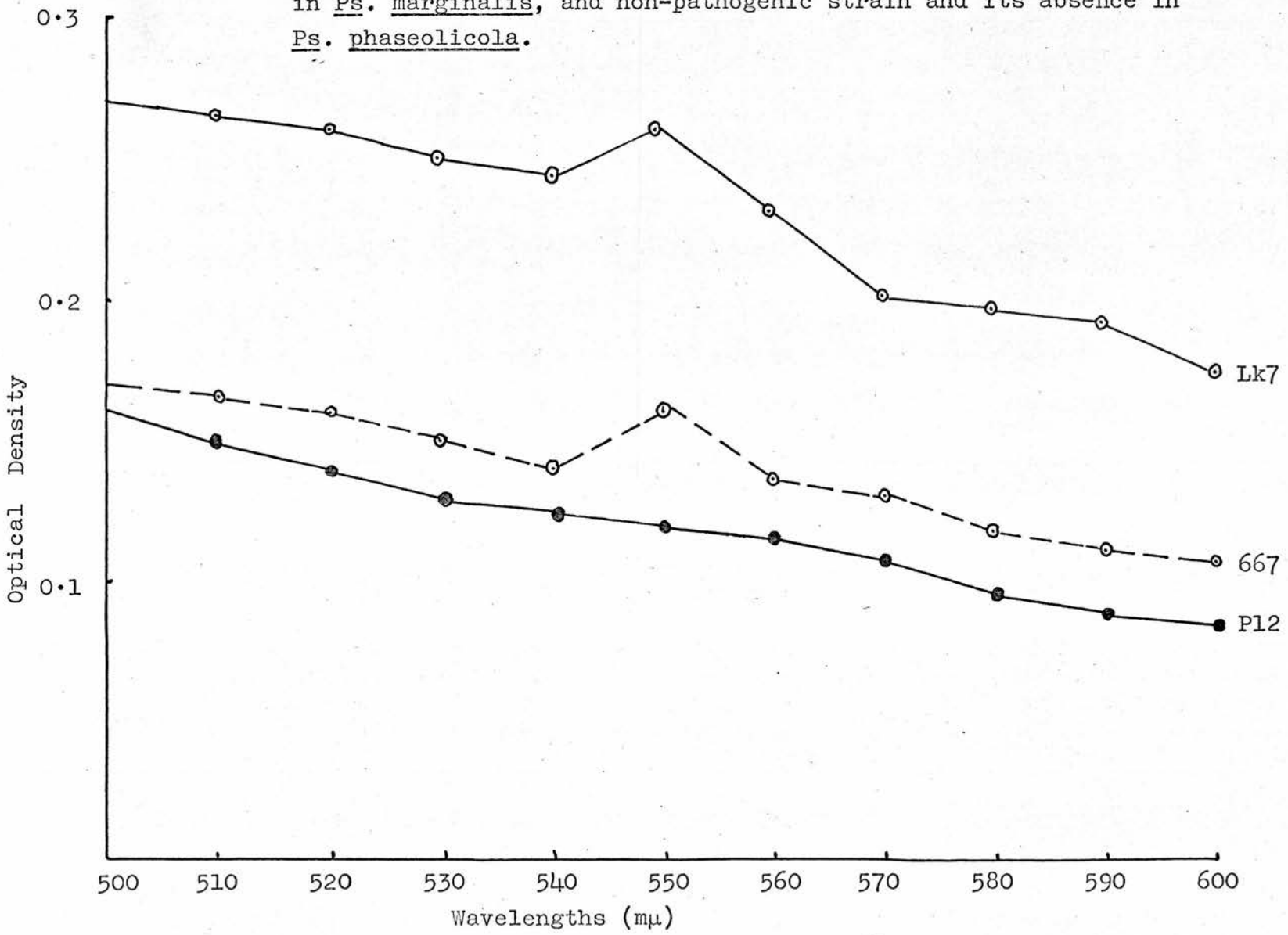
unbroken cells and cell debris. It was noticed that the cell free extracts of the non-pathogenic pseudomonad strains and of the two Ps. marginalis were pink in colour whereas those of the other plant pathogenic species were uncoloured or faintly straw-coloured. The soluble cytochromes were detected by measuring the absorption over a range of 500 to 600 mμ, using a Unicam sp. 600 spectrophotometer. The extracts were reduced immediately before the spectrophotometric observation by sodium dithionite.

Organisms tested:- Non-pathogenic Pseudomonas 4A3 - CS4 - Lk7
Ps. marginalis 667 and 1559
Ps. syringae S5 and S8
Ps. mors-prunorum D1 and D2
Ps. phaseolicola P12 and P14

Results:-

All the extracts of the non-pathogenic pseudomonads and Ps. marginalis strains contain an absorption band at about 550 which is the α- band of cytochrome c type, whereas the extracts of the other plant pathogenic pseudomonads tested contained no detectable soluble cytochrome, see figure 1.

Figure 1. Absorption spectra of cell free extracts of representative pseudomonads strains showing the presence of cytochrome c type in Ps. marginalis, and non-pathogenic strain and its absence in Ps. phaseolicola.



Experiment 27b. Determination of cytochrome spectra of intact cells of different pseudomonads grown on different media

The same representative strains as were used in experiment 27a were used in this study. The organisms were grown for 2 days on Roux bottles of nutrient agar + 2% Na succinate, on nutrient agar + 2% glycerol and on the glucose medium which was described in experiment 26.

A very heavy cell suspension was obtained and reduced by sodium dithionite and examined by Hartridge Reversion spectroscope.

Results:-

The non-pathogenic pseudomonads and Ps. marginalis strains have two absorption bands, an α- band at about 553 mμ and a β- band at about 521mμ. These two bands are characteristic of cytochromes of c type. Both the bands were very strong when these organisms were grown on succinate medium and barely detectable or undetectable when they were grown on glucose medium.

In the case of the plant pathogenic Ps. syringae, Ps. mors-prunorum and Ps. phaseolicola strains there was an α- band at about 558-560 and a weak β- band at about 530 which are characteristic of cytochromes of b type. These two bands were detected when these organisms were grown on glycerol or Na succinate medium but they were undetectable or very faint when the organisms were grown on glucose medium.

It was noticed also that in the case of the non-pathogenic strains, as well as with Ps. marginalis strains, the bacterial cell suspension was pink in colour when the organisms were grown

on glycerol or succinate but white when they were grown on the glucose medium. This white colour and the lack of absorption bands suggested that cells grown under these conditions (with glucose) possess a negligible amount of cytochromes. The inability to detect the cytochrome b type with the non-pathogenic pseudomonads and Ps. marginalis could be explained by the fact that the bands of cytochrome b type lies on the shoulder of the cytochrome c peak and cannot be easily located. These organisms contain large amounts of cytochrome c type and Smith (1961) stated that the presence of small amount of cytochrome b could be masked by a large amount of cytochrome c in spectrophotometric observations. Cytochrome of the b type was detected in the other plant pathogens presumably because of the absence of cytochrome c in these organisms.

Experiment 28. The presence of the blue protein in different pseudomonads

The blue protein is one of the copper enzymes which has been isolated from Ps. aeruginosa by Horio (1958a). Its physical and chemical properties were investigated by Horio (1958b) who reported that the blue protein can be oxidized and reduced repeatedly, losing its blue colour when reduced and regaining it on reoxidation. This blue protein has an absorption maximum at 625 mμ in the oxidized form. The absorption maximum disappear on its reduction. Ambler (1963) obtained the blue protein from Ps. fluorescens.

A similar blue protein has been isolated by Sutherland & Wilkinson (1963). They called it "azurin", and isolated it from Bordetella pertussis, B. bronchiseptica and B. parapertussis. They also isolated the azurin from Alcaligenes faecalis, A. denitrificans and Pseudomonas fluorescens.

Results obtained from experiment 24 indicated that all the non-pathogenic pseudomonads tested were cytochrome oxidase positive whereas the plant pathogenic pseudomonads tested were cytochrome oxidase negative with the exception of Ps. marginalis which was oxidase positive. Horio (1958b) suggested that the blue protein of Ps. aeruginosa could act as an alternative electron-transport system between a cytochrome of the c type (pseudomonas cytochrome 551) and cytochrome oxidase. Therefore it was of interest to study the presence of the blue protein in the non-pathogenic and the plant pathogenic pseudomonads in an

attempt to correlate a cytochrome oxidase reaction with the presence of the blue protein.

Organisms tested:-

The following representative strains were examined.

Ps. syringae S5 - S8 - S20

Ps. mors-prunorum D1 - D2 - D9

Ps. phaseolicola P12 - P13 - P14

Ps. marginalis 667 - 1559.

Non-pathogenic pseudomonads:- F300, 1A1, 4A3, Lk7, CS4 and S60 strain which was received as Ps. syringae but has since proved to be a non-pathogenic pseudomonas spp. Ps. aeruginosa also tested.

The method used for the detection of the blue protein:-

The method used in this study was generally the same as used by Sutherland & Wilkinson (1963) but the final purification steps were omitted.

The organisms were grown in the following medium:- Nutrient broth + 2% (v/v) glycerol + 0.004% (w/v) $CuSO_4$, pH7, sterilized at 121° for 15 mins. 3 litres of medium were used for each organism. The medium was incubated at 27°C on a shaker for 2 days after inoculation. The bacterial cells were harvested and washed twice with water and kept in a deep freeze until they were extracted for the detection of the blue protein.

The preparation of cell-free extract and the detection of the blue protein:-

The cell-free extract was obtained by grinding the bacterial cells with polishing alumina; the grinding was done in mortar in

an ice bath and in the cold room for about 10 mins. Then 50 mls. of phosphate buffer, 0.02 M pH 7.2, was added before centrifugation to get the cell-free extract. The grinding was repeated again with the sediment to ensure the breakdown of a high percentage of the bacterial cells.

The 100 ml. of the cell-free extract was dialysed against running tap water for 16 hours and then concentrated to 20 mls. by polyethylene glycol according to the method of Kohn (1959). After the extraction was concentrated it was noticed that there was a blue colour in the extracts of Ps. aeruginosa and in some of the non-pathogenic pseudomonads. No blue colour was noticed in the extracts of all the Ps. syringae, Ps. mors-prunorum and Ps. phaseolicola strains. The concentrated extracts were dialysed against 0.02 M phosphate buffer pH 7.2 for 24 hours at $4^{o}C$, then the extract was applied to columns of DEAE cellulose which was equilibrated against the same buffer. After the extracts were passed through the columns, the columns were washed with a further 20 mls. of the phosphate buffer, then the extracts were dialysed for 24 hours against acetate buffer 0.02 M pH 4.6. The precipitate, which appeared at that stage, was removed by centrifugation and finally the extracts were concentrated to 20 mls. and examined by Backman D.b. recording spectrophotometer for the detection of the blue protein.

Results:-

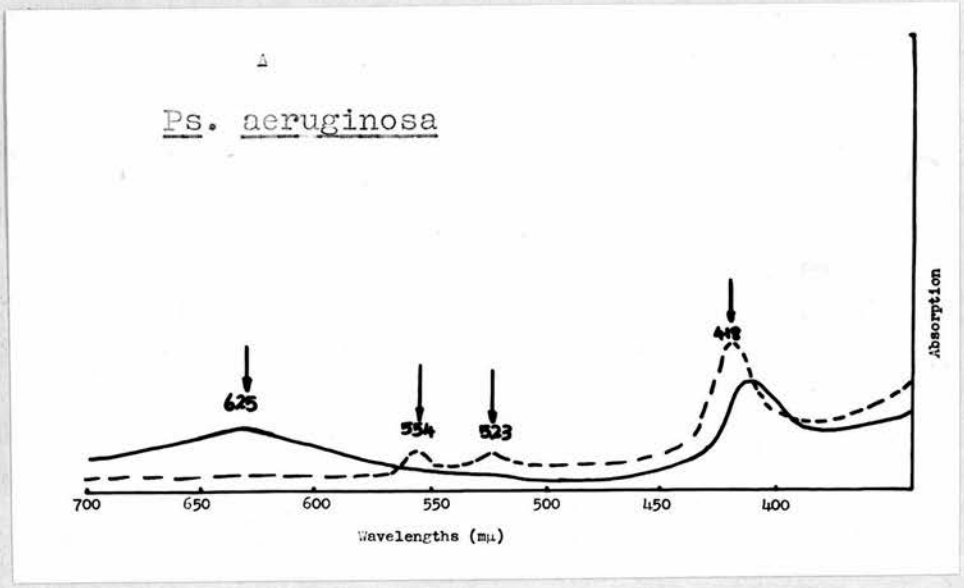
The blue protein was detected in the following organisms:- Ps. aeruginosa, Ps. marginalis 1559 and the following non-pathogenic

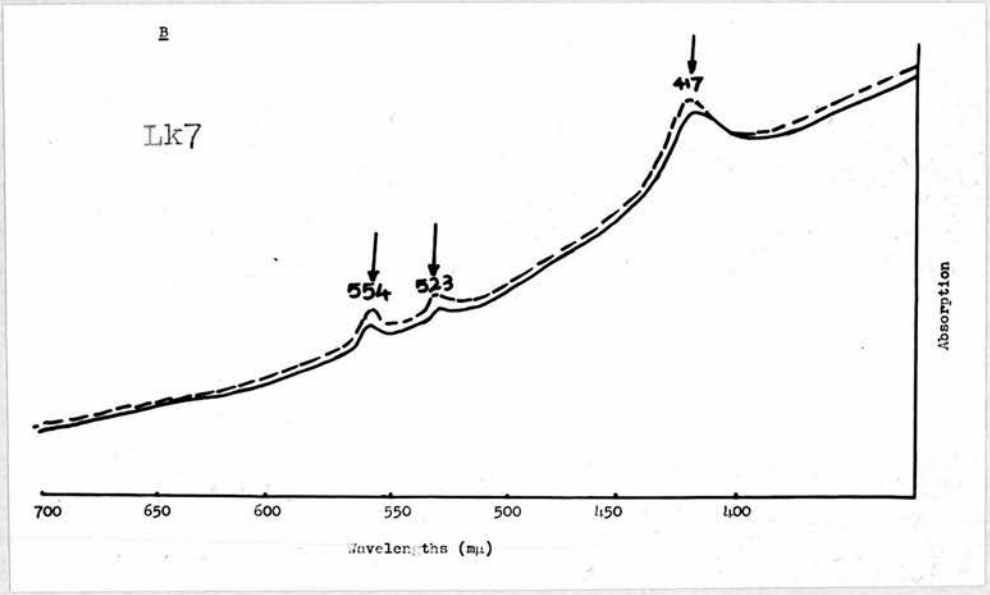
strains 1A1, F300 and S60. The blue protein isolated from these organisms was similar to that described by Horio (1958a) and Sutherland & Wilkinson (1963), since spectrophotometric examination showed that the extracts of the above organisms showed absorption at 625 mμ only when the blue material was in the oxidized form. No blue materials were detected in any of the other non-pathogenic pseudomonas strains tested nor in Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola strains which were tested.

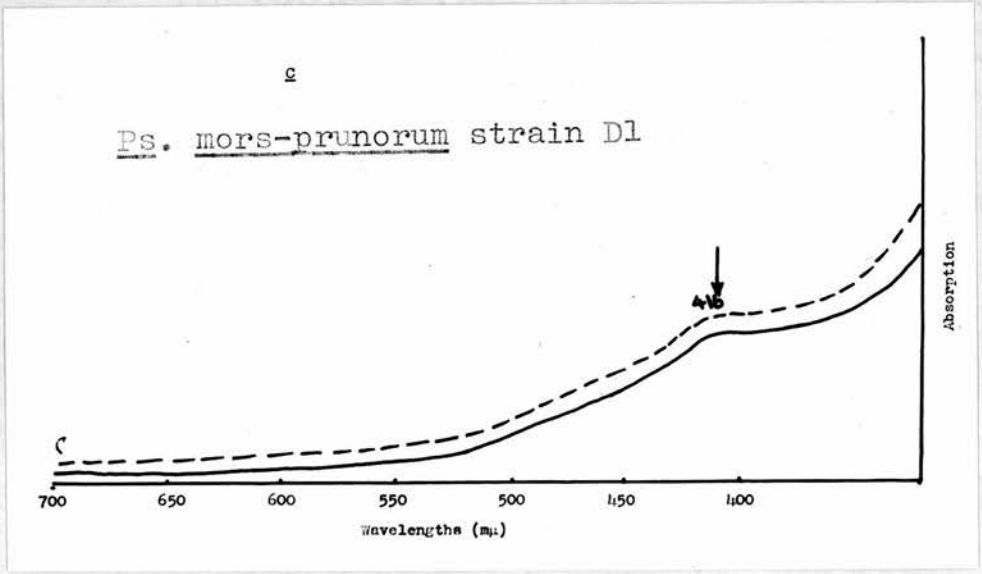
From these results it can be concluded that there was no correlation between the presence of blue protein and the cytochrome oxidase reaction since the blue protein could not be detected under the conditions described in some of the non-pathogenic pseudomonads strains, which are cytochrome oxidase positive. This was further confirmed when 1A1, non-pathogenic strain, was grown on nutrient broth + 2% glucose + 0.004% $CuSO_4$ a condition which gave a cell-free extract which had no cytochrome oxidase activity but in which the blue protein was still present.

However, it could be concluded that azurin was absent from all cytochrome oxidase negative plant pathogens, see figure 2.

Figure 2. Absorption spectra of cell free extracts of representative pseudomonads prepared for the detection of blue protein (azurin)







- - - = reduced

_____ = oxidized

Discussion:-

The results described above emphasized the close relationship between Ps. marginalis and the non-pathogenic pseudomonads, they differ from the other plant pathogenic pseudomonads studied, in that they are cytochrome oxidase positive. These results were in agreement with those reported by Billing (1963). It was also found that glucose inhibits the synthesis of cytochrome oxidase whereas Na succinate increased the synthesis of the enzyme. A similar effect of glucose and succinate on the cytochrome content of Salmonella typhimurium was observed by Richmond & Maaloe (1962). They found that organisms grown on compounds related to the Krebs cycle are pink whereas those grown on glucose are white and that the total cytochrome content was reduced when they are grown on glucose. The effect of glucose on cytochromes was also found by Strasters and Winkler (1963) when they studied the carbohydrate metabolism of Staphylococcus aureus.

In the case of pseudomonads, Klinge (1960) reported that the results of the cytochrome oxidase test were dependent on the composition of the medium but when solid media was used, the composition of the medium has no effect. The results described above were in disagreement with that statement since the presence of glucose in the solid medium still has effect on the results of the cytochrome oxidase test. The results described by Klinge (1960) also showed that the percentage of pseudomonads strains which gave a positive reaction was reduced when glucose was added

to one of his media. However he did not comment on the specific effect of glucose. It is clear that the composition of the medium must be carefully considered when this test is applied, as false negatives may result when too high a concentration of glucose is used.

In an attempt to correlate the cytochrome oxidase test with the cytochrome system component, namely azurin, it was suggested by Horio (1958b) that azurin acted as an alternative electron-transport system between a cytochrome of the c type and cytochrome oxidase of Ps. aeruginosa. No clear correlation, however, was found in the present study between the presence of the blue protein and the cytochrome oxidase activity. The blue protein, however, could not be detected in any of the plant pathogenic pseudomonads tested with the exception of Ps. marginalis. Azurin was detected in some of the non-pathogenic pseudomonads. Good correlation was found between the cytochrome oxidase results and the presence or absence of cytochrome c, only the oxidase positive organisms possessed cytochrome c.

The cytochrome c type of the non-pathogenic pseudomonads and Ps. marginalis could not be detected or was markedly reduced when these organisms were grown on glucose medium. The organisms also were negative in the cytochrome oxidase test when grown on the glucose medium. Stanier et al. (1966) found that Ps. maltophilia did not contain cytochrome c and was cytochrome oxidase negative.

When mammalian cytochrome c was added to ultrasonically broken-cell suspensions of the plant pathogenic pseudomonads and

the glucose grown cells of the non-pathogenic pseudomonads and Ps. marginalis strains, cytochrome oxidase activity could not be restored. This suggested that other components of the cytochrome system might not be present in these cases. It might also indicate that cytochrome c is an essential component of the oxidase reaction in that oxidase activity was only observed when pseudomonads were grown under conditions in which cytochrome c was obviously present. However without a complete analysis of cytochrome complement, no such conclusion can be drawn.

PART III

The role of phenolic compounds in the bacterial diseases caused by Pseudomonads

The role of phenolic compounds in the bacterial diseases caused by pseudomonads

Introduction:-

Phenolic compounds are widely distributed in the plant kingdom, and are second only to carbohydrates (Pridham 1965) in abundance in higher plants.

Bradfield, Flood, Hulm and Williams (1952) reported the presence of chlorogenic acid in mature and immature apple and pear fruits. Weurmann & Swain (1953) also found chlorogenic acid in the extract of apple and pear seedlings. They also reported chlorogenic acid to be one of the substances involved in the enzymic browning in both apple and pear. Williams (1960) noticed that phloridzin, the principal phenolic compound of apple leaves and bark, is absent from the flesh of the fruit, although some occurs in the seeds. He found that chlorogenic acid, isochlorogenic acid and arbutin are the main phenolic compounds of pear. Sondhemier (1964) reported the wide distribution of chlorogenic acid among higher plants (pears, apples, sweet potato seedlings, prunes, cherries, plums, peaches and other plants).

Phenolic compounds content appears to be correlated to disease resistance. The first indication in the literature is the work of Link, Angell & Walker (1929) who found that coloured onion varieties were resistant to smudge, which is caused by Colletotrichum circinens, while uncoloured varieties were susceptible. They suggested that catechol and protocatechuic acid, which is formed in the outer scales of the coloured varieties, were responsible for their resistance to the disease. Little,

Sportson & Foot (1948) isolated a quinone compound (2, methoxy-1, 4-naphthoquinone) from Impatiens balsamina, plant which is relatively free from fungus diseases, which was found to be toxic to phytopathogenic organisms. Sportson (1957) also isolated this quinone along with other phenolic compounds from Impatiens balsamina, but reported that these compounds are too toxic to be found in the plant in a free state. He suggested that they occur as glucosides and in other bound forms. Many workers have reported that phenols or tannins are responsible for disease resistance in plants (Walker & Stahmann, 1955). Phenols and quinones are stated to be natural plant chemotherapeutants by Dimond & Horsfall (1959) who also reported that quinones are more toxic than the phenols. A correlation between the phenolic content of host plants and their resistance to pathogenic fungi was found by Newton, Lehman & Clark (1929), who reported that the varieties of wheat resistant to stem rust disease were higher in phenolic content than the susceptible varieties. However, Kiraly & Farkas (1962) recently reinvestigated the position and found no clear cut correlation between the phenolic content and stem rust resistance of wheat varieties, but they did find resistance was correlated with a fast rate of accumulation of phenolic compounds and that susceptible varieties accumulate ascorbic acid, which decreases in content in the resistant varieties.

Johnson & Schaal (1952) tested a great number of potato varieties for the phenolic content of the periderm by the ferric chloride test and found a significant correlation between

increased resistance to the scab pathogen, Streptomyces scabies, and increased chlorogenic acid content. They suggested that resistance might be due either to the chlorogenic acid itself, which lowers the pH of the cells and makes conditions unfavourable for the pathogen, or to the formation of quinone by the oxidation of the acid by polyphenolase. The same authors (Schaal & Johnson, 1955) studied the inhibitory effect of six phenolic compounds on the growth of Streptomyces scabies, as related to the scab resistance. They found that increases of the pH also resulted in an increase in the rate of autooxidation of chlorogenic acid, caffeic acid, catechol and tetrahydroxybenzene and also in an increase of the inhibitory effect. It was concluded that the mechanism of scab resistance involves oxidation of chlorogenic acid to quinone which is toxic to the scab organism. The correlation between the high phenolic content of rice varieties and the resistance to fungus diseases was also found by Wakimoto & Yoshii (1958) who also found that the polyphenol content in the leaves increased when they were infected with rice blast fungus or the Helminthosporium leaf spot pathogen. Echandi & Fernandez (1962) found a correlation between the resistance of coffe varieties to canker disease and chlorogenic acid content.

Patil, Powelson & Young (1964) tested five varieties of potato and found that chlorogenic acid and free phenols in the roots of all varieties decreases 3 weeks after sprouting. This decrease of phenolic compounds was correlated with decrease of the resistance of the roots to Verticillium albo-atrum infection.

They found that the oxidation products of chlorogenic acid were toxic, but chlorogenic acid to be non-toxic. Kuc (1957) found that the pathogenic fungi of potato can grow well on extracts of potato peel, while the non-pathogenic fungi are inhibited. He found chlorogenic acid and coffeic acid in these extracts and concluded that the non-pathogenic fungi were more sensitive than pathogenic fungi to these acids.

Le Tourneau, McLean & Guthrie (1957) studied the effect of 13 different phenols and phenolic acids and 4 quinones on the growth of Verticillium albo-atrum. They found that of the 3 isomeric dihydroxybenzenes, catechol was the most inhibitory compound. The replacement of hydroxyl group with a carboxyl group or the introduction of a carboxyl group on the ring decreases the effectiveness of catechol. Quinones were found generally to be more toxic to the fungi than the phenols.

Phenolic compounds may also be important in the wilt diseases. For example Dimond, Waggoner & Davis (1954) found that the vascular discolouration, which is one of the symptoms in wilt diseases caused by Fusarium spp., was due to the oxidation of polyphenols released from glucosides by terminal oxidases of the host. More recently Mace (1964) found that dopamine, the major uncombined banana root phenol, can be used as a hydrogen donor by both the root and the Fusarium peroxidase in the presence of hydrogen peroxide. He concluded that the peroxidase along with the root polyphenol oxidase and cytochrome-c-cytochrome oxidase and Fusarium laccase may participate in the in vivo oxidation of

dopamine to produce vascular browning. Kuc, Barnes, Daftsios & Williams (1959) studied the effect of amino acids on the susceptibility of apple varieties to scab pathogen Venturia inaqualis and found that the infusion of D and DL-phenylalanine into some apple varieties induced resistance towards 3 races of the scab pathogen. More recently Halowczak, Kuc and Williams (1962) in an attempt to study the metabolism of DL- and L-phenylalanine in relation to resistance in Malus found that DL-phenylalanine caused an accumulation of phenolic compounds identified as phloretein and phloretic acid. The introduction of DL-phenylalanine failed to induce resistance in one variety in which the accumulation of phenolic compounds did not occur. Recently Noveroske, Williams & Kuc (1964) found that the oxidation products of phloretin was inhibitory to spore germination of Venturia inaqualis.

Oku (1960b) found that the toxicity of catechol against Cochliobolus miyabeanus, a pathogenic fungus to rice plant, is caused by the quinone formed by the fungal polyphenol oxidase. He concluded that the symptoms of leaf blight disease, in which the spotted area does not expand without limitation, results from the inhibition of the growth of pathogenic fungi by the accumulation of melanin like substances in the necrotic area. The phenolic compounds may also cause the inactivation of pectolytic enzymes; for example the work of Byrde, Fielding & Williams (1960) on the relationship between the rate of browning of injured tissues of apple and their resistance to infection with the brown rot fungi

Sclerotinia fructigena, revealed that the polyphenol-polyphenol oxidase system might be involved in the defence mechanism. Polyphenol extracts from the resistant tissue have not exerted an inhibitory effect on spore germination. Even the oxidation products of the phenol containing extract did not inhibit the germination of the spores. However, they found that the extracellular pectolytic enzymes of the fungi, which are necessary for its pathogenicity, were markedly inhibited by phenolic compounds and particularly by their oxidation products.

Deverall (1961) described the symptoms of chocolate spot disease of broad beans and suggested that the fungus polygalacturinase kills host cells rapidly and liberates the pectic substances which have been shown to activate host phenolase which was masked. This seems to be followed by the oxidation of host phenols and the formation of dark brown polymeric products which now inactivate the polygalacturinase of the pathogen and gave rise to these symptoms.

Lyr (1965) found that the oxidation products of catechol, protocatechuic acid and partly of coffeic acid, produce an increased inhibition of the fungal enzymes, pectinase and cellulase in comparison to the unoxidized compounds. Farkas & Kiraly (1962) reported that many workers found that aromatic compounds such as mono and dihydric phenols, phenolic glucosides, flavanoids, anthocyanin and aromatic amino acids, as well as the polyphenol oxidase and the peroxidase of the plant tissue were increased in plant tissue after it had been invaded by a parasite. They cited

many examples in the case of fungus diseases and virus diseases but few examples in the case of bacterial diseases. Umaerus (1959) found a positive correlation between peroxidase activity of potato varieties, Solanum tuberosum, and field resistance to late blight caused by Phytophthora infestans. He suggested that the peroxidase oxidizes a wide variety of mono- and dihydric-phenols and thus may play an important role in the accumulation of pathogen-induced fungistatic or fungicidal oxidation products. Patil, Powelson & Young (1964) reported that the phenol oxidase from potato roots of the highly resistant varieties to Verticillium albo-atrum was at least 50% more than of less resistant varieties.

In contrast to fungal diseases, little work has been done on the role of phenolic compounds in bacterial diseases. Maine & Kelman (1960) found that polyphenol oxidase activity increased in tobacco stem tissue infected by Pseudomonas solanacearum. The same authors (Maine & Kelman, 1961) found that glutathione and ascorbic acid increased the pathogenicity of Ps. solanacearum on tobacco plants treated with these reducing agents, they also tested the effect of some amino acids and found that only glutamic acid significantly reduced the resistance. They concluded that the effect of the reducing agents was not on the bacteria but on the metabolism of resistant plants, and glutamic acid stimulated the bacterial growth. Rudolph & Stahmann (1964) analysed the proteins of healthy bean leaves and infected leaves with Pseudomonas phaseolicola, and found the appearance of

bacterial catalase in the infected leaves, they suggested that bacterial catalase may be a factor of virulence, depressing the activity of peroxidases involved in the defence reaction of the host. They also found that the virulent strain of Ps.phaseolicola has more catalase activity than the avirulent strain.

Another interesting example in the case of bacterial diseases, is in the work of Hildebrand & Schroth (1964a) who found that pear leaves showed antibiotic activity against Erwinia amylovora, which causes the fire blight disease. They reported that the antibiotic action resulted from the function of β- glucosidase activity of the pear leaves which hydrolyse arbutin releasing the toxic hydroquinone. The same authors (Hildebrand & Schroth, 1964b) found that β-glucosidase activity was low in the tissues which are most susceptible to fire blight, and a higher β-glucosidase activity was found in the parts of the tree which are considered less susceptible to invasion by E. amylovora. They suggested that the arbutin hydroquinone complex is one of the factors that affects susceptability of pear tissue to fire blight.

Many workers have tried to break the host resistance to pathogenic fungi, for example Noveroske, Williams & Kuc (1962) found that the treatment of Malus plants with 4-chlororesorcinol and glucose, breaks the resistance of these plants to Venturia inaqualis. It was concluded that this polyphenol oxidase inhibitor may be inhibiting the oxidation and polymerization of naturally occurring phenols or prevent their accumulation in the plant. Oku (1960a) also broke down the resistance of the rice

plants against pathogenic fungi when he treated the plants with reducing agents such as ascorbic acid or glutathione. He concluded that the resistance of rice plants against hyphal penetration of Cochliobolus miyabeanus can be to some extent, attributed to fungal oxidation products, perhaps quinones, which are derived from the host cells. Farkas & Kiraly (1962) reported that phenolic compounds also accumulated upon mechanical damage of the plants in smaller amounts than in the diseased tissue; they also emphasized the fact that polyphenol oxidation products play a more important role than the phenols as a factor responsible for disease resistance. More recently Cruickshank & Perrin (1964) came to the same conclusion as Farkas & Kiraly (1962) and they suggested that phenolic compounds may be regarded as substrates of enzymes which convert them to other compounds more directly related to disease resistance.

From the above literature, it could be concluded that although the phenolic compounds have a definite role as the defence mechanism of the plants against fungal infection, very little similar work has been done in the case of bacterial diseases. Even though phenols may be involved the action of the microorganisms towards these compounds does not seem to have been studied.

It was of interest therefore to study the action of plant pathogenic and non-pathogenic pseudomonads on some phenolic compounds, hoping to find differences between these organisms.

A. The toxicity of phenolic compounds

Experiment 29. The toxicity of different phenolic compounds and of the quinone

This experiment was conducted to determine if the toxicity of phenolic compounds could differentiate between the plant pathogenic and the non-pathogenic pseudomonads.

The following phenolic compounds were tested:- Orcinol, m-aminophenol, p-aminophenol, pyrocatechol, hydroquinone, phloroglucinol, chlorogenic acid and p-benzoquinone.

The above phenolic compounds were sterilized by membrane filtration and added to the basal medium. The final concentration of these phenolic compounds was 0.01% (w/v), the quinones were also tested at 0.001% (w/v).

The basal medium has the following composition:- 0.5% (w/v) glucose, 0.008% (w/v) K_2HPO_4, 0.002% (w/v) KH_2PO_4, 0.02% (w/v) $mgSO_4$, 0.1% (w/v) yeast extract pH7. The medium was autoclaved at 121° for 15 mins. The medium was tubed in 5 mls. amount in $\frac{5}{8}$" sterile test tubes.

The inoculum:- All the organisms were grown for 24 hours on nutrient agar + 2% glycerol, slightly turbid cell suspensions were prepared in sterile distilled water. Pasteur pipettes were used and one drop of the slightly turbid suspension was added to each tube. The tubes were incubated at 27°C and observed for the growth.

Organisms tested:- The following representative organisms were tested.

Ps. syringae S5 - S8 - S20 - S36

Ps. mors-prunorum D1 - D2 - C9 - N5

Ps. phaseolicola P12 - P13 - P14 - P16

Ps. marginalis 667 - 1558 - 1559 - 1604

Ps. tabaci M45

non-pathogenic Pseudomonads 5A8 - 4A3 - CS4 - Lk7 - F300 - Bn3

Results:-

The results indicated that all the organisms tested were able to grow in the presence of the above phenolic compounds at a concentration of 0.01% (w/v). All the organisms tested were inhibited in the presence of p-benzoquinone at the same concentration. However when the concentration of p-benzoquinone was reduced to 0.001%, all were able to grow.

These results suggested that the quinone is more toxic to the organisms than are the phenolic compounds tested.

Experiment 30. The toxicity of pyrocatechol at different pH values

Schaal and Johnson (1955) studied the inhibitory effect of some phenolic compounds on the growth of Streptomyces scabies and found that increases of the pH value resulted in an increase of the inhibitory effects resulting from the increase in the rate of autooxidation of these phenolic compounds to the quinones, Sondheimer (1964) also reported that chlorogenic acid and related compounds are very readily oxidized either aerobically at alkaline pH values or enzymatically with polyphenol oxidase. Therefore

it was of interest to see if the inhibitory effect of the phenolic compounds increase when they are oxidized to the quinone by increasing the pH of the growing medium.

The method:-

The basal medium used in this experiment was the same as described in experiment (29), the pH values were pH5, pH6, pH 6.5, pH7 and pH 7.5. The phenolic compound used was pyrocatechol which was added to each of the above media to the final concentrations as 0.01% - 0.03% and 0.05% (w/v). The media was tubed and inoculated as described in experiment (29).

Organisms tested:- The following representative strains were examined:-

Ps. syringae S5 - S8

Ps. mors-prunorum D1 - N5

Ps. phaseolicola P12 - P14

Ps. marginalis 1558 - 1559

non-pathogenic Pseudomonads 5A8 - 4A3 - Cb4.

Results:-

The results (Table 15) indicated that all the organisms tested were able to grow on the control media at the different pH values. Pyrocatechol at 0.01% concentration has no inhibitory effect except when the pH of the medium was 7.5 since only (4) organisms out of the (11) tested were able to grow. Pyrocatechol at 0.03% (w/v) has no inhibitory effect at the lowest pH values (pH5 and pH6) but when the pH of the medium was increased the

Table 15. The toxicity of pyrocatechol at different pH values

Pyrocatechol concentration	media pH5				media pH6				media pH 6·5				media pH7				media pH 7·5			
	C	0·01%	0·03%	0·05%	C	0·01%	0·03%	0·05%	C	0·01%	0·03%	0·05%	C	0·01%	0·03%	0·05%	C	0·01%	0·03%	0·05%
Organism tested																				
Ps. syringae																				
S5	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	−	−
S8	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	+	−	−
Ps. phaseolicola																				
P12	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−
P14	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−
Ps. mors-prunorum																				
D1	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	−	−	−
N5	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−
Ps. marginalis																				
1558	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	−
1559	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	+	−	−
Non-pathogenic pseudomonads																				
5A8	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	+	−	−
Cb4	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	−	+	+	−	−
4A3	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	−	−	−

C = Control medium (no pyrocatechol added).

inhibitory effect increased and at pH 7.5 only one organism out of the (11) tested was able to grow. The same trend was also observed with pyrocatechol tested at 0.05% (w/v) concentration; none of the organisms tested were able to grow at that concentration when the pH of the medium was 7.5 although all the organisms were able to grow at that concentration when the pH value was 5 or 6. It was also noticed that with the same concentration of pyrocatechol a red brown colour developed and darkened (increased) when the pH of the medium increased. This was an effect of alkaline pH values, the phenols rapidly auto-oxidized to the quinones.

Experiment 31. The effect of L-ascorbic acid on the toxicity of p-benzoquinone

The results of the above experiments indicated that the quinones are more toxic than the phenols, therefore if the quinone is reduced to the phenol its toxicity against the organisms should be abolished or markedly reduced.

The following experiment was conducted to examine this. The method:- The basal medium used in this experiment was the same as described before (experiment 29). The toxicity of p-benzoquinone was examined in the presence and absence of L-ascorbic acid. P-benzoquinone was tested at 0.01% (w/v) final concentration, L-ascorbic acid was added from a Seitz filtered solution and the final concentration was 1 mM. The media were tubed aseptically 5 ml. amounts in sterile test tubes and inoculated as described before, (23) representative pseudomonads were tested, the tubes incubated for 7 days at 27°C.

Results:- (Table 16)

The results indicated that the addition of L-ascorbic acid to the basal medium had no inhibitory effect in most cases, whereas p-benzoquinone as shown before is toxic and none of the organisms were able to grow in that medium. However, when L-ascorbic acid is added to the quinone medium without exception all the organisms were able to grow in a concentration of p-benzoquinone which is toxic to them in the absence of L-ascorbic acid. These results indicated that the addition of L-ascorbic

acid to p-benzoquinone causes its reduction (disappearance of colour) to the phenol.

Table 16. The effect of L-ascorbic acid on the toxicity of p-benzoquinone

Organisms tested		L-ascorbic acid 1 mM	p-benzoquinone 0.01%	p-benzoquinone + L-ascorbic acid
Ps. syringae				
	S5	+	-	+
	S8	+	-	+
	S20	+	-	+
	S36	+	-	+
Ps. mors-prunorum				
	D1	+	-	+
	D2	+	-	+
	C9	+	-	+
	N5	(+)	-	+
Ps. phaseolicola				
	P12	+	-	+
	P13	(+)	-	+
	P14	+	-	+
	P16	+	-	+
Ps. marginalis				
	667	+	-	+
	1558	+	-	+
	1559	+	-	+
	1604	+	-	+
Ps. tabaci				
	M45	+	-	+
Non-pathogenic pseudomonads				
	5A8	+	-	+
	4A3	+	-	+
	CS4	+	-	+
	Lk7	+	-	+
	F300	+	-	+
	Bn3	+	-	+

Experiment 32. The oxidation of phenolic compounds

The ability of the organisms to oxidise the phenolic compounds to the quinones was tested. p-aminophenol and pyrocatechol was used in this experiment. The organisms were grown for 24-48 hours on glycerol nutrient agar, then they were spotted on a filter paper impregnated with 0.4% (w/v) p-aminophenol and on filter paper impregnated with 1% (w/v) pyrocatechol. The filter paper was kept in a Petri dish, and observed for 2 hours at room temperature.

Results:- (Table 17).

The results indicated that within 5 mins. of the addition of the organisms to p-aminophenol impregnated filter paper, a dark brown colour developed in the case of the non-pathogenic pseudomonads and Ps. marginalis strains, but that no colour developed with the other plant pathogenic pseudomonads. Even after 2 hours, they did not oxidize the p-aminophenol. The one pathogen exception was strain NZ21 received as Ps. syringae, which gave a positive reaction, but has since proved to be misnamed, the other exception was non-pathogenic strain P19 received as Pseudomonas spp. which gave a negative reaction. It was also noticed that after about 30 mins. the p-aminophenol was auto-oxidized and the filter paper was stained with a pale brown colour. It was noticed, however, that such an auto-oxidation did not occur around the plant pathogenic pseudomonads (see plate 1). This could be due to the ability of these organisms to reduce the quinone formed from the auto-oxidation of p-aminophenol. Similar results to the above were also obtained with pyrocatechol and o-aminophenol.

Plate 1.

The oxidation of p-aminophenol.

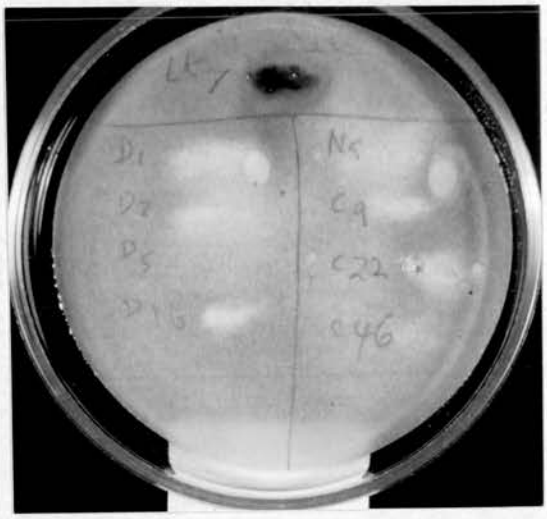


Table 17. The oxidation of p-aminophenol by different pseudomonads

Organism	number of strains tested	number gave positive reaction	number gave negative reaction
Ps. syringae	29	1	28
Ps. mors-prunorum	10	0	10
Ps. phaseolicola	6	0	6
Ps. tabaci	2	0	2
Ps. medicagines	1	0	1
Ps. marginalis	6	6	0
Ps. aeruginosa	1	1	0
Non-pathogenic pseudomonads	30	29	1

Experiment 33. The oxidation of p-aminophenol by other plant pathogenic bacteria belonging to Xanthomonads and Erwinia

Results of the above experiment indicated that apart from the Ps. marginalis strains, all the other plant pathogenic pseudomonads did not oxidize the phenolic compounds and this might explain the ability of these plant pathogenic pseudomonads to establish themselves in the plants. The non-pathogenic pseudomonads on the other hand oxidized the phenolic compounds and might, therefore, kill themselves in the plants. Therefore it was of interest to examine other plant pathogenic bacteria. The Xanthomonads strains (3) and Erwinia strains (26) were tested for their ability to oxidize p-aminophenol.

Results:-

The results indicated that all the organisms tested did not oxidize the p-aminophenol.

Experiment 34. The effect of sodium diethyldithiocarbamate on the cytochrome oxidase and the p-aminophenol oxidase

The results described above showed a positive correlation between the cytochrome oxidase reaction and the oxidation of phenols. Furthermore when representative organisms were grown on glucose medium, a medium which inhibited the synthesis of cytochrome oxidase (see page 67) it was also found that such a medium inhibited the oxidation of phenols. Assuming that the phenol oxidase of these organisms might be a copper enzyme (as most phenol oxidases seem to be) the effect of sodium diethyldithiocarbamate (a copper enzyme inhibitor) was studied on the phenol oxidase activity of these organisms. Representative strains (10) were grown on nutrient agar glycerol medium, and the effect of sodium diethyldithiocarbamate (Na dieca) was tested by growing the organisms on the above medium to which Na dieca was added from Seitz filtered solution at 1 mM final concentration. The organisms were grown on the above media for 2 days and tested for cytochrome oxidase and phenol oxidase.

Results:-

The results indicated that (Na dieca), an inhibitor of the copper enzymes, had no significant effect on the cytochrome oxidase reaction as examined by Kovac's method, but it had an inhibitory effect on the oxidation of phenol [illegible] although the inhibition was not complete and the organisms were able to oxidize the p-aminophenol after longer time. Table (18) showed

the results of the oxidation of p-aminophenol.

Table 18. The effect of Na dieca on the oxidation of p-aminophenol.

Organisms tested	Time required for the oxidation of p-aminophenol	
	Control medium	1mM (Na dieca)
5A8	5 mins.	40 mins.
4A3	5 "	45 "
CS4	4 "	50 "
Lk7	5 "	50 "
F300	3 "	45 "
1A1	5 "	50 "
Bt6	4 "	40 "
Cb1	5 "	55 "
667	5 "	40 "
1559	4 "	55 "

Experiment 35. The survival of plant pathogenic and non-pathogenic pseudomonads in the presence of p-aminophenol

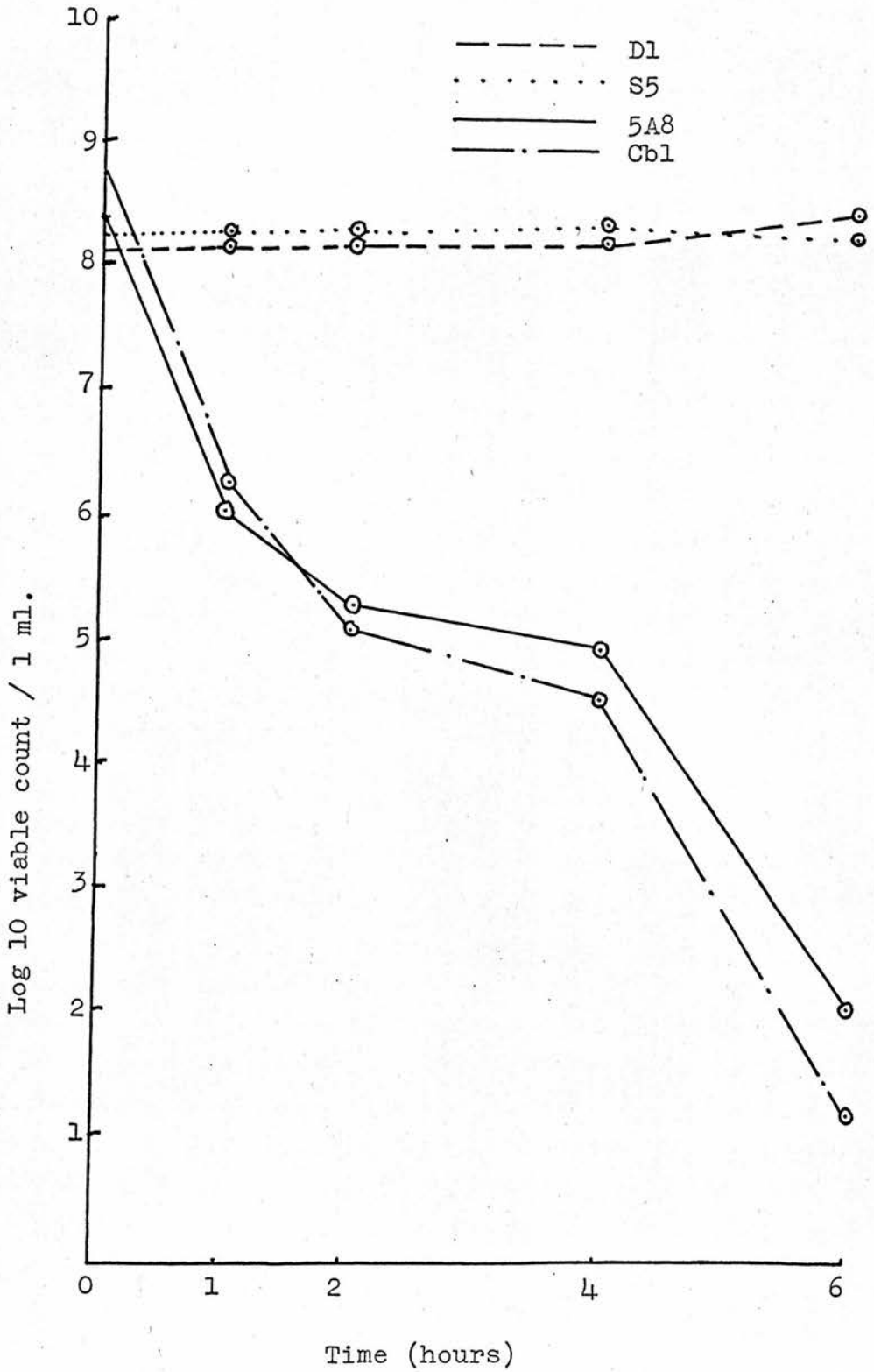
This experiment was conducted to see if non-pathogenic pseudomonads growing in the presence of phenolic compounds killed themselves, whereas the plant pathogenic pseudomonads, which did not oxidize the phenolic compounds, survived.

Two non-pathogenic pseudomonads strains, 5A8 and Cb1 and two phytopathogenic pseudomonads strains, D1 and S5, were tested. They were grown for 48 hours on glycerol nutrient agar. The cells were harvested and washed with sterile water, they were then added to 0.03 M sterile solution of p-aminophenol. The flasks were incubated at 27°C and a viable count was made according to the method described by Miles & Misra (1938) after 1, 2, 4 and 6 hours on glycerol nutrient agar as a recovery medium.

Results:-

The oxidation of p-aminophenol occurred in the flasks inoculated with the non-pathogenic strains but not in flasks inoculated with the plant pathogenic strains. Figure 3 shows that the viable count of the non-pathogenic pseudomonads strains markedly decreased during the test whereas the viable count of the plant pathogenic pseudomonads remained constant or slightly increased.

Figure 3. The survival of plant pathogenic and non-pathogenic pseudomonads in p-aminophenol



Experiment 36. The survival of Ps. syringae in p-benzoquinone

The plant pathogenic pseudomonads examined in this study were characterized by their ability to produce levan from sucrose. It was thought that this polysaccharide might protect the organisms from the toxic action of the quinones. Therefore this experiment was conducted to study the survival of a plant pathogenic organism grown on sucrose containing medium in p-benzoquinone.

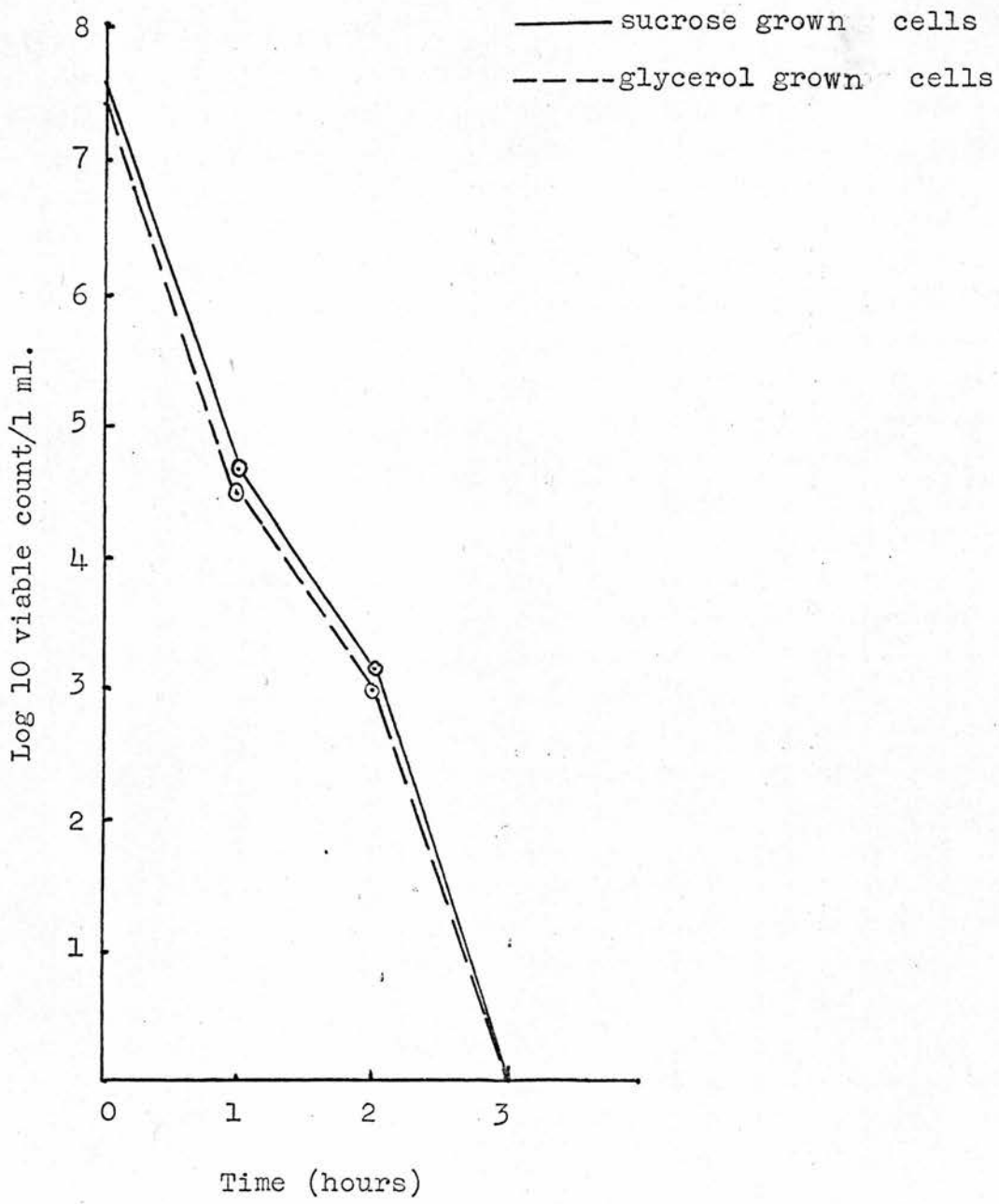
The method:-

Ps. syringae strain S5 was grown for 2 days on glycerol nutrient agar, and on nutrient agar containing 5% (w/v) sucrose. The cells were harvested with sterile phosphate buffer 0.02 M, pH7. The two samples of cells were adjusted to the same turbidity as measured by EEL colorimeter and 1 ml. of standard cell suspension was added to 100 ml. of sterile buffer containing 0.002% (w/v) p-benzoquinone. The survival of the organism was also studied in the phosphate buffer. The viable count was made as described before after 1, 2, 3, 4 and 24 hours on glycerol nutrient agar as a recovery medium.

Results:-

Figure 4 indicates that the p-benzoquinone is very toxic at the concentration used and that the organisms were dead after 3 hours. No differences were found between the survival rates of the organisms grown with or without sucrose.

Figure 4. The survival of Ps. syringae strain S5 in p-benzoquinone solution.



Experiment 37a. The reduction of quinone

The results obtained from the oxidation of p-aminophenol indicated that the plant pathogenic pseudomonads, apart from Ps. marginalis, did not oxidize that phenolic compound but that a colourless area developed around these organisms suggesting their ability to reduce the quinone formed by auto-oxidation of the phenol, see plate 1. Results of the above experiment also indicated that quinone is very toxic to these organisms and that such toxic effect could be abolished if the quinone was reduced back to the phenol by ascorbic acid. It was of interest therefore to study the ability of these organisms to reduce p-benzoquinone in the presence of different carbon sources as electron doners. The reduction of quinone was determined colourimetrically depending on the fact that p-benzoquinone loses its colour on reduction.

The method:-

The organisms were grown on glycerol nutrient agar for 2 days, the cells were harvested and washed with 0.2 M phosphate buffer pH7 and resuspended in the same buffer. The cell suspension was adjusted to 5.5 - 6 mg. dry weight per ml.

The ability of the organisms to reduce the quinone was tested in the following basal medium:-

0.1% (w/v) KNO_3

1% (w/v) of the carbon source.

The following carbon sources were tested:- glucose, Na succinate, Na gluconate and sucrose. The constituents of

the medium were dissolved in 0.2 M phosphate buffer pH 7.0. This strong buffer was used to minimize the changes in the pH of the media during the experiment since the pH of the medium was found to have an effect on the reduction or further oxidation and polymerization of the quinone. The media were dispensed in 50 mls. amounts in 100 ml. flasks and sterilized at 10 lb. for 10 mins. p-benzoquinone (final concentration 0.02% (w/v)) was added to each flask. Each flask was inoculated with 1 ml. of the adjusted cell suspension. Flasks were incubated at 27°C and after 18, 36 and 72 hours, 10 mls. from each flask was removed and centrifuged and the density of the red colour measured by EEL colourimeter (the green filter was used) and the control medium which had no quinone was used in the reference cell.

Organisms tested:-

The following representative organisms were tested:-

Ps. syringae S5 - S8 - S20 strains

Ps. mors-prunorum D1 - N5 - C9 strains

Ps. phaseolicola P12 - P14 - P16 strains

Ps. marginalis 1559 - 667 strains

Non-pathogenic pseudomonads CS4 - 1A1 - 4A3 - Lk7 - F300 strains

Results:- (Table 19)

Results indicated that the control uninoculated media which contained p-benzoquinone was red in colour and on further incubation the colour becomes more dark, this could be due to

the autooxidation and polymerization of the quinone.

Sodium succinate:-

The results indicated that all the non-pathogenic pseudomonads failed to reduce the quinone whereas the plant pathogenic pseudomonads, with the exception of Ps. marginalis, reduced the quinone.

Sucrose:-

The results indicated that the plant pathogenic pseudomonads except for Ps. marginalis reduced the quinone in sucrose medium, but that again the non-pathogenic pseudomonads did not reduce the quinone. The ability of the plant pathogenic pseudomonads apart from Ps. marginalis to reduce quinone in sucrose medium could be due to 2 factors, their inability to oxidize the phenolic compounds and their ability to produce 2-ketogluconate from sucrose, whereas the non-pathogenic pseudomonads and Ps. marginalis, although some were able to produce 2-ketogluconate from sucrose, were unable to reduce the quinone presumably because of their strong phenol oxidase activity which masks the reducing activity of 2-ketogluconate.

Sodium gluconate:-

The results indicated that apart from Ps. marginalis, the plant pathogenic pseudomonads were able to reduce the quinone whereas the non-pathogenic pseudomonads and Ps. marginalis strains did not after 18 hours. After 36 and 72 hours incubation, however, a slight reduction of the quinone was observed with the

Table 19. The reduction of p-benzoquinone by pseudomonads in different media

Organisms tested	Sodium succinate medium			Sucrose medium			Glucose medium			Sodium gluconate medium		
	18 hrs	36 hrs	72 hrs	18 hrs	36 hrs	72 hrs	18 hrs	36 hrs	72 hrs	18 hrs	36 hrs	72 hrs
Control	3·4*	5·5	6·6	3·7	5·9	6·3	4·4	4·6	4·8	3·6	5·9	6·4
Non-pathogenic pseudomonads												
CS4	7·5	8·0	9·0	6·4	7·3	9·0	5·6	6·2	7·0	7·1	7·7	8·3
1A1	4·8	5·9	7·0	5·1	6·2	7·1	3·6	3·7	3·8	4·0	5·0	5·7
4A3	7·2	7·4	8·2	6·1	6·8	7·8	3·8	4·0	4·3	4·9	6·6	7·8
Lk7	5·0	6·1	7·1	5·0	6·4	7·3	4·1	4·2	4·4	4·3	6·2	6·8
F300	5·1	6·0	7·2	5·1	6·2	7·0	3·5	3·5	3·7	4·3	5·0	5·7
Ps. marginalis												
667	3·4	5·6	6·8	3·5	5·7	6·2	3·3	3·8	4·0	3·7	5·0	5·9
1559	3·5	5·8	6·9	4·0	5·9	6·4	3·4	3·7	3·9	3·8	5·2	5·9
Ps. syringae												
S5	2·5	4·1	4·3	3·0	4·7	5·2	3·2	3·5	3·7	3·0	5·0	5·2
S8	2·7	3·9	4·5	2·9	4·5	5·0	3·2	3·4	3·5	3·0	4·8	5·1
S20	2·6	4·0	4·2	3·0	4·9	5·4	3·3	3·4	3·4	2·8	4·6	4·9
Ps. phaseolicola												
P12	2·8	4·0	4·5	3·2	5·0	5·3	4·0	4·0	4·2	3·2	5·1	5·5
P14	2·7	3·9	4·3	3·1	4·9	5·1	3·2	3·6	3·7	3·2	4·9	5·2
P16	2·8	4·3	4·8	3·2	5·1	5·5	3·5	3·5	3·6	3·1	4·8	5·0
Ps. mors-prunorum												
D1	3·1	4·4	5·1	3·2	5·3	5·4	3·6	3·7	3·8	3·5	5·2	5·6
N5	3·0	4·3	4·9	3·5	5·2	5·5	3·1	3·4	3·5	3·4	5·1	5·4
C9	2·9	4·7	5·0	3·4	5·0	5·3	3·5	3·5	3·7	3·2	4·9	5·3

* These figures are the absorption as determined by EEL colourimeter.

strains which were shown previously to produce 2 ketogluconate from gluconate.

Glucose:-

The results indicated that in glucose medium all the plant pathogenic and non-pathogenic pseudomonads were able to reduce the quinone, with the exception of one strain CS4 a non-pathogenic pseudomonad. The ability of the non-pathogenic pseudomonad to reduce the quinone in glucose medium could be due to the glucose effect on cytochrome oxidase and phenol oxidase as described before. With such activities repressed, the quinone reducing properties are observed, and probably reflect 2-ketogluconate formation from glucose. Figures 5 and 6 show general differences between the plant pathogenic and non-pathogenic pseudomonads in their ability to reduce p-benzoquinone in sucrose and sodium succinate media.

Figure 5. The reduction of p-benzoquinone in sucrose medium.

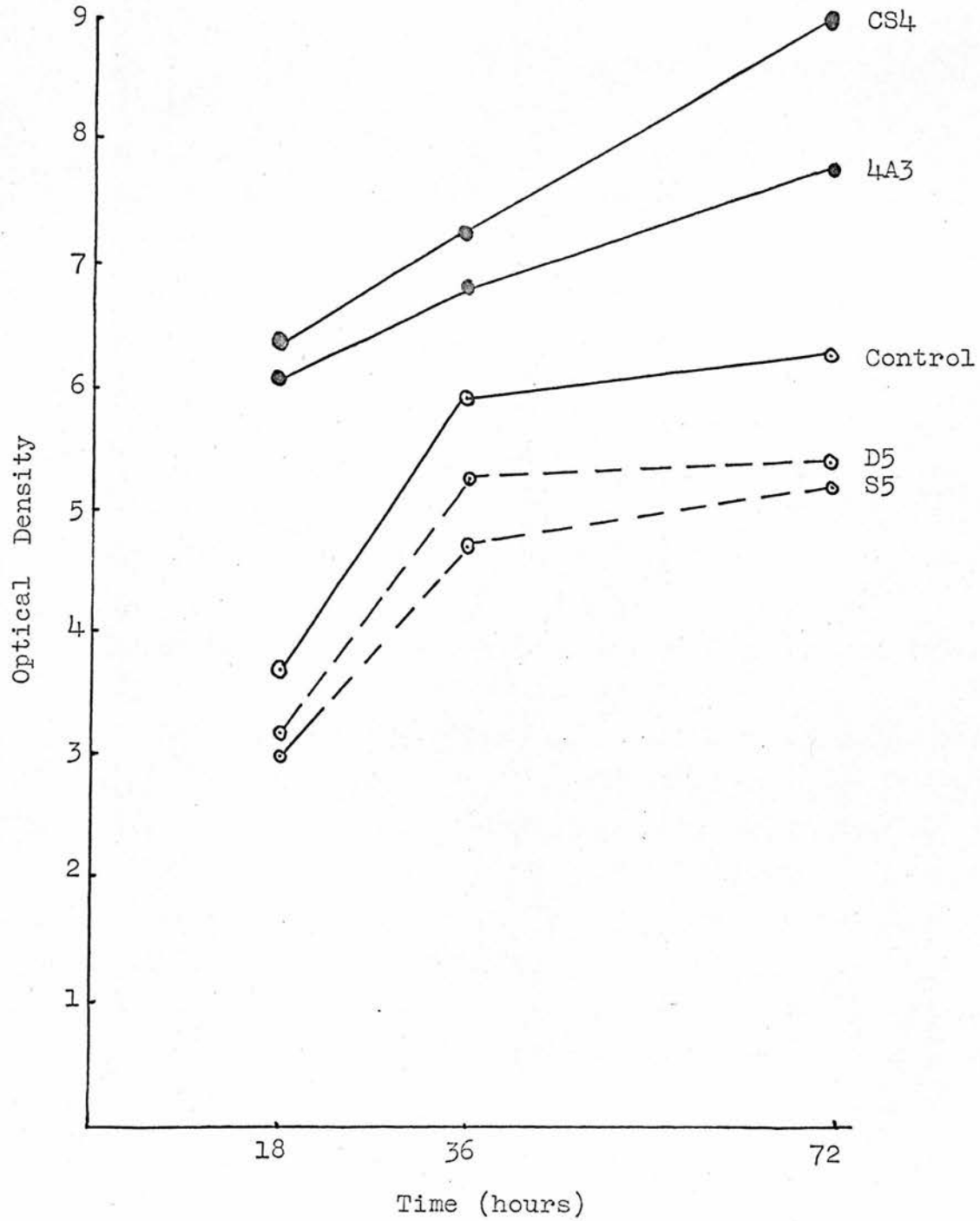
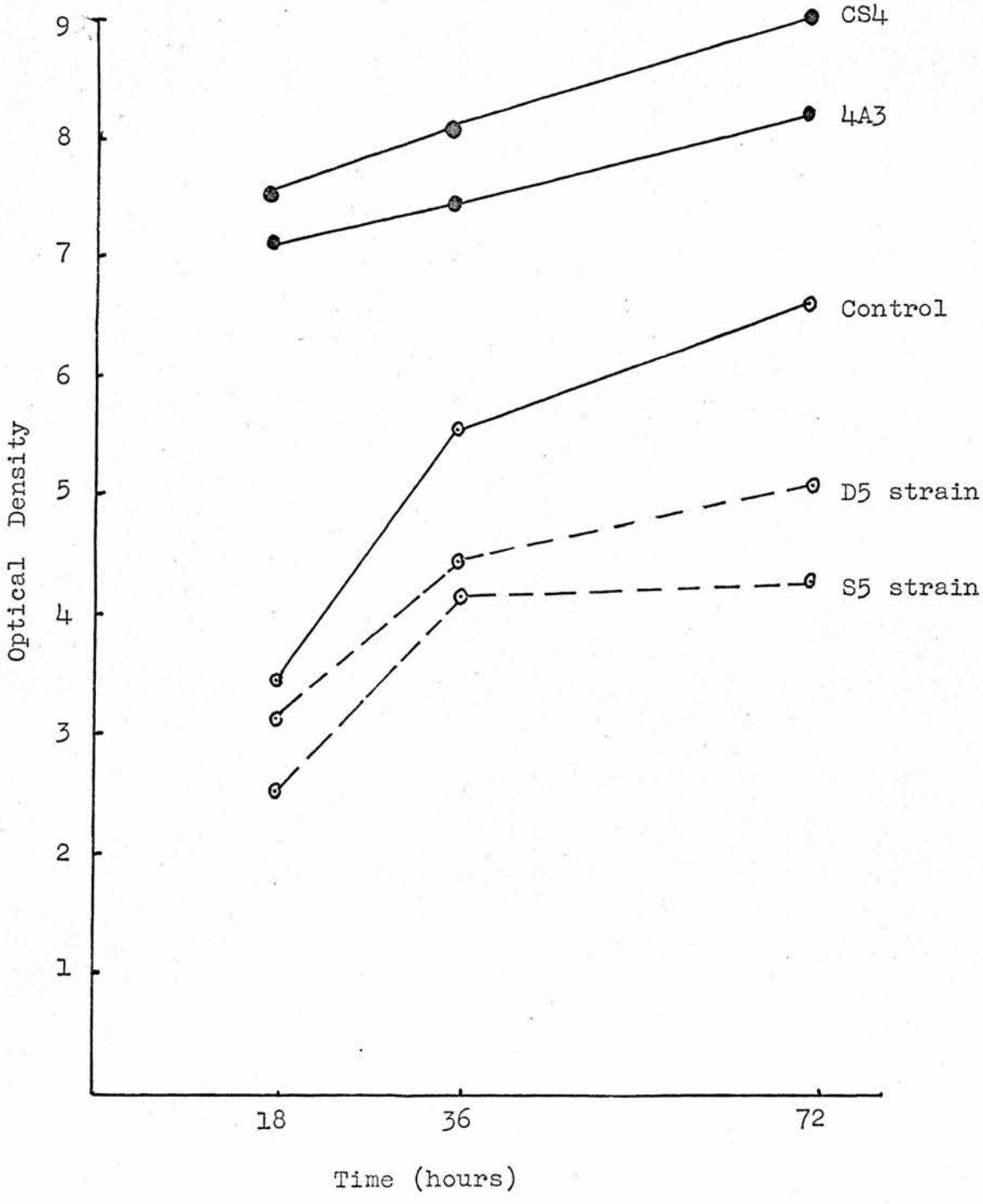


Figure 6. The reduction of p-benzoquinone in Na succinate medium



Experiment 37b. The reduction of naturally occurring quinones

The ability of the organisms to reduce the quinones extracted from potato tubers was studied.

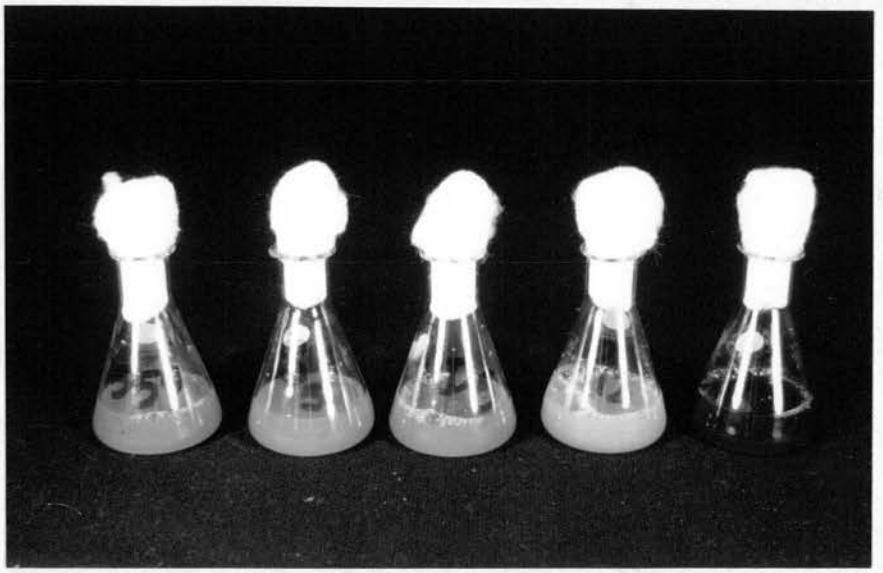
The potato tuber variety King Edward was used in this experiment. 300 gm. of the potato tubers were macerated with 350 mls. of 0.02 M phosphate buffer pH7 for 10 mins. The potato extract was squeezed through a piece of muslin and it was noticed that after 2 hours at room temperature the extract was red in colour. The colour became darker on standing at room temperature presumably because of the autooxidation of the phenolic compounds. The crude extract was distributed in 25 ml. amounts in flasks. Each flask was inoculated with 2 mls. of the bacterial suspension adjusted to 5 mg. dry weight per ml. The flasks were incubated at 27°C for 14 hours. The degree of the reduction was measured by using a UNICAM SP1300 colourimeter. Green filter No.3 was used and phosphate buffer was in the reference cell. After 14 hours, 10 ml. from each of the flasks was centrifuged and the degree of reduction was measured as described above.

Results:-

The results (Table 20 & plate 2) indicated that the plant pathogenic pseudomonads tested reduced the quinones formed from the autooxidation of the potato phenols. The Ps. marginalis strain as well as the non-pathogenic 4A3 strain were able also to reduce quinones slightly.

Plate 2.

The reduction of quinones extracted from potato tubers by pseudomonads.



1559 S5 D5 P12 Control

Table 20. The reduction of quinones extracted from potato tubers

Organisms tested	absorption
Control	0.37
Ps. syringae S5	0.25
Ps. mors-prunorum D5	0.22
Ps. phaseolicola P12	0.20
Ps. marginalis 1559	0.28
Non-pathogenic pseudomonad 4A3	0.30

The ability of xanthomonads and Erwinia strains to reduce the quinone extracted from potato was also tested and the results indicated that 2 Xanthomonads and the 3 Erwinia strains were able to reduce the quinone (see plates 3 & 4). It was noticed also that the 3 Erwinia strains reduced the quinone markedly after 15 mins. of incubation. The following experiments were conducted to be sure that the reduction of the colour was due to the quinone reduction.

1. When ascorbic acid was added to the extract the brown reddish colour disappeared, the same occurred when ascorbic acid was added to p-benzoquinone solution.

2. To flasks inoculated with Erwinia strains in which the quinone has been reduced, H_2O_2 was added and the red colour appeared again. The breakdown of H_2O_2 by the catalase activity of the Erwinia strains, caused rapid autooxidation of the phenolic compounds.

Plate 3.

The reduction of potato quinones by Erwinia species

H2 G110 G117 Control

Plate 4.

The reduction of potato quinones by xanthomonads

X1 X2 Control

Experiment 38. The phenolic content of bean plants as affected by the age of the plants and by the infection with Ps. phaseolicola

Bean plants variety Canadian Wonder were used in this experiment, they were grown in the greenhouse until the first trifoliate leaf was developed. The plants were then divided into two groups, one of them was a control group inoculated with sterile water, the other group was inoculated with Ps. phaseolicola strain P12. This strain was grown for 2 days on glycerol nutrient agar, the cells harvested and washed with sterile water and prepared as a bacterial cell suspension (10^8/ml), which was sprayed on the lower surface of the leaves with an atomizer. The plants were then kept under polythene bags for 7 days. Samples of 20 plants from each group were collected after 3, 7, 14 and 21 days after the inoculation for the determination of phenols.

The determination of phenols:-

The reagent which is used for the determination of phenols is that described by Arnow (1937), this reagent is very specific and reacted with o-dihydric phenols but did not react with tyrosine.

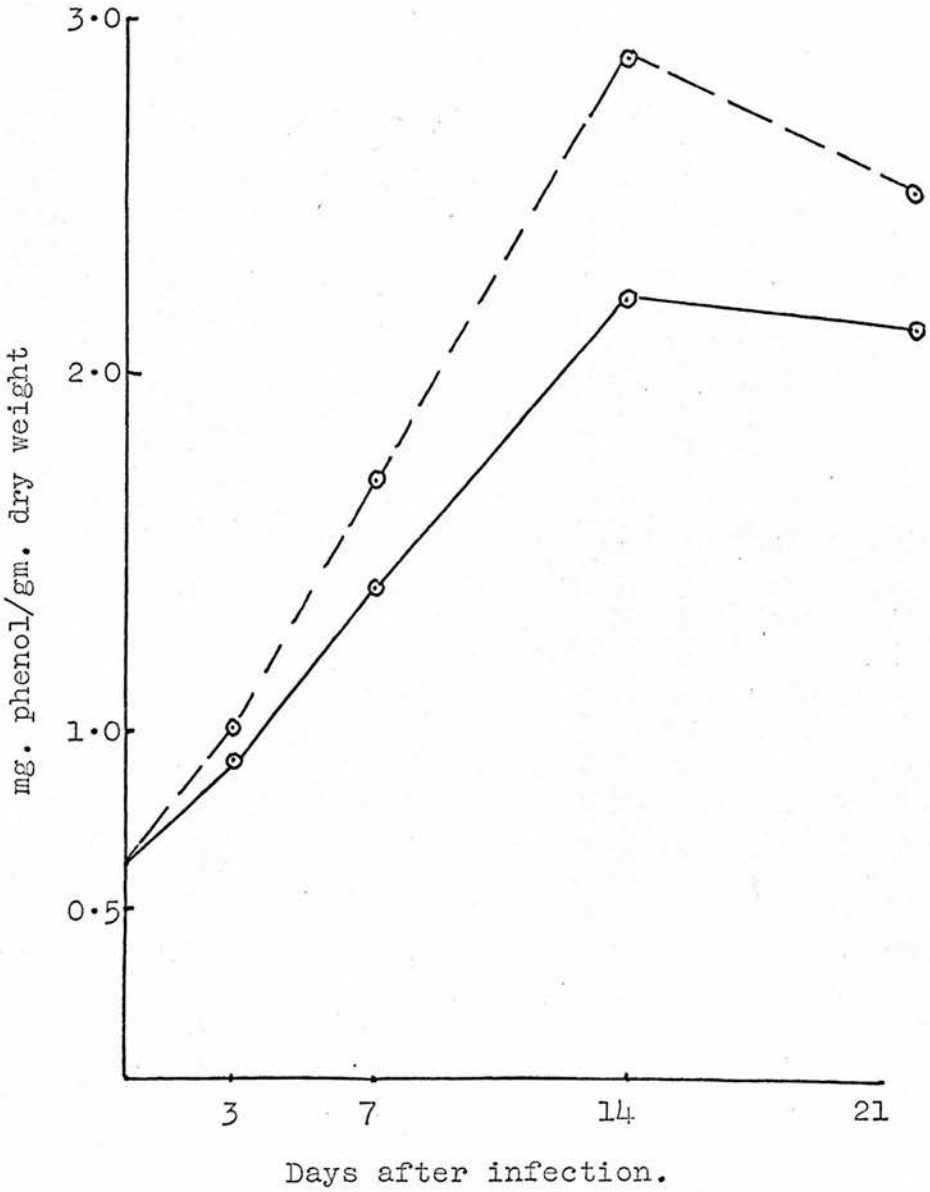
The plant materials were macerated in water for 10 mins. and the dry weight per/ml. was determined. To 1 ml. of the macerated plant material 1 ml. of 0.5 N HCl was added followed by 1 ml. of Arnow's reagent which is 10 gm. $NaNO_2$ and 10 gm. $Na_2M\ O_4$ in 100 ml. water. Then 5 mls.of distilled water was added,

followed by 1 ml. of 1 N NaOH. A red colour developed immediately after the addition of sodium hydroxide. The tubes were centrifuged for 10 mins. and the red solution read colourimetrically. A UNICAM SP 1300 with green filter No.3 was used. The reference cell contained the blank sample which was the solution used in the experiment with Arnow's reagent replaced by water. The standard curve was made by using pyrocatechol.

Results:- (Figure 7) showed that the phenolic content, as determined by Arnow's reagent increased with the increase of the age of the plant up to 14 days after the first trifoliate leaf was developed. After 21 days the phenols decreased slightly. Results also indicated the increase of the phenolic content of the plants after they had been infected.

The phenolic content, as determined by Arnow's reagent, is not a measure of the total phenols but only of the o-dihydric phenols.

Figure 7. The phenolic content of healthy (——) and Ps. phaseolicola (– – – –) infected bean plants.



Experiment 39. The phenol oxidase activity of bean plants as affected by the age of the plant and by the infection with Ps. phaseolicola

Bean plants were grown as described before, and inoculated with P12 Ps. phaseolicola strain. Samples of 20 plants, the leaves and the stems, were collected at the time of inoculation and 3, 7, 14 and 21 days after the inoculation. The samples were kept in the deep freeze until the end of the experiment. The preparation of the crude enzyme extract was as follows:- The samples were macerated with 0.1 M phosphate buffer pH7 for 10 mins., the dry weight was determined for each sample. The different samples were adjusted with the same buffer to give 14 mg. dry weight in 1 ml. of the plant extract. The phenol oxidase activity of the plant extract was determined by using 3,4 dihydroxyphenylalanine (DOPA) as substrate which when oxidized is converted to DOPA quinone which is black in colour. The degree of the oxidation of that phenol was determined as follows.

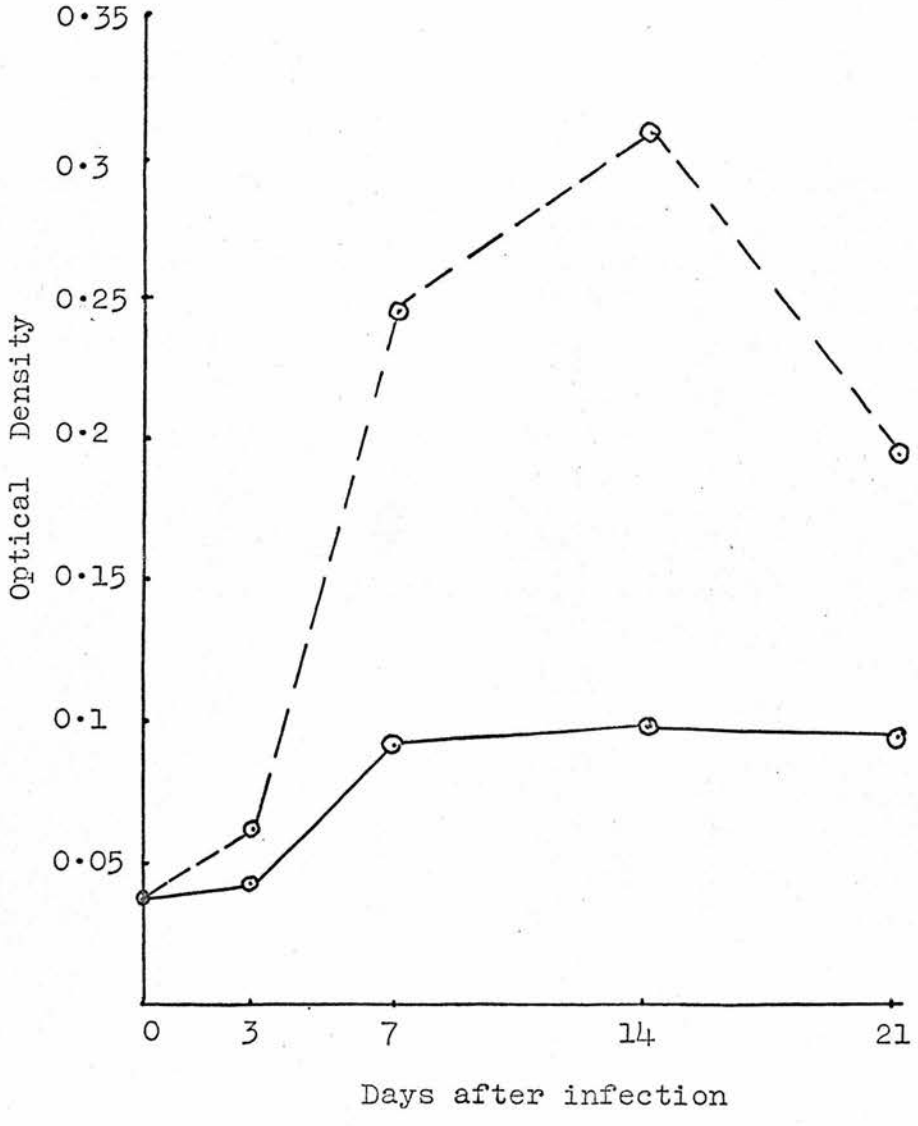
The reaction mixture:- consisted of 3 ml. of the crude enzyme extract and 3 ml. of DOPA (1 mg./ml.). One control was the crude enzyme extract 3 ml. + 3 ml. of water , and the other control was 3 ml. of DOPA + 3 ml. of water. The tubes were incubated at 30°C in a water bath for 18 hours and were then centrifuged. The degree of oxidation of DOPA was measured by using a UNICAM SP 1300 with green filter No.3. The reference cell contained the control sample. The absorption was

recorded and a correction was made because of the autooxidation of DOPA solution, (it is slightly autooxidized under these conditions).

Results:-

Figure 8 indicates that the phenol oxidase activity of the bean plants is increased up to the 7th day of the development of the first trifoliate leaf and then did not increase significantly after that. The results also indicated that the phenol oxidase activity of the plants was markedly increased when they were infected with Ps. phaseolicola.

Figure 8. The phenol oxidase activity of healthy (——) and Ps. phaseolicola (– – –) infected bean plants.



Experiment 40. The alteration of the resistance and susceptibility of the host plant to pseudomonad infection

The results described earlier indicated that quinones are more toxic than the phenols to the pseudomonads. Results also suggested that the inability of the soil pseudomonads (non-pathogenic) to infect the plants could be due to their ability to oxidise the plant phenolic compounds to the more toxic oxidized forms (quinones). Therefore it was of interest to study the effect of feeding plants with phenol oxidase inhibitors, such as sodium diethyldithiocarbamate (Na dieca) and with 4-chlororesorcinol, a compound used by Noveroske et al. (1962) to break down the resistance of Malus plants to Venturia inaqualis. The Na dieca compound has not been used previously for the breakdown of plant resistance to pathogenic organisms.

The effect of feeding plants with L-ascorbic acid, p-benzoquinone and pyrocatechol was also examined.

Bean plants were grown in the greenhouse for 10 days, then the plants were sprayed by atomizer with 1mM aqueous solution of the above compounds, which were also added to the compost on which the plants were grown (50 mls. of the above compounds were used each time). The plants were treated as above every 2 days for 10 days. The control plants were treated with sterile water. All the compounds were added from Seitz filter-sterilized stock solutions to sterile distilled water. After the plants had been treated with these compounds, they were inoculated 2 days after the last addition. Ps. phaseolicola

P12 strain was used in this experiment, and the plants were inoculated with bacterial cell suspension as described before. The plants were kept under polythene bags in the greenhouse and observed for 2 weeks. Each treatment consisted of 10 plants.

Results:-

The uninoculated plants treated with the above compounds were healthy. The results showed that plants inoculated with Ps. phaseolicola and treated with Na dieca and L-ascorbic acid had much greater lesions on them than did inoculated but untreated plants. These differences were marked 10-12 days after inoculation of the plants treated with Na dieca and L-ascorbic acid.

Results also indicated that 4-chlororesorcinol had no significant effect on the pathogenicity of Ps. phaseolicola on the treated plants. No symptoms developed on plants treated with p-benzoquinone, and the severity of the disease was markedly reduced on plants treated with pyrocatechol.

Plate 5 shows the effect of Na dieca on the pathogenicity of Ps. phaseolicola.

Plate 5.

The effect of Na dieca on the pathogenicity of *Ps. phaseolicola* on bean plants

A = Control plant

B = Treated plant inoculated with strain P12

Experiment 41. The effect of p-benzoquinone on the pathogenicity of Ps. marginalis

Lettuce plants variety Paris White were grown in the greenhouse and after 14 days from the transplanting, the plants were sprayed with p-benzoquinone 1mM and 0.1 mM. The p-benzoquinone was also added to the soil. The control plants were treated with water. These treatments were repeated every 2 days, 50 mls. were added each time. The plants were treated 4 times and after that they were inoculated with Ps. marginalis strain 1559 which was grown on glycerol nutrient agar. The bacterial cells were harvested and washed with sterile water and resuspended in sterile water and plants were sprayed with atomizer under pressure, the inoculated plants were kept under polythene bags for 2 days. The control plants were inoculated with sterile water.

Results:- (Plate 6) indicated that plants treated with 1mM p-benzoquinone (A) showed no symptoms, and that plants treated with 0.1 mM (B) had less severe symptoms than those appearing on control plants (C) which had been treated with water. This plate shows the results 3 days from the infection. At 7 days no difference was noticed between the plants treated with the lowest concentration of p-benzoquinone and the control plants, whereas no symptoms developed with plants treated with 1mM p-benzoquinone. Lettuce plants not sprayed but watered at the root with p-benzoquinone also resisted infection showing that the plants absorbed sufficient quinone to protect themselves from infection.

Plate 6.

The effect of p-benzoquinone on the pathogenicity of Ps. marginalis strain 1559 on lettuce plants

A = 1 mM p-benzoquinone treatment

B = 0.1 mM p-benzoquinone treatment

C = Control treatment

Experiment 42. The effect of feeding bean plants with L-ascorbic acid and (Na dieca) and their response to the infection with a non-pathogenic pseudomonads

The results obtained from the effect of L-ascorbic acid and Na dieca on the pathogenicity of Ps. phaseolicola, suggested a good possibility of obtaining a pathological response of the bean plants to infection by normally non-pathogenic pseudomonads, if the plants were fed with L-ascorbic acid, it was shown in experiment (31) that L-ascorbic acid abolished the toxic effect of the quinone. The dieca compound is a copper chelating compound and an inhibitor of copper enzymes.

The plants were fed with the above compounds as described before and inoculated with the following non-pathogenic pseudomonads 4A3 - F300 and Ps. 73. The last two strains are soft rotting strains as will be shown later. The plants were inoculated with these organisms (10 7/ml.) and observed for 3 weeks in the greenhouse.

Results:-

No pathological symptoms were noticed when these organisms were inoculated into the plants treated with water or on plants fed with dieca or L-ascorbic acid or plants fed with both compounds. Plants treated with 4-chlororesorcinol also showed no symptoms. These results do not exclude the possibility that these organisms were able to survive the defence mechanism of the treated plants, but do show that they cannot form pathological lesions.

Experiment 43. The pathogenicity of non-pathogenic pseudomonads strains on lettuce leaves

During this study a close relationship was found between the non-pathogenic pseudomonads and Ps. marginalis in many aspects. All were able to oxidize the phenolic compounds to the quinones. Ps. marginalis is the pathogenic organism to the lettuce plants and other related hosts. The ability of Ps. marginalis to infect and invade the lettuce plants, although the organism is able to oxidize phenolic compounds, could be due to the low content of phenolic compounds in such plants compared with other plants like plums and other stone fruits, therefore if this assumption was true one would expect that non-pathogenic pseudomonads would also be able to produce symptoms in lettuce plants, and the following experiment was conducted.

Lettuce seeds, variety Webbs Wonderful, were planted in the greenhouse and were grown for 3 weeks. The leaves of the plants were soaked in hypochlorite solution for 10 mins. and then washed with sterile water 3 times. The leaves were kept in sterile Petri dish which contained filter paper soaked with water.

Organisms tested:-

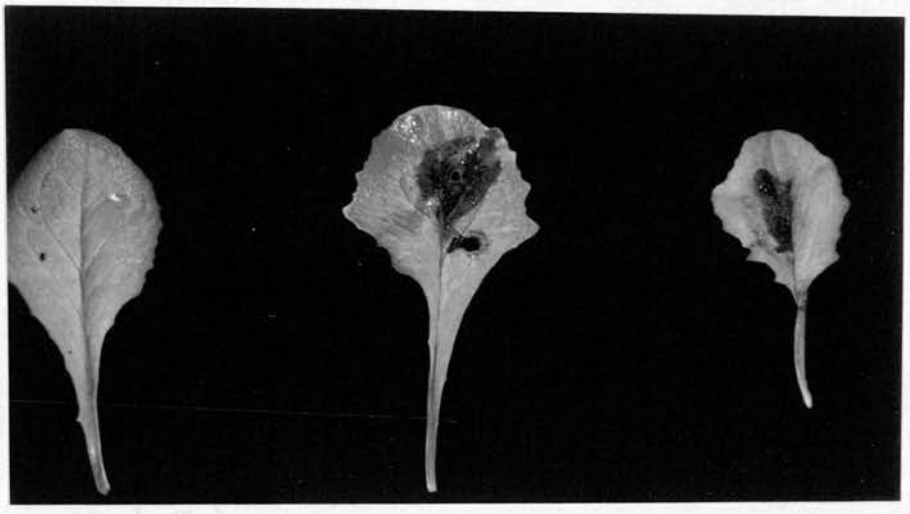
20 non-pathogenic pseudomonads strains as well as 6 Ps. marginalis. The organisms were grown for 24 hours on glycerol nutrient agar and the leaves were inoculated, half of the leaf was stabbed with the organism. The plates were incubated for 7 days at 27°C. Sterile water was added to the plates every 2 days to maintain a suitable humidity.

Results:-

Of the 20 non-pathogenic pseudomonads only 3 strains produced a lesion on the lettuce leaves. These strains were Ps. 73, 1A1 and F300 and the symptoms were similar to that produced by Ps. marginalis, (see plate 7). These 3 non-pathogenic strains were soft rotting organisms, as will be seen later, and are very similar to Ps. marginalis which is also a soft rotting organism. The symptoms were produced by the above organisms 2-3 days after the inoculation.

Plate 7.

The pathogenicity of *Ps. marginalis* strain 1559 and non-pathogenic *Pseudomonas* sp. strain Ps.73 on lettuce leaves



Control 1559 Ps73

Experiment 44. The pathogenicity of different pseudomonads on tobacco plants

Tobacco plants, variety White Burley, were used in this experiment and were grown in the greenhouse. They were inoculated after 3 weeks from the date of transplanting with the following representative strains.

Ps. syringae S5 - S8

Ps. mors-prunorum D1 - D2

Ps. phaseolicola P12 - P14

Ps. marginalis 667 - 1559

Ps. tabaci M44 - M45

Non-pathogenic pseudomonads 5A8 - Cb1 - CS4 - F300 - 1A1 - 4A3 and Ps. 73.

The above organisms were grown for 24-48 hours on glycerol nutrient agar. The bacterial cell suspension used contained about 10^7 cells/ml. and was sprayed into the lower surface of the leaves. The plants were kept under polythene bags and observed for the development of the symptoms for 10 days.

Results:-

Results indicated that Ps. marginalis strains as well as the non-pathogenic pseudomonads did not produce any pathological symptoms on to the plants, whereas all the other plant pathogenic pseudomonads tested caused the necrosis of the leaves within 6 days from the infection.

Discussion:-

The results described above indicated that quinones are more toxic than phenols to the organisms tested. This is in agreement with the results obtained by many workers in relation to pathogenic fungi (Farkas & Kiraly, 1962), Cruickshank & Perrin (1964).

Results also showed that the non-pathogenic pseudomonads, as well as Ps. marginalis strains, oxidized the phenolic compounds to quinones, whereas the other plant pathogenic pseudomonads did not oxidize the phenolic compounds. A positive correlation was found between the cytochrome oxidase activity and the ability of the organisms to oxidize the phenolic compounds. The differences between the two groups of the organisms, as indicated before, are that the plant pathogenic pseudomonads possess a cytochrome of b type but not of the c type, whereas the non-pathogenic pseudomonads and Ps. marginalis possess cytochrome c, and possibly b. Smith (1954) reported the ability of the cytochrome oxidase cytochrome c system to oxidize hydroquinone as well as the "Nadi" reagent which could also be oxidized by peroxidases. Nagasawa & Gutmann (1959) also reported the oxidation of o-aminophenol by cytochrome c, cytochrome oxidase enzymes. However the results obtained from the effect of Na dieca, a copper chelating compound, on the oxidation of phenols suggested a participation of a copper enzyme (phenoloxidase) in the oxidation of phenols. However Na dieca is able to chelate other metals (James, 1953).

Klement et al. (1964) found that all the phytopathogenic pseudomonads they tested (they did not examine Ps. marginalis) multiplied in tobacco plants whereas saprophytic pseudomonads are unable to do so. This suggests that tobacco plants are useful test plants. The inability of the non-pathogenic pseudomonads to multiply in the plants could be due to their ability to oxidize the phenolic compounds to the quinones, which is shown in this study to be toxic to pseudomonads. Results described in this study indicated that Ps. marginalis did not infect tobacco plant and this could be attributed to the quinone toxicity.

Many workers have tried to break down the resistance of plant against fungi by using reducing agents, Oku (1960a), or by phenol oxidase inhibitors, Noveroske et al. (1962). It was of interest here to note that the above authors were able to break down the resistance of Malus plants when they were fed with 4-chlororesorcinol plus glucose. The results described in this study indicated that feeding bean plants with 4-chlororesorcinol had no effect on the pathogenicity of Ps. phaseolicola, therefore the results obtained by Noveroske et al. could be due to the combined effect of 4-chlororesorcinal and glucose. As indicated in this study glucose represses cytochrome oxidase and phenol oxidase activity in some bacteria. The pathogenicity of Ps. phaseolicola was greatly increased when plants were treated with Na dieca and with ascorbic acid. Maine & Kelman (1961) also reported that the pathogenicity of Ps. solanacearum was increased on tobacco plants treated with

reducing agents.

The accumulation of phenolic compounds in bean plants after they have been infected with Ps. phaseolicola, as well as the increase in the phenol oxidase activity of the plant, seems to be a general response of the plants to diseases. Many workers also found similar results in the case of fungal diseases (Farkas & Kiraly, 1962), (Cruickshank & Perrin, 1964) and Hare (1966). Maine & Kelman (1960) also found that the phenol oxidase activity of tobacco plants was much greater in plants infected with Ps. solanacearum than in healthy plants. The mechanism of this increase of phenol oxidase in infected plants is not known. However this increase could be due to the activation of latent phenol oxidase of the plant by the acids produced during the metabolism of the pathogen in the plant tissue. Kenten (1957) showed that the phenol oxidase of broad bean can be greatly increased by brief exposure to mild acid or alkaline conditions. Therefore the increase of the phenol oxidase activity of the infected plants could also be due to such activation.

B. The peroxidase activity and its role in the oxidation of phenolic compounds

The plant phenol oxidase systems are slow to act and frequently require marked changes in the plants to bring about their activity. However, a fast reaction has been attributed to the plant peroxidase system as will be seen later. In the presence of H_2O_2, phenols are rapidly peroxidized to quinones by the plant, this reaction could constitute a rapid defence system to infection, provided H_2O_2 was available. A study was made of H_2O_2 production by pseudomonads on different substrates, to determine whether the non-pathogenic pseudomonads might possibly provide a substrate for the plant peroxidase and for their own destruction in the plant.

Experiment 45. The production of hydrogen peroxide from amino acids

It is known that H_2O_2 could be produced from some amino acids when the oxidative deamination occurs. Oxidative deamination is catalysed by a group of flavin enzymes, which become reduced during this reaction and then reoxidized by molecular oxygen to form H_2O_2.

The results obtained from the nutritional studies indicated that the following amino acids were utilized as carbon and nitrogen sources by all the pseudomonads tested, with the exception of 3 atypical strains; DL-alanine, L-glutamic acid, L-aspartic acid, L-proline. These acids and vitamin-free casaminoacids were tested in the following experiment. The basal medium used in this study has the following composition:-

0.1% (w/v) amino acid
0.02% (w/v) $MgSO_4$
0.008% (w/v) K_2HPO_4
0.002% (w/v) KH_2PO_4
1.2% (w/v) agar

The prepared media were adjusted to pH7 and autoclaved at 115° for 10 mins. The methods used for the detection of hydrogen peroxide was the same as described by Whittenbury (1964). Both methods were used.

(a) Heated blood o-dianisidine medium:-

The agar medium containing the amino acid was melted and 5 ml. of a 1 + 1 mixture of defibrinated ox blood and water added and the whole heated at 100°C for 15 mins. o-dianisidine (0.1 gm. in 5 ml. sterile water was heated at 100°C for 15 mins.) was then added to the melted agar medium, and the complete medium poured in plates.

(b) Haematin medium:-

This medium was prepared as above with haematin, prepared as described by Whittenbury (1964), added in the place of blood, to give a final concentration of 20µg/ml. The organisms were grown on nutrient agar + 2% (v/v) glycerol medium for 24 hours and then the organisms were spotted on to the above media. Production of H_2O_2 was indicated by the dark brown to black colour of the growth and the surrounding medium.

Results:-

Results (Table 21) indicated that all the plant pathogenic pseudomonads, with the exception of Ps. marginalis, failed to produce H_2O_2 from these amino acids, whereas all the non-pathogenic pseudomonads accumulated H_2O_2 from these amino acids. The reaction given by the non-pathogenic pseudomonads as well as Ps. marginalis strains was very strong and occurred after 36-48 hours of incubation. After a long time (7 or more days) of incubation some of the plant pathogenic pseudomonads produce a trace amount of H_2O_2 but this reaction is very weak compared with that produced by the non-pathogenic pseudomonads.

Table 21. The production of Hydrogen peroxide from different amino acids

Organisms tested	DL-alanine		L-glutamic acid		L-aspartic acid		L-proline		Casamino acids	
	heated blood medium	haema-tin medium	heated blood medium	haema-tin medium	heated blood medium	haema-tin medium	heated blood medium	haema-tin medium	heated blood medium	haema-tin medium
Ps. syringae (10)	0	0	0	0	0	0	0	0	0	0
Ps. mors-prunorum (9)	0	0	0	0	0	0	0	0	0	0
Ps. phaseolicola (6)	0	0	0	0	0	0	0	0	0	0
Ps. tabaci (2)	0	0	0	0	0	0	0	0	0	0
Ps. marginalis (6)	6	6	6	6	6	6	6	6	6	6
Non-pathogenic pseudomonads (20)	20	20	20	20	20	20	20	20	20	20
Xanthomonads (3)	0	0	0	0	0	0	0	0	0	0

These figures are the number of the strains which produced H_2O_2

Results also indicated that Xanthomonads do not accumulate H_2O_2 from the above amino acids. On heated-blood media a clear zone appeared round the growth of the Ps. marginalis strains and the strains of Ps. tabaci, and the Xanthomonads, this reaction could be due to the proteolytic activity of these organisms.

Similar results were also obtained in mineral salt medium containing 0.5% (w/v) glucose and 0.5% (w/v) yeast extract.

Experiment 46. The catalase activity of the organisms

The results obtained on the production of H_2O_2 from different amino acids suggested that the plant pathogenic pseudomonads, apart from Ps. marginalis, did not accumulate H_2O_2 from amino acids. This could possibly be a result of the higher catalase activity of the pathogenic organisms over the non-pathogenic pseudomonads. Rudolph & Stahmann (1964) found that the more virulent strains of Ps. phaseolicola showed higher catalase activity than the less virulent strains. It was of interest, therefore, to examine the pathogenic and non-pathogenic pseudomonads strains for their catalase activity, quantitatively.

The method:-

The organisms were grown for 24 hours on nutrient agar + 2% (v/v) glycerol, the bacteria were harvested and washed twice and resuspended in 0.01 M phosphate buffer pH 6.8. The bacterial cell suspension was adjusted to give 5 to 5.3 mg. dry weight/ml. The method of Herbert (1955) was used to determine the catalase activity of the organisms. This involved adding bacterial suspension (1 ml.) to 5 ml. phosphate buffer 0.01 M pH 6.8 which contained 200 μMole H_2O_2 in a test tube at 30°C, (in a water bath), the termination of enzyme activity was brought about after 15, 30, 45 and 60 seconds by the addition of H_2SO_4; KI and catalytic amounts of ammonium molybdate were added the free iodine released by the action of residual peroxide titrated with 0.05N $Na_2S_2O_3$ with starch as indicator.

Organisms tested:-

The following representative strains were tested:-

Ps. syringae S5 - S8

Ps. mors-prunorum D1 - N5

Ps. phaseolicola P12 - P14

Ps. marginalis 1559 - 667

Non-pathogenic pseudomonads 4A3 - CS4 - 1A1

Results:-

The results (table 22) indicated that generally there was no significant differences between the plant pathogenic and the non-pathogenic pseudomonads in their catalase activity. In some cases, the catalase activity of the non-pathogenic strains was greater than some of the pathogenic pseudomonads strains.

Great variation was found between the different strains of the same species.

Therefore the inability of the plant pathogenic pseudomonads to accumulate H_2O_2 from the different amino acids could not be due to their catalase activity.

Table 22. Percentage of H_2O_2 decomposed by different pseudomonads

Organisms tested	Time 15 sec.	30 sec.	45 sec.	60 sec.
Ps. syringae				
S5	17	28	50	61
S8	5.5	10	15.5	18.5
Ps. mors-prunorum				
D1	28.5	52.5	64.0	77.0
N5	11.4	24.3	31.0	40.0
Ps. phaseolicola				
P12	8.5	15.5	24	28.5
P14	2.5	5.5	7	11
Ps. marginalis				
667	30	48.5	62.5	75.5
1559	34	50.0	65.0	79.0
Non-pathogenic pseudomonads				
CS4	18.5	30	40	47
4A3	11	21	27	38.5
1A1	18.5	28.5	40	52

The determination of peroxidase activity of the plants

The following method was found to be very useful for the determination of the plant peroxidase. It was noticed when the plant materials were tested for their phenol oxidase activity using DOPA as substrate, that the phenol oxidase activity is very slow, whereas when H_2O_2 was added to the reaction mixture the red colour of the Dopa oxidation product developed very rapidly within 10 mins. of the addition of H_2O_2. Such a reaction did not occur when H_2O_2 was added to DOPA solution alone. This reaction of the plant peroxidases was studied further.

The following reaction mixture was used:-

2 ml. of the macerated plant materials

2 ml. of H_2O_2 0.6% (v/v)

2 ml. of DOPA 1 mg./ml.

The controls:-

Control 1

2 ml. of DOPA

2 ml. of H_2O_2

Control 2

2 ml. of the macerated plant materials

2 ml. of DOPA

2 ml. of water

The tubes were incubated at 30°C in water bath for 20 mins. The red colour developed in the tubes which contained H_2O_2. No colour developed in the control treatments.

Experiment 47. The peroxidase activity of bean plants and its change during infection with Ps. phaseolicola

The method:-

Kidney beans, variety Canadian Wonder, were used. They were grown as described before. After the first leaf had developed the plants were divided into two groups; one group was inoculated with Ps. phaseolicola strain (P12) as described before, the other group was kept as controls which were sprayed with sterile water. The stems and the leaves of 20 plants were collected before inoculation and from each group after 3, 7, 14 and 21 days of inoculation. The samples were kept in the deep freeze until the end of the experiment.

The preparation of the crude enzyme extraction:-

The samples were macerated with 0.1 M phosphate buffer pH7 for 10 mins, then the dry weight of the different samples was determined. They were then adjusted with phosphate buffer to 14 mg. dry weight in 1 ml. of the macerated plant materials.

The determination of peroxidase activity:-

The method which was described before was used. The reaction mixture was as follows:-

2 ml. of the macerated plant materials

2 ml. of H_2O_2 0.6% (v/v)

2 ml. of Dopa 1 mg./ml.

The control was:-

2 ml. of the macerated plant materials

2 ml. of Dopa 1 mg./ml.

2 ml. of water.

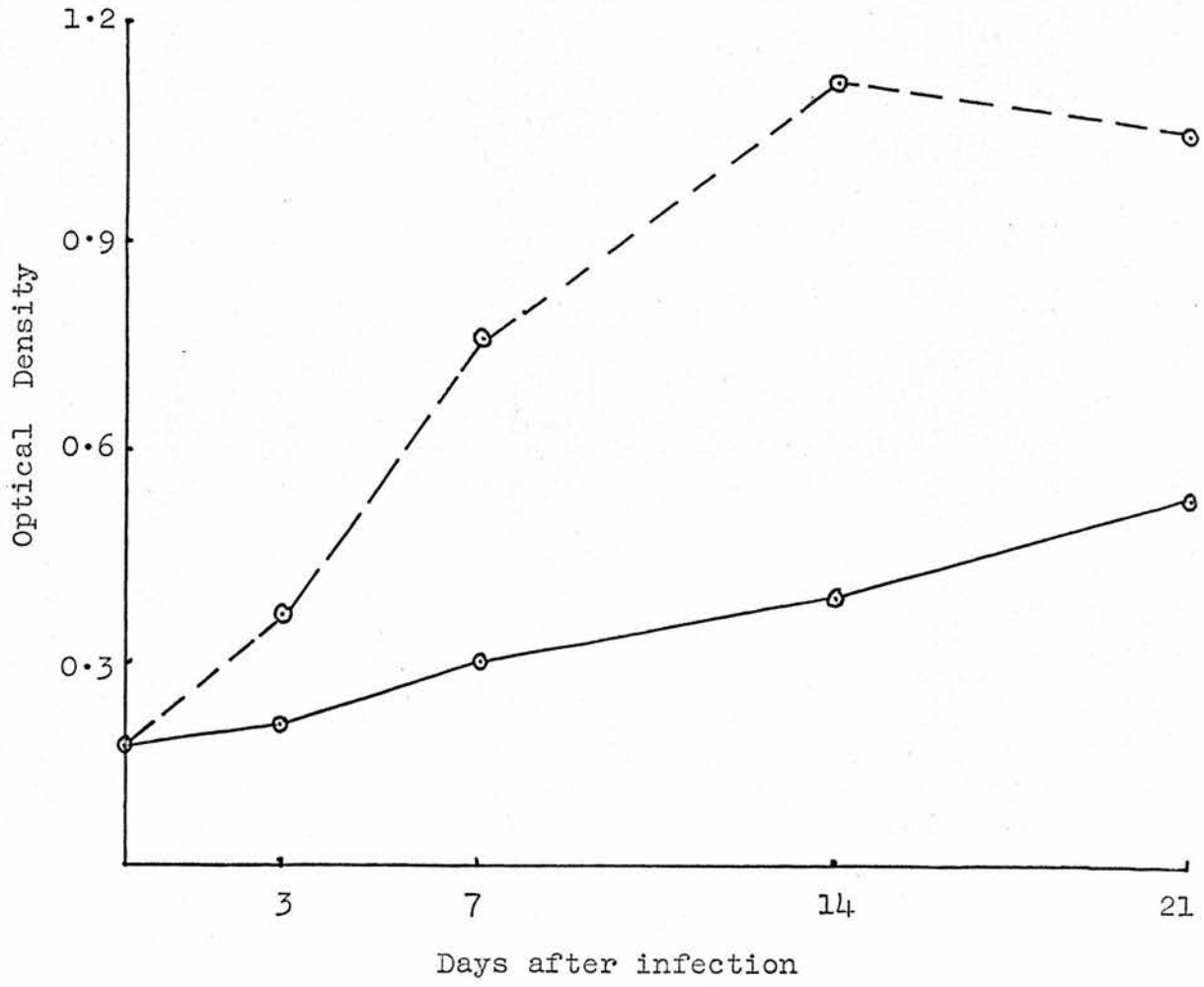
The tubes were incubated at 30°C for 20 mins. The red colour developed in the tubes which contained H_2O_2. No colour developed in the control treatments. The tubes were centrifuged for 15 mins. and the density of the colour measured colourimetrically using Unicam Sp. 1300 colourimeter (green filter No.3 was used). The colour was read against the corresponding control in the reference cell.

Results:-

Results (figure 9) indicated that the peroxidase activity increased in the diseased plants.

Results also indicated that between 14 and 21 days after the inoculation slight decreases in the peroxidase activity occurred, this could be a result of new leaves appearing which were not infected. The peroxidase activity, however, remained greater than the healthy plants. Results also indicated that the peroxidase activity of the plants increased when the age of the plants increased.

Figure 9. The peroxidase activity of healthy (——) and Ps. phaseolicola infected (— — —) plants.



Experiment 48. The peroxidase activity of bean plants infected with Ps. mors-prunorum, Ps. syringae, a non-pathogenic pseudomonad and in mechanical damaged plants

Method:-

Bean plants were grown and inoculated with Ps. mors-prunorum strain D5, and Ps. syringae strain S5 as well as a non-pathogenic strain 4A3 as described above. The mechanical damage treatment was conducted by inoculating the plants with a sterile needle every 2 days during the time of the experiment and by rubbing with sterile carborundum every 2 days. The stems and the leaves of 10 plants were collected before the inoculation and from each treatment and from the control plants after 7, 14 and 21 days after incubation.

The preparation of the crude enzyme extract and the determination of peroxidase activity was conducted as described before, the extracts were adjusted to 10 mg. dry weight. per ml.

Results:-

Results (figs. 10a & b) indicated that the peroxidase activity of the plants increased when the plants are infected with plant pathogenic pseudomonads, results also indicated that the peroxidase activity of plants inoculated with non-pathogenic pseudomonad strain 4A3 increased slightly as did that of mechanical damaged, but were shown not to be infected. The increase in peroxidase activity of the plant inoculated with the non-pathogenic strain could be a result of mechanical damage, no disease symptoms appeared. However as both these increases were slight compared with the control, they may not be significant.

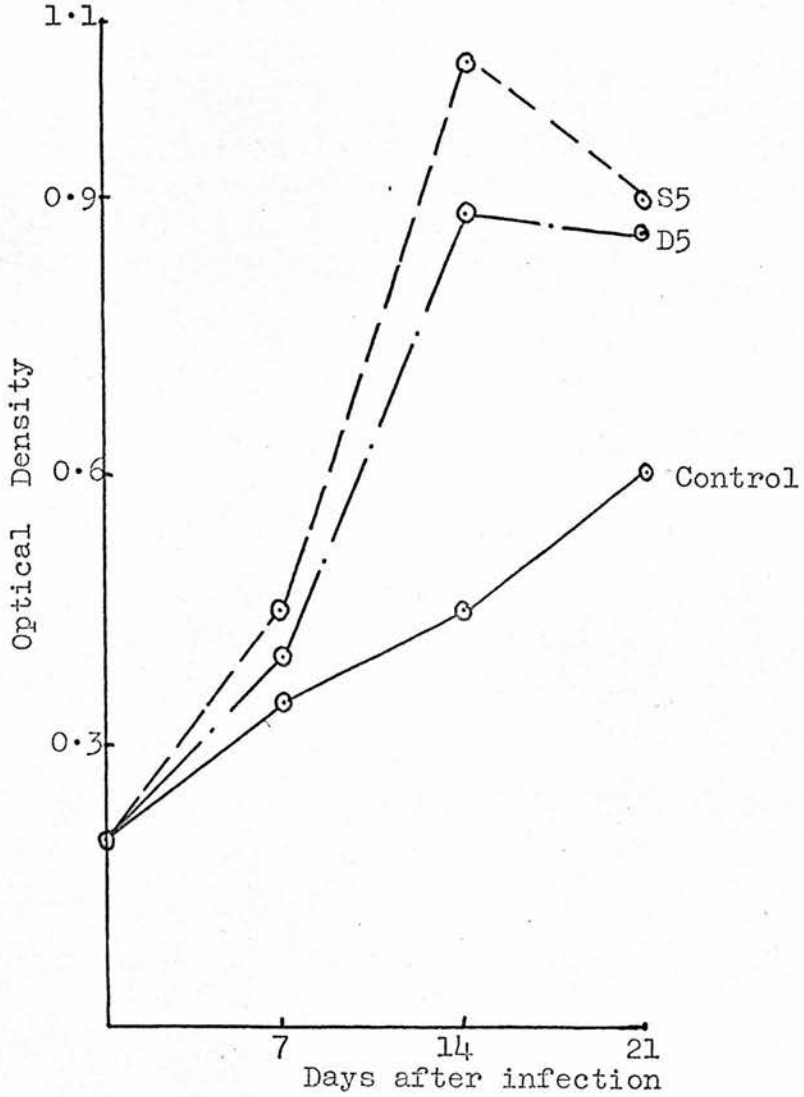


Figure 10a. The peroxidase activity of bean plants infected with Ps. syringae strain S5 and Ps. mors-prunorum strain D5.

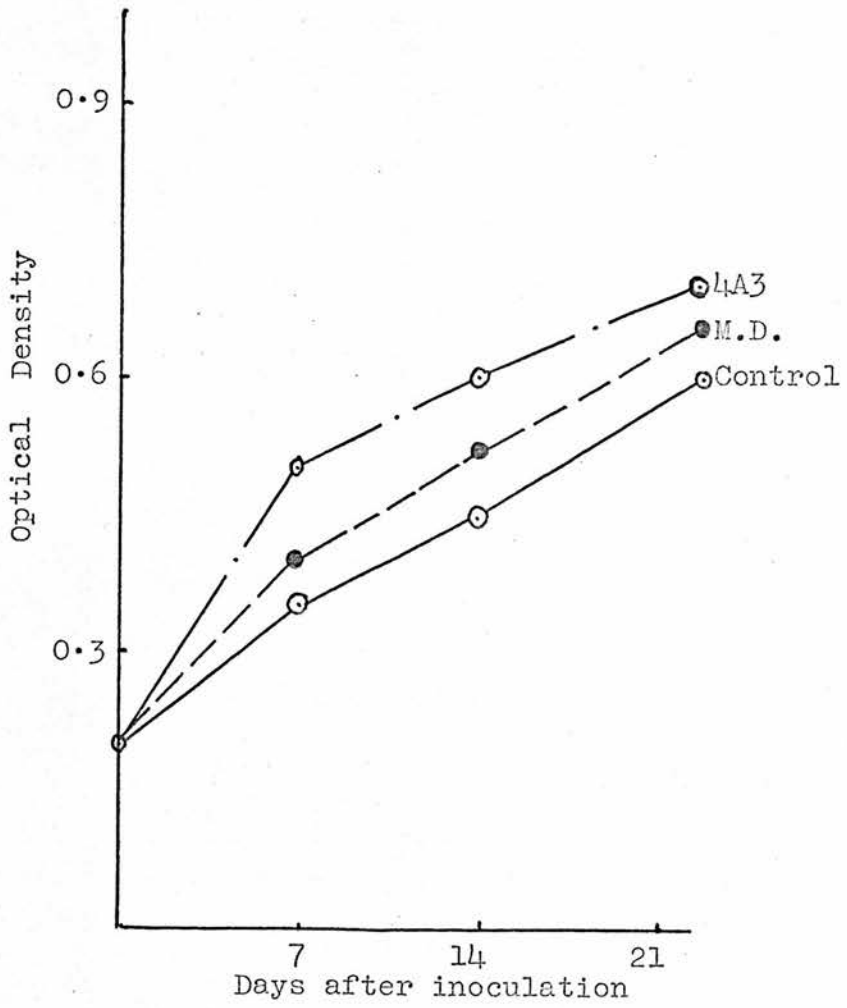


Figure 10b. The peroxidase activity of bean plants inoculated with non-pathogenic pseudomonas strain 4A3 and in mechanical damaged plants M.D.

Experiment 49. The peroxidase activity of healthy lettuce plants and plants infected with Ps. marginalis

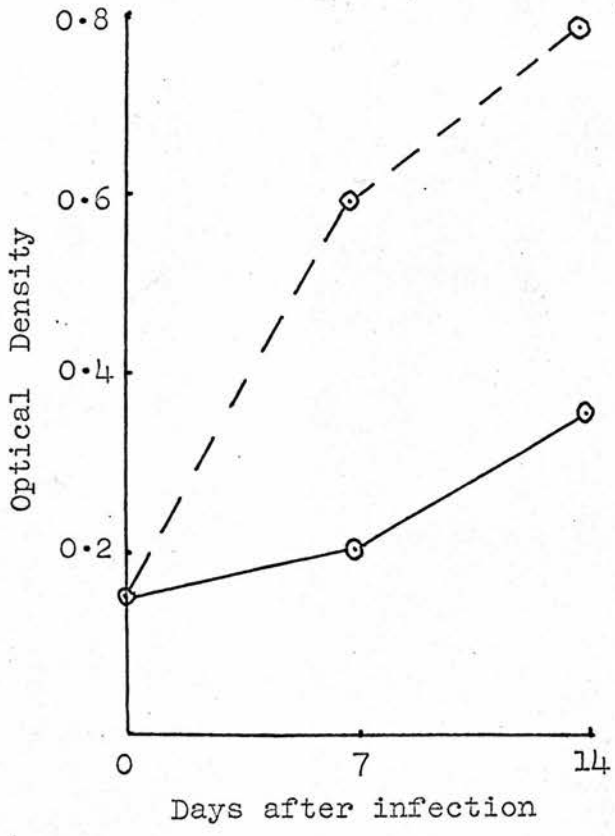
Lettuce seedlings, variety Paris White, were planted in pots containing greenhouse compost and after 2 weeks from the date of the transplanting, the plants were divided into two groups. One of them was inoculated with Ps. marginalis 1559 strain cell suspension made in sterile water, the other group (control) was inoculated with sterile water. Samples were collected before the inoculation and 7 and 14 days after inoculation.

The preparation of the crude enzyme extract and the determination of peroxidase activity was as described before. The different samples were adjusted to 10 mg. d.w/ml.

Results:-

Results (figure 11) indicated that in the diseased plants there was great increase in the peroxidase activity of the plants.

Figure 11. The peroxidase activity of healthy (——) and Ps. marginalis infected (- - - -) lettuce plants.



Experiment 50. The catalase activity of healthy bean plants and plants infected with Ps. phaseolicola P12 strain

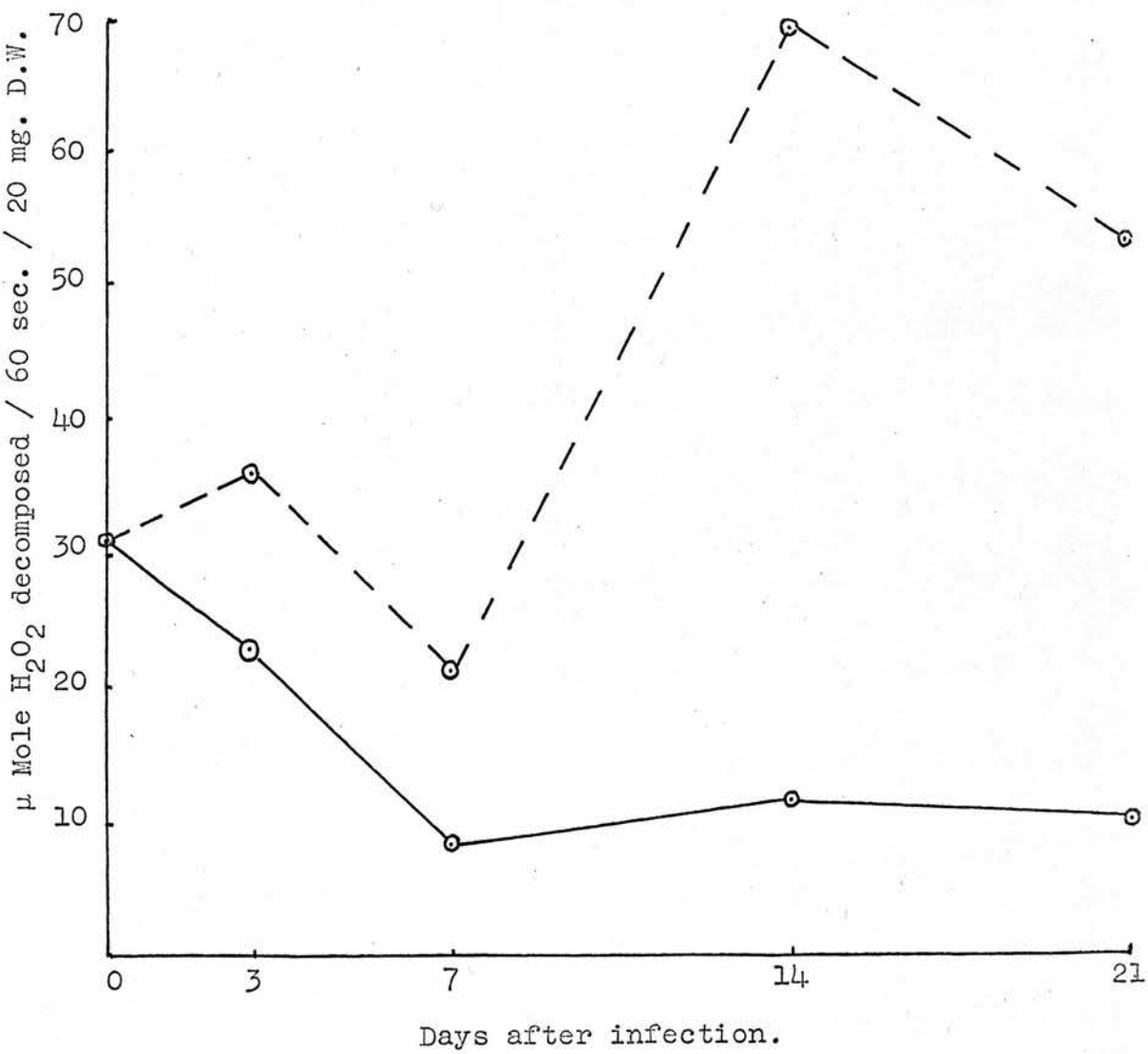
The method of growing the plants and method of inoculation were as described before.

Samples of 20 plants were collected at the time of the inoculation and 3, 7, 14 and 21 days after infection. The crude enzyme extract was obtained as described before. The determination of catalase activity was by the method of Herbert (1955), to 200 μmole H_2O_2 in 5 ml. phosphate buffer 0.01 M pH 6.8. 1 ml. of the crude extraction was added and after 60 seconds at 30°C in water bath, the reaction stopped by adding H_2SO_4. The residual H_2O_2 was determined by titration with $Na_2S_2O_3$ as described before.

Results:-

Results (Fig. 12) indicated that in the healthy plants, the catalase activity decreased generally as the age of the plant increased. Results also showed that a great increase in the catalase activity of the diseased plants over the control healthy plants, the maximum was after 14 days of infection.

Figure 12. The catalase activity of healthy (——) and Ps. phaseolicola infected (- - -) plants.



Experiment 51. Electrophoretic study of healthy bean leaves and Ps. phaseolicola infected leaves

The method:-

Bean plants, variety Canadian Wonder, were used in this experiment. They were grown as indicated above and inoculated with Ps. phaseolicola strain P12. They were inoculated when the first trifoliate leaf developed and kept in the greenhouse under polythene bags for 5 days. The diseased leaves were harvested 2 weeks after inoculation; leaves from the control healthy plants were collected at the same time.

The preparation of the crude extract:-

The diseased leaves of 12 plants, and the healthy leaves, were macerated for 15 mins. with 100 mls. of tris-citrate buffer pH 8.6 (Half the concentration used by Poulik, 1957). The macerated plant materials were then treated with the ultrasonic disintigrator for 10 mins. in an ice bath and then centrifuged at 20,000 g. for 30 mins. at 5°C. The supernatent fluid was then concentrated by polyethyleneglycol in the cold as described by Kohn (1959) to 20 mls. Finally they were dialysed against the same buffer for 24 hours. Starch gel was used in this study, and the method described by Lund (1965) was used. The electrophoresis was continued until the brown line marking the line of discontinuity between the buffers migrates through the gel from cathode to anode during electrophoresis for 10 cm. past the sample inserts. Under these conditions the results proved to be highly reproducible. The catalase and peroxidase bands were

described by a numerical system similar to the R_f value system used in chromatography. The brown line was given as "E_f value" of 100 and each band was given an E_f value which, under the conditions described, was its distance in m m from its insert.

The methods for the detection of the enzymes:-

The detection of catalase:-

The following method was found to be very useful for the detection of catalases, and was developed by Dr, R. Whittenbury (personal communication). An 0.3% (w/v) solution p-aminophenol is added to the gel slices and after 5 mins., H_2O_2 (2 vol.) is added. Within 15 mins. the catalase positions appear as dark brown bands which are also marked by oxygen bubbles trapped in the gel released by catalase from the H_2O_2. The oxygen is assumed to have oxidized the p-aminophenol to a dark brown quinone compound. This method was found to be superior to the KI, H_2O_2 method described by Baillie & Norris (1963) in many aspects.

The detection of peroxidases:-

The following method was found to be very useful for the detection of peroxidases and was developed by Dr. R. Whittenbury (personal communication).

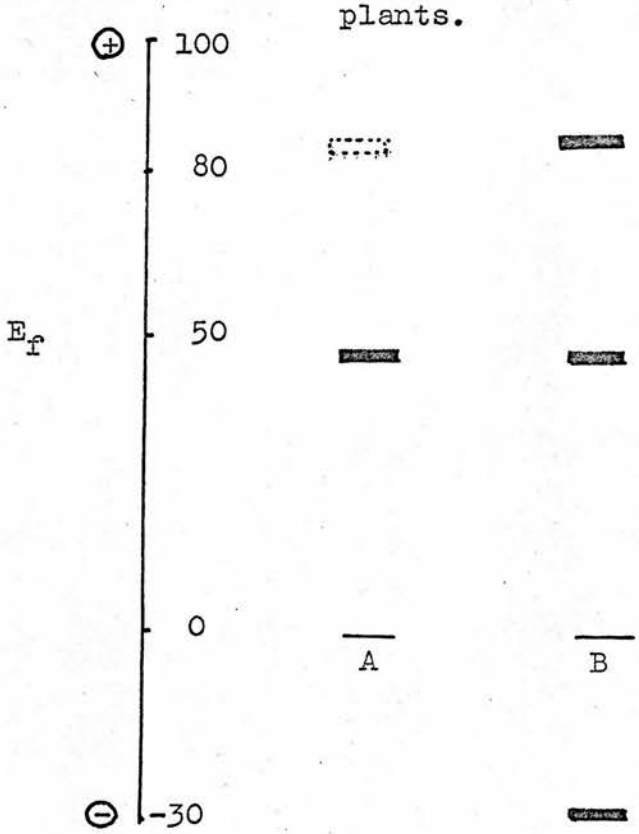
o-Dianisidine 0.2 gm. was dissolved in 1 ml. acetic acid, diluted to 200 ml., and added to the gel slices, followed by the addition of H_2O_2(2 vol.). The peroxidases appear as green bands which rapidly changed to a dark brown colour on the

further oxidation of o-dianisidine.

Results:-

The results (Figs. 13 & 14) indicated that the healthy plants did not contain any catalase, whereas the diseased plant showed a catalase band of bacterial origin since the E_f of that band is identical with that of the organism as will be seen later. Results of the peroxidase analysis indicated a marked increase in the peroxidase second band at $E_f 83$ which was very weak in the healthy plant materials.

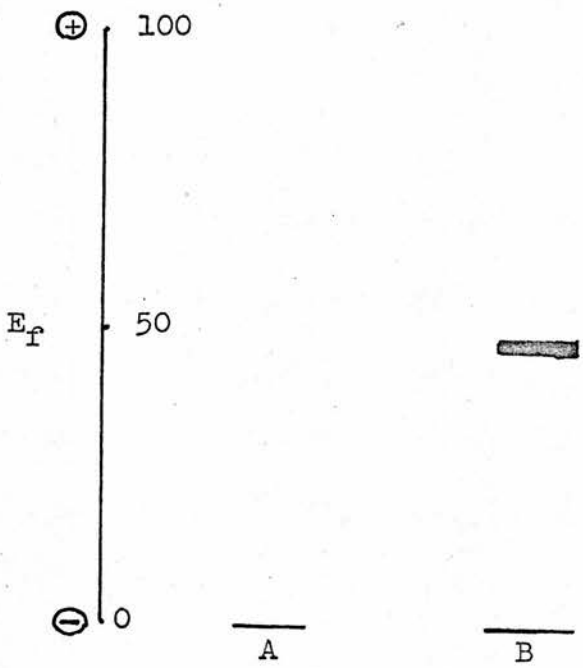
Figure 13. The peroxidase pattern of healthy and Ps. phaseolicola infected bean plants.



A = Healthy bean plants.

B = Diseased bean plants.

Figure 14. The catalase pattern of healthy and Ps. phaseolicola infected bean plants.



A = Healthy bean plants.

B = Infected bean plants.

Discussion:-

Phenolic compounds can be oxidised to quinones by peroxidases in the presence of H_2O_2. A number of authors have found a correlation between the activity of phenol oxidizing enzymes in healthy plants and their resistance to microorganisms (Farkas & Kiraly, 1962). Umaerus (1959) reported that the peroxidase activity of Solanum tuberosum was found to be positively correlated with field resistance to phytophthora infestans. He also reported that the youngest leaves, the more susceptible to infection, had less peroxidase than the old leaves. Peroxidases was also found to participate in the vascular browning of banana roots infected with fusarium (Mace, 1964). Results described above indicated that non-pathogenic pseudomonads as well as Ps. marginalis strains were able to accumulate H_2O_2 from different amino acids, whereas the other plant pathogenic pseudomonads did not accumulate H_2O_2. The production of free H_2O_2 by the non-pathogenic pseudomonads but not by pathogens except Ps. marginalis, may be another factor in their failure to infect plants as the H_2O_2 is a substrate for plant peroxidases which may then peroxidise phenols to quinones preventing non-pathogenic pseudomonads from establishing themselves in the plants. The results described above also indicated that the peroxidase activity of bean and lettuce plants was increased when the age of the plant increased. Their peroxidase content also increased when they were infected with

pseudomonads. The peroxidase activity of bean plants also increased, although slightly upon mechanical damage. These latter results were in agreement with those reported by many workers (Farkas & Kiraly, 1962). Deverall & Walker (1963) found that healthy bean plants resistant to halo blight had a higher oxygen uptake than those of healthy susceptible plants. The tentative suggestion was made that the oxygen uptake was caused by an oxidative peroxidation. The hydrogen peroxide destroying activity of the bean plants was found to decrease when the age of the plant increased whereas it was increased when the plants were infected with Ps. phaseolicola. This was due to the catalase activity of the organism as the electrophoretic study indicated. The results obtained from the analysis of the catalase pattern of healthy and Ps. phaseolicola infected bean plants were in agreement with those reported by Rudolph & Stahmann (1964). The role of the bacterial catalases in the establishment of the plant pathogenic pseudomonads could be important, in an attempt by the organism to destroy H_2O_2 before plant peroxidase activity oxidises phenols.

Farkas & Kiraly (1958) reported that the catalase has certainly an important role in some host-parasite relations. However they failed to detect any significant difference between the catalase activity of highly resistant and susceptible wheat varieties to rust disease.

C. The β-glucosidase activity of the organisms and of the plants

Phenolic compounds occur in the plants as free compounds or as glucosides (Harborne, 1964).

The β-glucosidase enzyme is not substrate specific and will hydrolyse most phenols β-D-glucosides. It was of interest, therefore, to study the activity of that enzyme in the organisms and in the plants, and its possible role in the plant defence mechanism against bacterial infection.

The β-glucosidase activity of the organisms.

Experiment 52a. The hydrolysis of aesculin

The medium used in this study was the same as described by Sneath (1956). The medium was tested also in the presence of 0.5% (w/v), 1% (w/v) and 2% (w/v) glucose, the medium was adjusted to pH7 and autoclaved at 121° for 15 mins. The organisms were grown for 2 days on glycerol nutrient agar and the plates inoculated and incubated at 27°C for 10 days. The blackening of the medium around the mass of growth (due to the liberation of the phenol which reacted with ferric citrate to the black colour) indicated that aesculin was hydrolysed.

Results:-

Table 23 showed the results after 7 days incubation and indicated that Ps. syringae strains hydrolyse aesculin whereas Ps. mors-prunorum strains do not, except for strain P47. However, when glucose was added (at 0.5% w/v) another 2 strains of

Table 23. The hydrolysis of aesculin by different pseudomonads

Organisms tested	Sneath medium	medium + 0.5% glucose	medium + 1% glucose	medium + 2% glucose
Ps. syringae				
S5	+	++	+	-
S8	+	++	+	-
S20	+	++	+	-
S36	+	++	+	-
W1	+	++	+	-
W183	+	++	+	-
G2	+	++	+	-
Ps. mors-prunorum				
D1	-	-	-	-
D2	-	-	-	-
D5	-	-	-	-
D16	-	+	-	-
N5	-	-	-	-
C9	-	+	-	-
C22	-	-	-	-
C46	-	-	-	-
P47	+	++	+	-
Ps. phaseolicola				
P12	-	-	-	-
P13	-	-	-	-
P14	-	-	-	-
P16	-	-	-	-
P18	-	-	-	-
NZ41	-	-	-	-
Ps. tabaci				
M44	+	++	-	-
M45	+	++	(+)	-
Ps. marginalis				
667	(+)	++	++	+
1558	-	++	-	-
1559	-	++	++	+
1604	-	++	-	-
Non-pathogenic pseudomonads				
20 strains	3 ++	3 ++	3 ++	3 ++

Ps. mors-prunorum were able to hydrolyse aesculin, and the other strains gave doubtful results. Ps. marginalis strains hydrolysed aesculin only in the presence of 0.5% (w/v) glucose. Glucose at the higher concentrations, in most cases, inhibited the hydrolysis of aesculin. Results also indicated that of the 20 non-pathogenic pseudomonad strains, only 3 hydrolysed aesculin. These strains are Pt2 - Bn3 and 7A9 which were considered to be atypical pseudomonads.

Experiment 52b. The hydrolysis of arbutin

The medium used for this study was the same as described by Crosse & Garrett (1963) and was used as an agar medium, the same organisms were inoculated into that medium as described above.

Results:-

Results (Table 24) indicated that Ps. syringae strains but not Ps. mors-prunorum strains with the exception of P47 strain are able to hydrolyse arbutin. Of the non-pathogenic pseudomonads strains tested (20 strains) only 3 were able to hydrolyse arbutin and they were the same strains described in the previous experiment.

Table 24. The hydrolysis of arbutin by different pseudomonads

Organisms tested	arbutin hydrolysis	Organisms tested	arbutin hydrolysis
Ps. syringae		Ps. tabaci	
S5	++	M44	++
S8	++	M45	++
S20	++	Ps. marginalis	
S36	++	667	+
W1	++	1558	+
W183	++	1559	+
G2	++	1604	(+)
Ps. mors-prunorum		Non-pathogenic pseudomonads	
D1	-	20 strains	3 +
D2	-		
D5	-		
D16	-		
N5	-		
C9	-		
C22	-		
C46	-		
P47	++		
Ps. phaseolicola			
P12	-		
P13	-		
P14	-		
P16	-		
P18	-		
NZ41	-		

The β-glucosidase activity of plant materials

The following method was developed and found to be useful for the determination of B-glucosidase activity of plant materials. It depends on the incubation of the crude plant extracts with aesculin in the presence of ferric citrate for 3 hours at 30°C in water bath. The B-glucosidase activity of the plants released phenol from aesculin which gives a black colour in the presence of ferric citrate. The reaction could be followed colourimetrically against the control, the plant extract which contained ferric citrate but no aesculin. No black colour developed in the mixtures which had no ferric citrate.

Preliminary work indicated that the reaction was inhibited if 1:5-glucono-lactone (β-glucosidase inhibitor) was added to the reaction mixture.

Experiment 53. The β-glucosidase activity of bean plants and its change during infection with Ps. phaseolicola

The bean plants used in this study were the variety as described before. The plants were grown in the greenhouse and inoculated with Ps. phaseolicola P12 strain as described before. Samples of 20 plants were collected at the time of inoculation (when the first trifoliate leaf developed) and 3, 7, 14 and 21 days after inoculation. Samples of the control plants were also collected at each date. The preparation of the crude extract was as described before. All the samples were adjusted to give

14 mg. dry weight in 1 ml. of the crude preparation.

The reaction mixture was as follows:-

4 ml. crude extract

1 ml. aesculin 0.5% (w/v)

1 ml. ferric citrate final concentration 0.1% (w/v)

The control tubes contained 4 ml. of the plant extract, 1 ml. ferric citrate and 1 ml. of buffer.

After 5 hours incubation at 30°C in a water bath, the tubes were centrifuged. The density of the black colour, which is related to the amount of phenol released, was measured by Unicam Sp. 1300 colourimeter. Filter No.3 (green filter) was used against the corresponding control reaction mixture.

Results:-

Results (Figure 15) indicated that the B-glucosidase activity of the plant, which is a mechanism responsible for the liberation of phenols from the glucosides, is increased when the plants were infected with Ps. phaseolicola.

The results also indicated that increase of the plant's age resulted in increase in the B-glucosidase activity of the plants.

Experiment 54. The β-glucosidase activity of bean plants infected with Ps. mors-prunorum, Ps. syringae, non-pathogenic pseudomonads and in mechanically damaged plants

The method:-

Bean plants were grown and inoculated with Ps. mors-prunorum D5 strain, and Ps. syringae S5 strain as well as non-pathogenic strain 4A3 as described before. The mechanical damage treatment was as described before. The stems and the leaves of 10 plants were collected at the time of inoculation (when the first leaf had developed) and from each treatment and the control plants 7, 14 and 21 days after the inoculation.

The preparation of the crude enzyme preparation was as described before. The different preparations were adjusted to 10 mg. dry weight in 1 ml. of the preparation. The method used for the determination of the β-glucosidase activity was the same as described before.

Results:-

Results (Figs. 16a & b) indicated that the β-glucosidase activity of the plants increased upon the infection with Ps. mors-prunorum and Ps. syringae. The increase in β-glucosidase activity of the plants infected with Ps. syringae was greater than that of Ps. mors-prunorum infected plants. The β-glucosidase activity of the plants increased slightly over the control upon mechanical damage. The slight increase of β-glucosidase activity of plants infected with non-pathogenic pseudomonad strain 4A3 could also be explained by the mechanical damage effect of inoculation. However the significance of the slight increase of β-glucosidase with damaged and non-pathogen infected plants is doubtful.

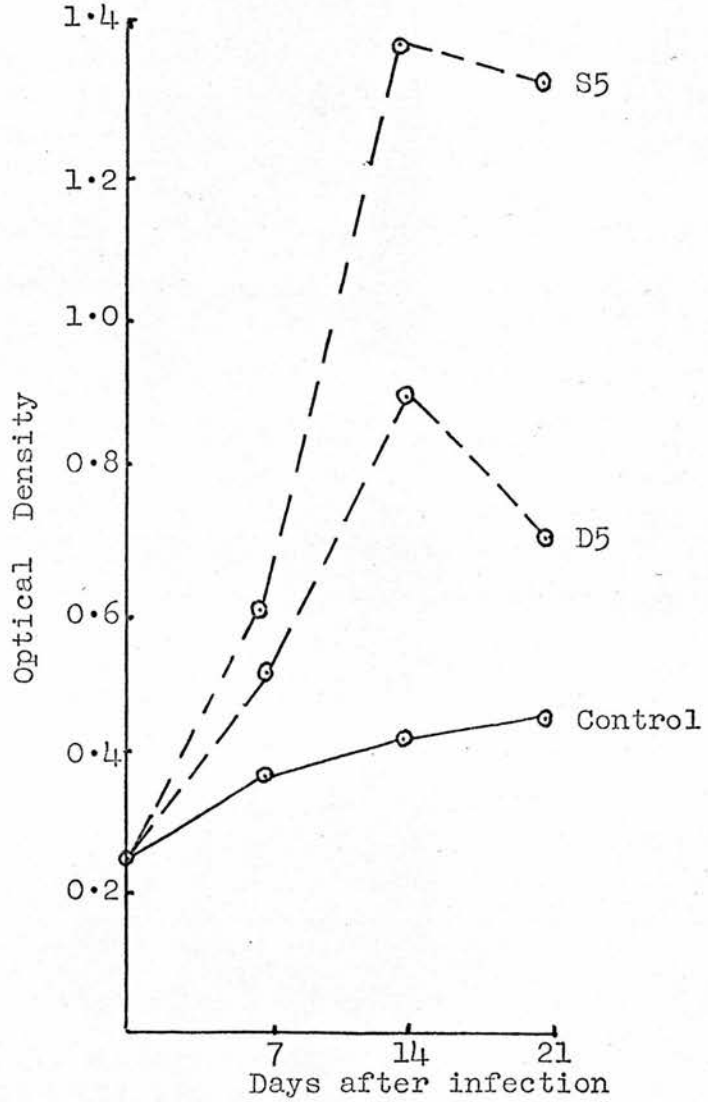


Figure 16a. B-glucosidase activity of bean plants infected with Ps. syringae strain S5 and Ps. mors-prunorum strain D5.

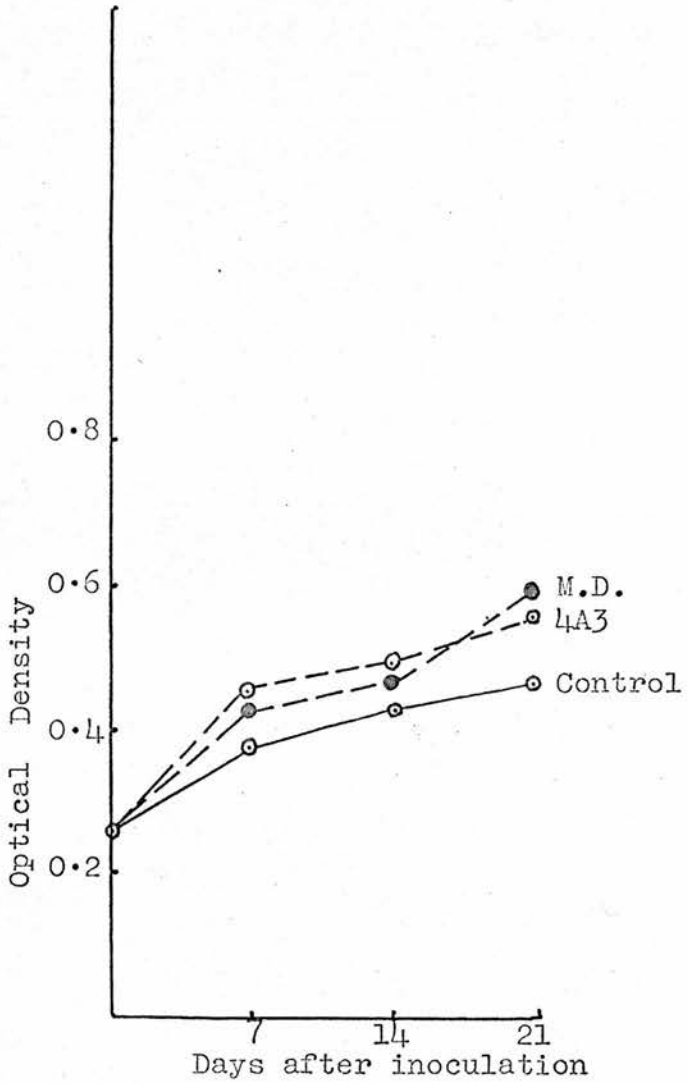


Figure 16b. B-glucosidase activity of bean plants inoculated with non-pathogenic pseudomonas strain 4A3 and in mechanical damaged plants M.D.

Experiment 55. The β-glucosidase activity of healthy lettuce plants and plants infected with Ps. marginalis

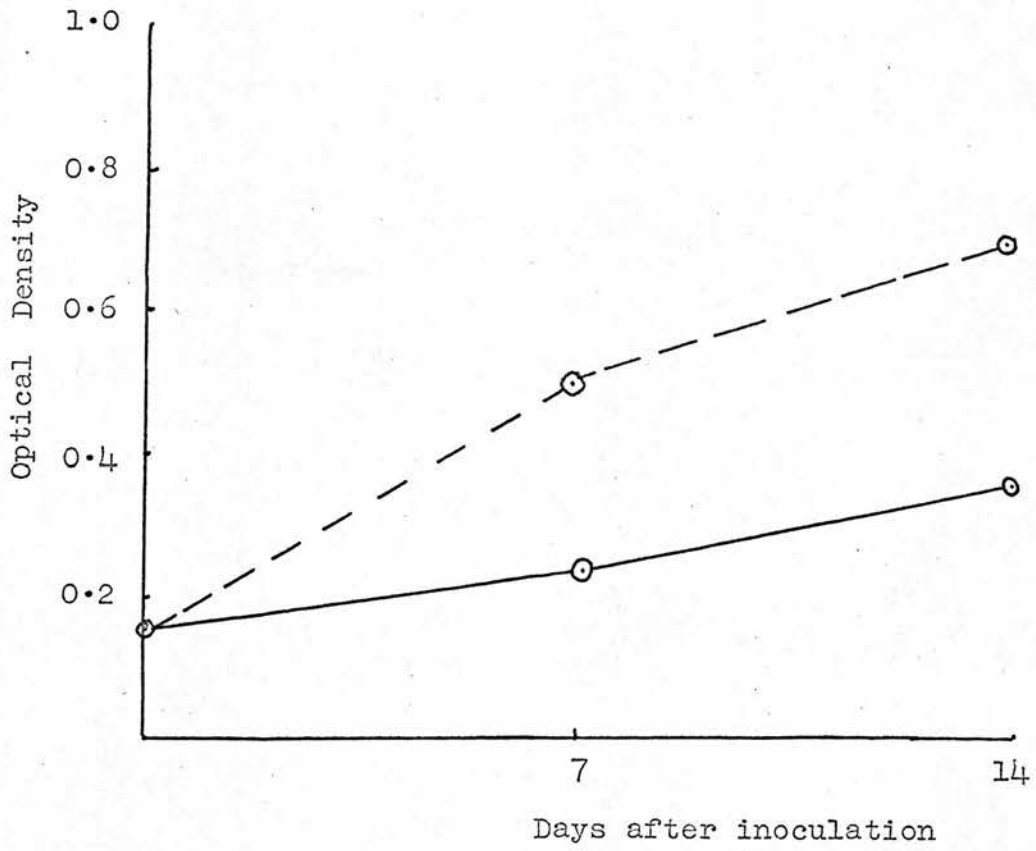
Lettuce seedlings, variety Paris White, were planted as described before. After 2 weeks from the date of transplanting, the plants were inoculated with Ps. marginalis 1559 strain. Samples were collected before the inoculation and 7 and 14 days afterwards.

The preparation of the plant extracts was conducted as described before, the enzyme preparations were adjusted to 10 mg. dry weight in 1 ml. of the preparation. The method used for the determination of β-glucosidase activity was the same as described before.

Results:-

Results (Fig. 17) indicated that the β-glucosidase activity of the lettuce plants also increased when they were infected with Ps. marginalis (1559 strain).

Figure 17. B-glucosidase activity of healthy (———) and Ps. marginalis infected (— — —) lettuce plants.



Discussion:-

Results described above indicated that apart from the 3 atypical non-pathogenic psuedomonads strains, all the other non-pathogenic pseudomonads had no β-glucosidase activity and in that, they are different from Ps. marginalis strains which had the B-glucosidase enzyme. Hildebrand & Schroth (1964) also found that Ps. fluorescens exhibited no β-glucosidase activity. Crosse & Garrett (1963) and Garrett et al. (1966), reported that Ps. syringae was able to hydrolyse aesculin and arbutin whereas Ps. mors-prunorum did not exhibit B-glucosidase activity. The results described above indicated that on the addition of glucose to aesculin medium, some of the Ps. mors-prunorum strains were able to hydrolyse the aesculin, although the reaction given by these strains was weaker than that given by Ps. syringae.

The results described above also indicated that the β-glucosidase activity of bean plants increased with the age of the plants and slightly upon mechanical damage. It also increased when the plants were infected with pathogenic pseudomonads. These results could explain the results reported in this study concerned with the increase of phenolic compounds by increase of the plant age or by bacterial infection.

The B-glucosidase activity of the plants could be in part, an explanation of the results of many workers about the accumulation of phenolic compounds in the diseased tissue and upon injury (Farkas & Kiraly, 1962), Cruickshank & Perrin (1964), Johnson & Schaal (1957). The increase of the β-glucosidase activity of the diseased plants compared with healthy plants has not been reported before.

PART IV

The Toxicity of some metals against some Gram-negative bacteria with special reference to pseudomonads

The toxicity of some metals against some Gram negative bacteria with special reference to pseudomonads

Introduction:-

The study of the toxicity of metals against the Gram negative bacteria has received little attention. It is well known that certain cations are toxic to the growth of micro-organisms at high concentrations. Hotchkiss (1932) studied the effect of certain cations upon the growth of Escherichia coli, and divided the metals into two groups based on their toxicity against that organism, a less toxic group, which included Mn^{++}, Ba^{++}, Ca^{++}, Mg^{++}, Sr^{++}, Li^{+}, NH_4^{+}, Na^{+} and K^{+}, a more toxic group including Zn^{++}, Ni^{++}, G^{++}, Fe^{++}, Fe^{+++} and Cu^{++}. Some of these metals were stimulatory at very low concentrations. The actual inhibitory concentration of the metals depended upon many factors especially the composition of medium (Schade, 1949), Schade studied the toxicity of cobalt against Proteus vulgaris. The pH of the medium was also found by Macleod (1954) to affect metal toxicity; he examined the toxicity of Li^{++} and Zn^{++} against lactic acid bacteria and observed that the toxicity of these metals was markedly decreased when the pH of the medium was reduced from pH 6.0 to 5.0 in the case of Lactobacillus arabinosus and Lactobacillus pentosus. The same results were obtained for the toxicity of Li^{++} for Leuconostoc mesenteroides. In contrast, however, the inhibitory action of Zn^{++} for L-mesenteroides is increased when the pH of the medium is reduced.

It has been found that the addition of Mn^{++} to nutrient broth and other complex media stimulates the sporulation but not the growth of many species of the genus Bacillus. The requirement of Mn^{++} by these organisms is specific and cannot be replaced by the addition of other metals tested. Charney, Fisher & Hegarty (1951), Curran & Evans (1954). Casida & Santoro (1961) also found that the growth of several soil sporeforming bacilli and some other soil organisms including Pseudomonas aeruginosa, Rhizobium meliloti, Agrobacterium tumefaciens, Agrobacterium radiobacter, Erwinia tracheiphila and Sarcina ureae, was stimulated by manganese when these organisms were grown on complex agar media. Weinberg (1956) confirmed the requirement of Mn^{++} for sporulation in Bacillus subtilis and also found that Mn^{++} reversed the toxicity of oxy-chloro- and tetracycline slightly in B. subtilis. More recently the same author Weinberg (1964) found that the concentration of manganese required was in excess of that required for normal vegetative growth by Bacillus sp. for the synthesis of some other metabolites and structures. Schade (1949) found that cobalt was toxic against both aerobic and anaerobic Gram-positive and Gram-negative bacteria. Inhibitory concentrations of cobalt ranged from 1 to 100 PPM. Of the 17 amino acids, he tested, only histidine and cysteine were capable of overcoming the growth inhibition of Proteus vulgaris by cobalt. McIntire, Riker & Peterson (1941) studied the role of certain metals in the nutrition of Agrobacterium tumefaciens, a plant pathogenic organism,

and found that Fe^{++}, Mn^{++} and Zn^{++} were important elements in its nutrition. However, they did not study the tolerance of Agrobacterium tumefaciens to these metals, but did find that Mn^{++} had a very interesting effect in that Mn^{++} increased both the rate of sugar fermentation and the amount of sugar converted to gum and cells. MacLeod & Snell (1950) found that Zn^{++} was toxic to Lactobacillus arabinosus, and that toxicity was relieved completely by Mn^{++}. They also found that Mg^{++}, Ca^{++} and Sr^{++} counteracted the inhibitory effect of Zn^{++} on Lactobacillus arabinosus and Lactobacillus pentosus.

Another example of ion antagonism in microorganisms is in the work of Abelson & Aldous (1950) who found that the toxicity of Ni^{++}, Co^{++}, Cd^{++}, Zn^{++} and Mn^{++} on E. coli was markedly lowered in the presence of an excess of magnesium. The toxicity of Ni^{++} and Co^{++} was similarly diminished by Mg^{++} in the case of Aerobacter aerogenes, Torulopsis utilis and Aspergillus niger.

A few comparisons have been made of the requirement for and tolerance of metallic ions of virulent and avirulent bacterial strains, Higuchi, Kupferberg & Smith (1959) found that virulent strains of Pasteurella pestis, but not the avirulent strains, required 2×10^{-3} to 4×10^{-3} M Ca^{++}, Sr^{++} or Zn^{++} for invitro growth at $37^{o}C$. However, reduction of the concentration of Mg^{++} in the growing medium from 20×10^{-3} to 2.5×10^{-3} M resulted in a decreased need for Ca^{++} by the virulent strains and an increased Ca^{++} requirement by the avirulent strains.

The work of Firshein (1962) with pneumococci showed that

Mn^{++} stimulated the multiplication rate of virulent strains, produced in cases an inhibitory effect on avirulent strains. He related the stimulatory effect of Mn^{++} to the enhancement of respiration of the virulent strains, whereas the toxic effect of Mn^{++} on multiplication of avirulent pneumococci oppositely involved a depressing effect on respiration. A contradictory effect of Mn^{++} was found in Lancefield Group A streptococci by Firshein & Zimmerman (1964). In their studies they found that the virulent streptococci showed a depressed rate and extent of multiplication in the presence of Mn^{++}. The same authors Firshein & Zimmerman (1964) found that in this virulent strain of Lancefield Group A beta haemolytic streptococci (Streptococcus pyogenes) that Mn^{++} depressed both nucleic and protein synthesis whereas in the avirulent strains only nucleic acid synthesis was depressed and protein synthesis largely unaffected. They also found that the oxidation of glucose was depressed below control values by Mn^{++} to a much greater extent in virulent strains than in avirulent strains. Studies on Rhizobium, a genus probably related to some plant pathogenic bacteria, have been carried out by Singer (1964) who studied the toxicity of Cu^{++}, Ni^{++}, Al^{+++} and Co^{++} on effective and ineffective strains of Rhizobium trifolii. He found that, in general, the effective rhizobia were no more susceptible to heavy metals toxicities than were the ineffective strains, although he did find that the effective rhizobia were more susceptible to Mn^{++} toxicity than were the ineffective strains. As his results were taken from an

examination of only a few strains, it remains to be elucidated in how far one could generalize from such a conclusion.

There are no data concerning the differential toxicity of heavy metals against the plant pathogenic bacteria, although it is general practice to spray plants with Cu^{++} and other metals in different forms to reduce the incidence of funal and bacterial diseases on plants.

It was of interest therefore to study the toxicity of heavy metals against pseudomonads of both pathogenic and non-pathogenic variety. Other organisms belonging to other genera, especially the plant pathogenic genera, were also examined for a comparative study.

Materials and methods:-

The basal medium used in this study was found to support the growth of all the strains examined and has the following composition:- (W/v)

0.5% glucose - 0.008% K_2HPO_4 - 0.002% KH_2PO_4

0.02% $MgSO_4$ - 0.1% yeast extract, the pH of the medium was adjusted to pH7 and the medium in 100 mls. amounts was autoclaved at 121° for 15 mins. To 99 ml. of the medium, 1 ml. of the metal solution, which had been sterilized by autoclaving, was added aseptically to give the required final concentration. The complete medium was tubed aseptically in 5 ml. amount in $\frac{5}{8}$" sterile test tubes.

The inoculum:- All the organisms were grown for 24 hours on nutrient agar + 2% glycerol. Slightly turbid suspensions were prepared in sterile distilled water, Pasteur pipettes were used and one drop of the bacterial suspensions was added to each tube. The tubes were incubated at 27°C and observed for growth.

The metals used in this study were as follows:-

1. Zinc Zn^{++} as $ZnSO_4$, $7H_2O$
2. Manganese Mn^{++} as $MnSO_4$, $4H_2O$
3. Copper Cu^{++} as $CuSO_4$, $5H_2O$
4. Cobalt Co^{++} as $CoSO_4$, $7H_2O$
5. Nickel Ni^{++} as $NiSO_4$, $7H_2O$

Experiment 56. The toxicity of some metals against different pseudomonads and other Gram-negative bacteria

The following representative strains were tested:-

I. Plant pathogenic pseudomonas

NZ1 - NZ10 - P6 - NZ41 - M45 - PsP3

II. Non-pathogenic pseudomonads

CS4 - 4A3 - Pt2 - 3D13 - Lk7 - Ps73

III. Achromobacter

7A14
Cb11

IV. Alcaligenes

CS8
CS11

V. Agrobacterium

Lk11
ZM1

The toxicity of the metals was studied at the following concentrations after preliminary work indicated the significant ranges of concentration.

Zinc 0.2 m.g.e/L. Zn^{++} - 0.1 m.g.e./L and 0.05 m.g.e./L.

Copper 0.1 m.g.e./L. Cu^{++} - 0.2 m.g.e./L and 0.3 m.g.e./L.

Cobalt 0.04 m.g.e./L. Co^{++} - 0.06 m.g.e./L and 0.08 m.g.e./L.

Nickel 0.2 m.g.e./L. Ni^{++} - 0.4 m.g.e./L and 0.6 m.g.e./L.

Manganese 4 - 6 - 8 - 10 - 20 and 30 m.g.e./L Mn^{++}

Results:- (Table 25)

There were no significant differences between the plant pathogenic and the non-pathogenic pseudomonads in their ability

Table 25. The toxicity of some metals against some Gram-negative bacteria.

Organism tested	Zn^{++} m.g.e./L			Cu^{++} m.g.e./L			Co^{++} m.g.e./L			Ni^{++} m.g.e./L		
	0.05	0.1	0.2	0.1	0.2	0.3	0.04	0.06	0.08	0.2	0.4	0.6
Plant pathogenic pseudomonads												
NZ1	+ 2	-	-	+ 2	-	-	-	-	-	-	-	-
NZ10	+ 2	+ 2	-	+ 2	-	-	-	-	-	+ 3	-	-
P6	+ 3	-	-	+ 1	-	-	-	-	-	-	-	-
NZ41	-	-	-	-	-	-	-	-	-	-	-	-
M44	-	-	-	+ 1	+ 2	-	-	-	-	+ 2	-	-
Ps.P3	+ 1	+ 1	+ 3	+ 1	+ 1	+ 2	+ 3	+ 3	+ 5	+ 2	-	-
Non-pathogenic pseudomonads												
4A3	+ 2	-	-	+ 1	+ 2	-	+ 1	+ 1	+ 2	+ 3	-	-
CS4	-	-	-	+ 1	+ 1	-	+ 1	+ 1	+ 1	+ 2	-	-
Pt2	+ 1	+ 1	+ 2	+ 1	+ 2	-	-	-	-	-	-	-
3D13	-	-	-	-	-	-	+ 1	+ 1	+ 3	+ 2	+ 3	-
Lk7	+ 1	+ 1	-	+ 1	+ 1	-	+ 1	+ 1	+ 2	+ 2	-	-
Ps.73	+ 2	+ 2	+ 3	+ 1	+ 3	-	+ 2	+ 2	+ 3	+ 3	-	-
Achromobacter spp.												
7A14	-	-	-	+ 3	-	-	-	-	-	-	-	-
Cb11	+ 1	+ 2	-	+ 2	+ 3	-	+ 1	+ 1	+ 3	-	-	-
Alcaligenes spp.												
CS8	+ 1	+ 1	+ 2	+ 1	+ 1	+ 2	+ 1	+ 1	+ 1	+ 3	-	-
CS11	+ 1	+ 1	+ 2	+ 1	+ 1	+ 1	+ 1	+ 1	+ 1	-	-	-
Agrobacterium spp.												
Lk11	+ 2	+ 2	+ 3	+ 2	+ 2	+ 2	+ 2	+ 2	+ 2	+ 3	+ 4	-
ZM1	+ 2	+ 3	+ 3	+ 2	+ 2	+ 2	+ 2	+ 2	+ 3	+ 3	-	-

+ = The organism grown in the medium. The number indicated the days of incubation when the growth was first observed.

- = No growth in the medium after 10 days incubation.

Table 25 (Contd.)

Organism tested	Manganese concentrations m.g.e./L Mn^{++}					
	4 Mn^{++}	6 Mn^{++}	8 Mn^{++}	10 Mn^{++}	20 Mn^{++}	30 Mn^{++}
Plant pathogenic pseudomonas						
NZ1	+ 1	+ 2	+ 2	+ 3	-	-
NZ10	+ 1	+ 2	+ 2	+ 2	+ 5	-
P6	+ 1	+ 2	+ 3	+ 3	-	-
NZ41	+ 1	+ 3	-	-	-	-
M44	+ 1	+ 1	+ 4	+ 4	-	-
Ps.P3	+ 1	+ 2	+ 3	-	-	-
Non-pathogenic pseudomonas						
4A3	+ 1	-	-	-	-	-
CS4	+ 1	-	-	-	-	-
Pt2	+ 2	+ 4	-	-	-	-
3D13	-	-	-	-	-	-
Lk7	-	-	-	-	-	-
Ps.73	+ 2	+ 3	-	-	-	-
Achromobacter spp.						
7A14	+ 2	+ 4	-	-	-	-
Cb11	+ 1	+ 1	+ 4	+ 4	-	-
Alcaligenes spp.						
CS8	+ 1	+ 1	+ 1	+ 1	+ 1	+ 1
CS11	+ 1	+ 1	+ 1	+ 1	+ 1	+ 2
Agrobacterium spp.						
Lk11	+ 2	+ 2	+ 2	+ 2	+ 2	+ 2
ZM1	+(1)	+ 2	+ 2	+ 2	+ 2	+ 3

to tolerate Zn^{++}, Cu^{++}, Ni^{++}. However the results obtained from Co^{++} and Mn^{++} may be significant in that all the plant pathogenic pseudomonads with the exception of Ps. marginalis strain Ps.P3 were inhibited by cobalt whereas non-pathogenic pseudomonads with the exception of Pt2 strain were able to grow on such media. An opposite was found with Mn^{++}, all the non-pathogenic pseudomonads were inhibited at 10 m.g.e./LMn^{++} whereas the plant pathogenic pseudomonads were able to grow in that medium, with the exception of Ps. phaseolicola NZ41 strain and Ps. marginalis Ps.P3 strain.

Results also indicated that the Agrobacterium strains as well as the Alcaligenes strains were able to tolerate higher concentrations of manganese than any of the other organisms tested. They were able to tolerate as high concentration of Mn^{++} as 30 m.g.e. /L Mn^{++}. These results were considered worthy of further investigation.

Experiment 57.

Results from the above experiment showed that the effect of manganese and cobalt was interesting from the point of view of the differences between the plant pathogenic and non-pathogenic pseudomonads. The following experiment was conducted to find out to what extent it is possible to generalise about such differences and to extend the test to a greater number of strains. Strains of plant pathogenic pseudomonads (54) and non-pathogenic pseudomonads (38) from different sources were examined.

Results:-

Table 26 results indicated that the addition of manganese at 10 m.g.e./L Mn^{++} to the basal medium had an inhibitory effect on the growth of most of the non-pathogenic pseudomonads tested, whereas most of the plant pathogenic pseudomonads tolerated that concentration of manganese and grew after a period of 2-4 days from the inoculation. The exceptions were 3 strains of Ps. syringae NZ3, NZ21 and P38. NZ21 strain was found to be both cytochrome oxidase and phenol oxidase positive, and was considered, therefore, unlikely to be a pathogen.

The Ps. phaseolicola strains tested grew on the manganese medium after a longer time of incubation than the above strains; growth occurred between 6 and 8 days and one strain, P13, however, failed to grow even after 10 days of incubation. Neither Ps. marginalis nor Ps. medicaginis strains grew in the medium.

The effect of manganese on non-pathogenic pseudomonads was interesting, therefore, in that generally they were more sensitive to manganese than were the plant pathogenic pseudomonads. Manganese at 10 m.g.e./L Mn^{++} appeared to be the significant differential concentration under these conditions. Results obtained from the effect of cobalt on the two groups revealed that, overall, the plant pathogenic pseudomonads were more sensitive to cobalt than were the non-pathogenic pseudomonads.

Table 26. The toxicity of manganese and cobalt against plant-pathogenic and non-pathogenic pseudomonads

Organism and number of strains tested		number tolerate 10 m.g.e./L Mn^{++}	number tolerate 0.08 m.g.e./L Co^{++}
Ps. syringae	(29)	26	6
Ps. mors-prunorum	(10)	10	0
Ps. phaseolicola	(6)	4	1
Ps. tabaci	(2)	2	0
Ps. medicaginis	(1)	0	0
Ps. marginalis	(6)	0	6
Non-pathogenic pseudomonads	(38)	6	31

Experiment 58.

This experiment was designed to study the viability of original inoculum of both pathogenic and non-pathogenic pseudomonads in manganese and cobalt media.

The following representative strains were examined.

Non-pathogenic pseudomonads 4A3 - CS4 - Lk7 - Pt2 - 5A8 and 3D13

Ps. marginalis 1559, 667

Ps. syringae S5, S8

Ps. mors-prunorum C9, D1

Ps. phaseolicola P12, P14

The above organisms were inoculated into the basal medium which contained manganese at concentration 10 m.g.e./L Mn^{++}, and also into the basal medium which contained cobalt at 0.08 m.g.e./L Co^{++}. The tubes were incubated at 27°C and observed up to 10 days for growth. After 10 days incubation, the tubes showing no visible growth were inoculated back to the control medium, and the tubes incubated again at 27°C for 7 days to record viability of the original inoculum.

Results:-

The non-pathogenic strains and the two Ps. marginalis strains tested did not grow on the manganese medium, when they were inoculated back on the control medium, all of them failed to grow except 667 strain. This suggested that manganese has a bactericidal effect on the non-pathogenic strains examined and on one of the Ps. marginalis strains, the inoculum of the other strain still survived. However, as indicated in the previous experiment Ps. phaseolicola grew on medium with 10 m.g.e./L Mn^{++} after a long incubation time, but none grew at 15 m.g.e./L Mn^{++}. It was found also that manganese at that concentration had no bactericidal effect on Ps. phaseolicola; all the strains were found to grow in the control medium when the inoculum was taken from the medium which contained 15 m.g.e./L Mn^{++}.

The results obtained from the effect of cobalt on the plant pathogenic pseudomonads suggested that the effect was not bactericidal in nature since the original inoculum still survived.

Experiment 59. The reversion of the toxicity of manganese and cobalt

Introduction:-

Results from the previous experiment indicated that manganese has a bactericidal effect against the non-pathogenic pseudomonads while the inhibitory effect of cobalt to the plant pathogenic pseudomonads is not a bactericidal in nature since the inoculum was still able to survive. These results suggested that the plant pathogenic pseudomonads were able to tolerate manganese possibly by their ability to synthesize chelating compounds and thereby removing some of the metals from the medium.

An attempt was made to reverse the toxicity of manganese against the non-pathogenic pseudomonads and to overcome the toxicity of cobalt against the plant pathogenic pseudomonads, by testing naturally occurring chelating compounds.

Albert (1950, 1952) has demonstrated the considerable chelating ability of aminoacids. Schade (1949) was able to reverse the toxicity of cobalt against Porteus vulgaris by cysteine and histidine. Singer (1964) tested a number of naturally occurring chelating compounds in an attempt to overcome the inhibitory effect of yeast autolysate in the medium against effective strains of Rhizobium trifolii. He found that most of these chelating agents failed to stimulate the growth of these organisms, while ascorbic acid and cystine stimulated the growth of effective rhizobia.

Experiment 59a. The reversion of manganese toxicity

Representative strains of the non-pathogenic pseudomonads as well as Ps. marginalis were inoculated into the basal medium which contained 10 m.g.e./L Mn^{++}. The following amino acids were tried in an attempt to reverse the toxicity of manganese against these organisms; arginine, glycine, cysteine and histidine and L-ascorbic acid. These compounds were added to give final concentrations of 0.01% (w/v), which was non-toxic in each case. The following representative strains were tested:-

Non-pathogenic pseudomonads 4A3 - CS4 - Lk7 - 5A8 - 3D13 and Pt2

Ps. marginalis 1604 and 1559 strains

Results:-

None of the amino acids tested, nor ascorbic acid, were able to overcome the toxicity of manganese against these non-pathogenic and Ps. marginalis strains.

Experiment 59b. The reversion of cobalt toxicity against plant pathogenic pseudomonads

Representative (9) strains of plant pathogenic pseudomonads were inoculated into the basal medium which contained 0.08 m.g.e./L Co^{++}. The amino acids which were used in the above experiment, were tested for their ability to reverse the toxicity of cobalt against the plant pathogenic pseudomonads; L-ascorbic acid was also tested.

Organisms tested:-

Ps. syringae S5 - S8 - S20

Ps. mors-prunorum D1 - D2 - C9

Ps. phaseolicola P12 - P14 - P16

Results (Table 27):-

From the compounds tried only cysteine and histidine were able to overcome the toxicity of cobalt against all the strains examined. All strains grew very well over-night in the presence of a concentration of cobalt which normally inhibits the growth of all these organisms.

Table 27. The reversion of cobalt toxicity against plant pathogenic pseudomonads spp. Results after 7 days.

Organisms tested	Co^{++}	Co^{++} + histidine	Co^{++} + cysteine	Co^{++} + arginine	Co^{++} + glycine	Co^{++} + L-ascorbic acid
Ps. syringae						
S5	-	+ 1	+ 1	-	-	-
S8	-	+ 1	+ 1	-	-	-
S20	+ 4*	+ 1	+ 1	+ 4	+ 4	+ 4
Ps. mors-prunorum						
D1	-	+ 1	+ 1	-	-	-
D2	-	+ 1	+ 1	-	-	-
C9	-	+ 1	+ 1	-	-	-
Ps. phaseolicola						
P12	-	+ 1	+ 1	-	-	-
P14	+ 4	+ 1	+ 1	+ 4	+ 4	+ 4
P16	-	+ 1	+ 1	-	-	-

* The numbers indicated the number of days in which growth was first observed.

Discussion:-

None of the amino acids tested nor L-ascorbic acid overcame the toxicity of manganese against the non-pathogenic pseudomonads and Ps. marginalis strains, and only cysteine and histidine overcame the toxic effect of cobalt against the plant pathogenic pseudomonads tested. Similar results were obtained by Schade (1949) who found that cysteine and histidine were capable of overcoming the toxicity of cobalt against Proteus vulgaris. These results could be explained by the results of Michaelis and Barron (1929) who showed that cobalt forms a stable complex with cysteine and, as indicated by Schade (1949) that histidine also formed a complex compound with cobalt.

From this experiment, there was no evidence obtained in relation to the synthesis of chelating agents by the plant pathogens which enable them to tolerate the high concentrations of manganese, when the naturally occurring chelating compounds were tested. The tolerance of synthetic chelating agents was studied in an attempt to find any difference between the plant pathogenic and non-pathogenic pseudomonads. 8,hydroxyquinoline (oxine) and bio-hydroxyphenylethylenediaminediacetic acid (Chel.D.P.) were studied, and the results showed that there were no differences between the plant pathogenic and non-pathogenic pseudomonads in their ability to tolerate the chelating agents tested. These results exclude the idea that the plant pathogenic pseudomonads are able to tolerate the high concentration of manganese because they are able to synthesize chelating compounds.

Experiment 60. The manganese content of healthy and Pseudomonas infected plant materials

This investigation was done in an attempt to find out why a plant pathogenic pseudomonad can tolerate high concentrations of manganese. Healthy plum leaves, variety Victoria and Ps. mors-prunorum infected leaves and plum bark were kindly analysed by Dr. D. Purves for manganese content.

Results:- (Table 28) showed that the infected plum leaves contained significantly lower concentrations of Mn^{++} than did the healthy plum leaves collected from the same tree. The manganese determination of the bark revealed no significant differences between the healthy and the diseased bark. The relatively low concentration of manganese in the diseased leaves is possibly a result of an increase in the translocation when the infection occurs. Although the healthy leaves contained higher concentration of manganese than did the diseased ones, the concentration was too low to inhibit the growth of the non-pathogenic pseudomonads in laboratory medium. No further attempts were made to explain the effects of manganese.

Table 28. The manganese content of healthy and diseased plant materials

Sample No.	Source of the sample	Mn PPM
1	healthy plum leaves	57
2	" " "	52
3	" " "	47
4	" " "	49
5	" " "	53
6	Pseudomonas infected plum leaves	34
7	" " "	34
8	" " "	40
9	" " "	21
10	" " "	23
11	healthy plum bark	44
12	" " "	16
13	" " "	25
14	" " "	20
15	" " "	26
16	Ps. infected plum bark	18
17	" " " "	26
18	" " " "	31
19	" " " "	32
20	" " " "	26

Experiment 61. The manganese tolerance of plant pathogenic bacteria belonging to other genera

It was of interest to study the tolerance of manganese by other plant pathogenic species in other genera, (Agrobacterium, Erwinia and Xanthomonas). Results are presented in Tables 29 and 30.

Results:-

The basal medium was found to support the growth of all the organisms tested.

Agrobacterium:- All the agrobacterium strains tested tolerated manganese at 20 m.g.e./l Mn^{++}. At 30 m.g.e./L, however, all but 223 and 794 strains of A. tumefaciens grew. These two strains did not produce 3 keto lactose from lactose, a character which is shown by Bernaerts & DeLey (1963) to be common among A. tumefaciens and A. radiobacter. At 40 m.g.e./L A. rhizogenes failed to grow but, apart from two previous exceptions the other Agrobacterium strains grew well.

Xanthomonas:- The two strains of Xanthomonas phaseoli and one X. phaseoli var. fuscans studied grew on the basal medium contains 5 m.g.e./L Mn^{++}, but not the 10 m.g.e./L.

Erwinia:- Results indicated that all the Erwinia strains tested were able to tolerate manganese up to 20 m.g.e./L, with the exception of two of the E. atroseptica strains which were inhibited by 10 m.g.e./L Mn^{++}. These results, however, did not help to distinguish between the different species.

Table 29. The toxicity of manganese against Agrobacterium spp.

Results after 7 days incubation.

Organism tested	20 m.g.e./L Mn^{++}	30 m.g.e./L Mn^{++}	40 m.g.e./L Mn^{++}
4	++	+	+
5	++	+	-
223	++	-	-
396	++	+	+
397	++	+	+
398	++	+	+
794	++	-	-
925	++	++	++
930	++	+	+
1001	++	++	++
8149	++	++	++
8150	++	++	++
Lk11	++	+	+
ZM1	++	+	+

++ = very good growth

+ = good growth

- = No growth after 10 days.

Table 30. The toxicity of manganese against different Erwinia spp.

Results after 7 days incubation at 27°C.

Organism tested	Manganese concentration m.g.e./L 10 Mn	20 Mn	30 Mn
Erwinia atrosepticum			
76.V	-	-	-
QB4	-	-	-
G110	++	+	+
E. caratovorum			
1172	++	++	++
G147	++	+	-
340	++	++	++
J6	++	+	-
E. aroideae			
911V	++	++	-
A8IV	++	++	-
ENA10	++	+	-
E. chrysanthemi			
EP3	++	++	++
402	++	++	++

++ = very good growth

+ = good growth

- = no growth after 10 days

Experiment 62. The toxicity of manganese against Alcaligenes spp.

Results from experiment 56 revealed that the two alcaligenes strains tested were exceptionally tolerant of manganese, up to 40 m.g.e./L Mn^{++}, therefore, it was of interest to study the manganese tolerance by some known Alcaligenes species.

The organisms tested were obtained from NCIB.

Manganese concentrations tested were 10, 20, 30 and 40 m.g.e./L.

Results:-

The basal medium was found to support good growth for all the strains tested. None of the NCIB cultures were able to tolerate manganese at any concentration tested, and in that result they differ from the Alcaligenes strains isolated from soil.

The isolation of plant pathogenic pseudomonads from infected plant materials

Introduction:-

Members of the genus Pseudomonas are of widespread occurrence and in many cases they are a dominant organism in the plant environment.

An attempt to devise a medium which was selective for a particular species of phytopathogenic pseudomonads was made in the case of Ps. phaseolicola by Wilson (1938). This medium was not entirely specific since certain saprophytes grew in the selective medium. Crosse & Bennett (1955) described a selective medium for the isolation of Ps. mors-prunorum. The selectivity of that medium depended on the ability of Ps. mors-prunorum to utilize tartrate as the sole source of carbon and the inhibitory effect of Di-octyl sodium sulphosuccinate on saprophytes. They reported that the medium was not entirely specific for Ps. mors-prunorum (other unidentified species of pseudomonads are able to grow on that medium). Erikson (1945), however, found that not all strains of Ps. mors-prunorum utilized tartrate. Paton (1956) developed a method for the isolation of pseudomonads from infected plants. He enriched plant material in sodium tartrate and β. hydroxybutyrate, in inorganic salts media. The subsequent bacterial growth was plated on sucrose agar and the levan producing colonies picked and checked for their ability to produce the fluorescent pigment. This method was found to be very useful for the isolation of pseudomonads.

Paton (1960) emphasized the fact that both pathogenic pseudomonads and saprophytic pseudomonads may be isolated from infected plant materials. Goth (1965) described a puncture method for isolating pathogenic bacteria from beans. This consists of puncturing through active lesions onto glucose nutrient agar, then streaking the bacteria from the stab site with a sterile glass rod. He found this method to be rapid, efficient, and simple for isolating the major bacterial pathogens of beans including Ps. phaseolicola, Ps. syringae, Xanthomonas phaseoli, and X. phaseoli var. fuscans. He also claimed that there was little possibility of obtaining surface contaminants because of the minute surface area involved in this method. There is, however, a possibility of picking non-pathogenic pseudomonads associated with the surface which could be confusing in an attempt to isolate the pathogenic Pseudomonas.

It was found earlier in the present study that the phytopathogenic Ps. syringae and Ps. mors-prunorum, among some other plant pathogenic species, can tolerate manganese while most of the non-pathogenic pseudomonads are inhibited. It was of interest, therefore, to study the possibility of using a manganese medium to isolate the phytopathogenic pseudomonads.

Experiment 63. The toxicity of manganese in L-proline medium against some pseudomonads

It has been described before that L-proline was utilized as the only source of carbon and nitrogen by the majority of the pseudomonads strains with the enhancement of the production of the fluorescent pigment produced by some members of the genus. It was of interest, therefore, to study the toxicity of manganese against plant pathogenic and non-pathogenic pseudomonads in proline medium which has the following composition:- L-proline 0.5% (w/v) - K_2HPO_4 0.008% (w/v); KH_2PO_4 0.002% (w/v) - $MgSO_4$ 0.02% (w/v); agar 1.5% (w/v). The pH of the medium was adjusted to pH7 and autoclaved at 115° for 10 mins. Manganese was tested at 10 and 20 m.g.e./L Mn^{++}. Representative strains (43) of the plant pathogenic and non-pathogenic pseudomonads were streaked onto the above medium. The plates incubated at 27°C and examined for the growth up to 7 days.

Results:- Table 31 shows the results after 4 days incubation and indicated that apart from most of Ps. marginalis strains, all plant pathogenic pseudomonads were able to grow on that medium when manganese was added at 20 m.g.e./L Mn^{++}, whereas the majority of the non-pathogenic pseudomonads were inhibited. The growth of the plant pathogenic pseudomonads on that medium was very good after 2-3 days of the incubation. The same results were obtained after 7 days. It was also noticed that cloudy areas developed in the medium around the growth of the organisms in the

Table 31. The toxicity of manganese in L-proline medium against pseudomonads strains

Organisms tested	Control medium	10 m.g.e./L Mn^{++}	20 m.g.e./L Mn^{++}
Ps. syringae			
S5	+	+	+
S8	+	+	+
S20	+	+	+
S36	+	+	+
W1	+	+	+
W183	+	+	+
G2	+	+	+
Ps. mors-prunorum			
D1	+	+	+
D2	+	+	+
D5	+	+	+
D16	+	+	+
C9	+	+	+
C22	+	+	+
N5	+	+	+
C46	+	+	+
P47	+	+	+
Ps. phaseolicola			
P12	+	+	(+)
P13	+	+	(+)
P14	+	+	+
P16	+	+	(+)
P18	+	+	+
NZ41	+	+	(+)
Ps. tabaci			
M44	+	+	+
M45	+	+	+
Ps. marginalis			
607	+	+	-
1558	+	+	(+)
1559	+	-	-
1604	+	-	-

Table 31 (Contd.)

Organisms tested	Control medium	10 m.g.e./L Mn^{++}	20 m.g.e./L Mn^{++}
Non-pathogenic pseudomonads			
5A8	+	-	-
4A3	+	-	-
1A1	+	-	-
F300	+	+	-
Lk2	+	+	-
Lk7	+	-	-
Bt6	+	-	-
Cb1	+	+	+
Cb4	+	+	(+)
Ps.73	+	+	+
6A12	+	-	-
CS4	+	-	-
7A9	-	-	-
Bn3	-	-	-
Pt2	-	-	-

presence of manganese. This could be due to the precipitation of some of manganese salt probably phosphate in the manganese caused by the alkali reaction which is normally produced when these organisms are grown in the presence of L-proline and other amino acids. The addition of manganese to L-proline medium reduced the production of the fluorescent pigment.

Experiment 64. The comparison of potentially selective media

Infected plum leaves, Victoria variety, were used in this study. The suspected pathogen was Ps. mors-prunorum. The infected area of the leaves were macerated with a small amount of sterile water in a mortar. Three media were compared for the isolation of the organism.

1. The enrichment in Na tartrate medium as described by Paton (1956).
2. The enrichment in the manganese medium which is the same medium used in the study on the metal toxicity. Manganese was added at 10 m.g.e./L Mn^{++}, and will be referred to as GYE manganese medium.
3. Direct plating on the L-proline medium which contained 20 m.g.e./L Mn^{++} as described in experiment (63).

The first two media were inoculated and incubated for 2 days, and the resulting growth plated onto L-proline medium containing no manganese. The plates were incubated for 2 days at 27°C and then examined.

Results:-

The L-proline medium plates inoculated from the enrichment in Na tartrate medium were found to contain large numbers of fluorescent colonies when examined under the U.V. lamp. Other organisms also grew on the plates but it was easy to detect the fluorescent pseudomonads colonies.

Results obtained from the plate inoculated from the

enrichment in (GYE) manganese medium indicated that few fluorescent colonies were present, and that some other organisms also grew on the medium. The enrichment in manganese medium markedly reduced all these contaminants. The direct plating on L-proline manganese medium indicated that a fluorescent organism was obtained in practically pure culture. Tests were now made to separate the plant pathogenic from the non-pathogenic fluorescent pseudomonads colonies growing on these plates.

Representative colonies were picked and tested for cytochrome oxidase and phenol oxidase as described before. The results indicated that the majority of the pseudomonads isolated from the enrichment in Na tartrate medium were positive in both tests, and only a few isolates were negative in both tests.

The majority of the fluorescent pseudomonads isolated from the enrichment in (GYE) manganese medium were found to be cytochrome oxidase and p-aminophenol oxidase negative, and very few isolates were positive in both tests.

The majority of the fluorescent pseudomonads isolated from the direct plating on L-proline manganese medium were found to be negative when they were tested for cytochrome oxidase and p-aminophenol oxidase. Only one isolate out of 14 was found to be positive in both tests.

The fluorescent pseudomonad isolates, which were cytochrome oxidase and phenol oxidase negative, were found eventually to be the pathogenic Ps. mors-prunorum. They produced symptoms on

bean pods comparable to those of known Ps. mors-prunorum strain, and they were biochemically indistinguishable.

The proline manganese medium was used for the isolation of plant pathogenic pseudomonads from different hosts, and the following method was routinely used for the isolation:-

The infected plant material was crushed with small amount of sterile water and after 10-15 mins. streaked on L-proline medium which contained manganese as indicated above. The control L-proline medium was also used. After 2-3 days incubation, the plates were examined under the U.V. lamp and the fluorescent colonies tested for the phenol oxidase (p-aminophenol oxidase). The fluorescent pseudomonads which were negative were found to be the plant pathogenic organism when the pathogenicity tests were made.

The method described above was used for the isolation of Ps. mors-prunorum and Ps. syringae.

The method described by Goth (1965) for the isolation of plant pathogenic bacteria from beans was also tested and in some cases this method was found very useful in that it reduced the contamination, but was improved, when applied to delphinium leaves and bean leaves, by using L-proline agar medium instead of glucose nutrient agar as was originally recommended. Here the predominant organisms were fluorescent pseudomonads and some of them were non-pathogenic as shown by the cytochrome oxidase and phenol oxidase tests applied to representative strains. In all cases, the fluorescent pseudomonads which were negative to the cytochrome oxidase and phenol oxidase test were found to be the pathogenic organism.

A selective medium for the isolation of Agrobacterium spp.

Introduction:-

It was found that Agrobacterium species were exceptional among the Gram-negative bacteria tested, in that they tolerated higher concentrations of manganese than did the other Gram-negative bacteria. This finding was used in devizing a selective medium for the isolation of Agrobacterium spp. from soil and infected plant materials. A search in the literature revealed that there was no satisfactory selective medium described for the isolation of these organisms.

Patel (1926) used a medium containing peptone, glucose, sodium taucholate and crystal violet. Because of the high concentration of glucose and peptone used, many fungi and other organisms grew (Schroth, Thompson and Hildebrand, 1956), and the fungi grew rapidly and hindered the development of Agrobacterium colonies. Ark & Schroth (1958) suggested the use of carrot slices for the isolation of A. tumefaciens from soil. The limitation of such method is that the soil contains soft rotting organisms, which cause soft rot of the carrot.

Recently Schroth et al. (1965) described a medium which they showed to be very useful for the isolation of A. tumefaciens and A. radiobacter from soil. The selectivity of the medium depended mainly on the following constituents added to a mannitol, $NaNO_3$ mineral salts agar medium; berberine, sodium selenite, penicillin, streptomycin, cyclohexamide, tyrothricin and bacitracin. The above medium is laborious to prepare, and

some of these compounds are not easily obtainable and are expensive. Another limitation is that it contains antibiotics which could not be tolerated by all strains of Agrobacterium (Dickey, 1965).

The following experiments were conducted to develop a selective medium for Agrobacterium spp.

Experiment 65. The toxicity of manganese against Agrobacterium spp.

The ability of agrobacteria to tolerate high concentration of manganese was confirmed in different media with different carbon and nitrogen sources. The following experiment illustrated this fact and resulted in the development of a medium useful for the isolation of Agrobacterium and studying the survival of these organisms in soil.

The basal medium has the following composition:-

0.5% (w/v) Lactose

0.008% (w/v) K_2HPO_4

0.002% (w/v) KH_2PO_4

0.02% (w/v) $MgSO_4$

0.5% (w/v) $NH_4H_2PO_4$

1.5% (w/v) agar

The lactose (filter sterilized) and the ammonium phosphate (autoclaved) were added to the basal medium which was adjusted to pH8 before autoclaving. Manganese was added to give final concentrations of 20, 30, 40 and 50 m.g.e./L Mn^{++}. The organisms examined were grown for 2 days on nutrient agar containing 2% glycerol. A slightly turbid suspension was made in sterile distilled water. One drop of that suspension was added on the surface of dried plates which were incubated at 27°C for 4 days.

Results:- (Table 32)

Results showed that all the Agrobacterium strains tested were able to tolerate 30 m.g.e./L Mn^{++}. The results also

showed that 223 and 794 strains were able to tolerate manganese at 50 m.g.e./L Mn^{++}. These two strains were also found to be inhibited in the presence of 30 m.g.e./L Mn^{++} (Table 29). These different results could be due to the different media used.

Representative organisms from the following genera Pseudomonas, Erwinia, Alcaligenes, Achromobacter, Xanthomonas and Bacillus were found to be unable to grow on that medium which contained 20 m.g.e./L Mn^{++}.

Table 32. The manganese tolerance by Agrobacterium spp. in lactose ammonium, phosphate medium

Strain examined	manganese concentration m.g.e./L				
	0	20	30	40	50
4	++	++	++	-	-
5	++	+	(+)	-	-
223	++	++	++	++	++
396	++	++	++	-	-
397	++	++	+	-	-
398	++	++	+	-	-
794	++	++	++	++	++
925	++	++	++	-	-
930	++	++	++	-	-
1001	++	++	++	-	-
8149	++	++	++	-	-
8150	++	++	+	-	-
Lk11	++	++	++	-	-
ZM1	++	++	+	-	-

++ = good growth
+ = moderate growth
(+) = weak growth
- = no growth

Experiment 66. A comparison of media for the isolation of Agrobacterium from soil

The following media were tested as control media and with different concentrations of manganese.

1. glucose yeast extract medium. The same medium as described in the study of the metal toxicity.

2. glucose ammonium phosphate medium. As medium 1 but ammonium phosphate at 0.5% used instead of yeast extract.

3. Lactose ammonium phosphate medium. As described before.

4. Patel's medium.

5. Nutrient agar containing 2% (w/v) glycerol.

A gram of soil was added to 9 ml. of sterile distilled water and the tubes were shaken vigorously for 1 hour and then streaked on the above media. The agar plates were incubated at 27°C for 7 days.

Results:-

Medium (1). The control plates were covered with sporeformers and other organism including fungi, however the addition of manganese at 20 and 30 m.g.e./L Mn^{++} reduced the population considerably. Even so, of the bacteria which grew none were Agrobacterium.

Medium (2). The results were slightly better than the above but some organisms grew even when manganese was present at 30 m.g.e./L Mn^{++}.

Medium (3). The lactose ammonium phosphate medium gave the best results in that very few soil organisms grew. At 20 and

30 m.g.e./L of Mn^{++}, the soil population was often reduced to just one or two colonies per plate. Moulds and actinomycetes were the most frequently encountered organisms other than agrobacteria.

Medium (4). Patel's medium. This medium had no significant selective effect on the soil flora.

Medium (5). Nutrient agar contained glycerol. This medium was included for comparison and many types of soil organisms grew on this medium.

Experiment 67.

From the above experiment, no agrobacteria were isolated from the soil. This was probably due to the fact that this soil sample contained no agrobacteria. For this reason a garden soil inoculated with agrobacteria was used. To 1 gm. of the soil the following A. tumefaciens strains 8150, 1001, 794 and 223 were inoculated. The final concentration of agrobacteria was about 2×10^4 per/gm. of soil. The infected soil was shaken vigorously with sterile water (1 gm. of soil to 9 ml. of water). A viable count was made according to Miles & Misra (1938) on the lactose ammonium phosphate medium, which contained 20 and 30 m.g.e./L of Mn^{++}. The plates incubated for 4 days. Results indicated that the agrobacteria were recovered in a pure culture on the manganese media. The same samples were streaked

onto the above media and on Patel's medium, the results are as shown in plates 8 & 9 which indicated that Agrobacterium strains were recovered in pure culture on the manganese medium but not on Patel's medium. Occasionally, however, the odd fungus grew on the medium, therefore the following experiment was conducted to increase the selectivity of that medium by the use of some antifungal agents and crystal violet.

Experiment 68.

The effect of the funal inhibitors actidione, di-octyl sodium sulphosuccinate and crystal violet on the growth of agrobacteria in lactose ammonium phosphate manganese medium was studied. Actidione was added as final concentration 100 PPM, di-actyl sodium sulphosuccinate as 0.01% and crystal violet at 1/500000. The plates were inoculated as indicated above and incubated at 27°C for 7 days.

Results:- (Table 33)

Results indicated that di-actyl sodium sulphosuccinate is very toxic to the agrobacterium strains tested. The agrobacteria tolerated 0.001% concentration but this was too low to inhibit fungi. The addition of the above compounds did not improve the selectivity of the medium significantly.

Plate 8.

Plate 9.

The recovery of Agrobacterium tumefaciens mixed with soil on Patel's medium and on manganese medium

Table 33. The effect of the addition of antifungal agents and crystal violet on the growth of agrobacteria in lactose-ammonium phosphate manganese medium

Strain tested	Actidione 100 PPM				Crystal violet 1/500000				Di-octyl sodium sulphosuccinate .01%
	Control	20 m.g.e./L Mn^{++}	30 m.g.e./L Mn^{++}	40 m.g.e./L Mn^{++}	Control	20 m.g.e./L Mn^{++}	30 m.g.e./L Mn^{++}	40 m.g.e./L Mn^{++}	
4	++	++	+	-	++	++	+	-	No growth occurred even in the control medium which had no manganese
5	++	+	(+)	-	++	+	(+)	-	
223	++	++	++	++	++	++	++	++	
396	++	++	+	-	++	++	+	-	
397	++	++	+	-	++	++	+	-	
398	++	++	+	-	++	++	+	-	
794	++	++	++	++	++	++	++	++	
925	++	++	+	-	++	++	(+)	-	
930	++	++	+	-	++	++	+	-	
1001	++	++	+	-	++	++	+	-	
8149	++	++	+	-	++	++	-	-	
8150	++	++	+	-	++	++	-	-	
Lk11	++	++	+	-	++	++	-	-	
ZM1	++	+	+	-	++	++	-	-	

++ = good growth

+ = moderate growth

(+) = weak growth

- = no growth

Experiment 69. The isolation of Agrobacterium tumefaciens from infected plant materials

A Dahlia plant suspected to be infected with A. tumefaciens was macerated in a small amount of sterile water and plated onto lactose-ammonium phosphate medium containing 20 and 30 m.g.e./L Mn^{++} and on Patel's medium as well as MacConkey's bile-salt medium and nutrient agar, which contained 2% (v/v) glycerol. The plates were incubated at 27°C for 4 days.

Results:-

Results indicated that a pure culture of A. tumefaciens was obtained on the lactose ammonium phosphate manganese medium 30 m.g.e./L Mn^{++} but not on the other media.

Similar results were obtained when $NaNO_3$ was used instead of ammonium phosphate as described by Clark (personal communication). This modification was proved to be slightly better than the medium described in this study and proved to be very useful and better in some cases for the isolation of A. tumefaciens from infected plant materials.

Plates 10 & 11 show the results of the isolation of A. tumefaciens from infected plant materials on different media and on manganese medium.

Plate 10.

Plate 11.

The isolation of Agrobacterium tumefaciens from infected plant on different media and on manganese medium

Summary:-

The fact that Agrobacterium species can tolerate a high concentration of manganese was used to develop a selective medium which proved very useful in the isolation of these organisms from soil and from infected plant materials.

The selectivity of the medium depends on its high concentration of manganese and also on lactose as the only source of carbon, which is not utilized by many soil organisms.

In most cases this medium gave a pure culture of Agrobacterium but in rare instances fungi appeared in very small numbers. The antifungal agents were found to be of no use in this media. This manganese medium was clearly superior to Patel's medium as a selective medium for the isolation of these organisms.

PART V

Miscellaneous properties of the organisms

The pectolytic activity of the organisms

The ability of some members of the genus Pseudomonas to cause a soft rot of plant tissue and to hydrolyse pectin is well known. Paton (1958) examined 400 pseudomonad strains, from a variety of sources, for their soft rotting ability and reported that 10% of the strains were able to cause the soft rot of cucumber slices. Smith (1958) reported that a number of saprophytic bacteria, as well as some plant pathogens, produced enzymes which degrade pectic substances but production of pectin methylesterase is confined to a small number of the pathogens.

Experiment 70. Liquefaction of pectin

The ability of the organisms to liquffy pectate gel was studied; the method used for the preparation of pectate gel was as described by Paton (1959) and consisted of a plate of calcium agar base over which the pectin solution, containing disodium salt of ethylenediamine tetra acetic acid was poured. The pectin used in this study was sodium polypectate. After 24 hours, the poured plates were inoculated, 4 organisms to each plate. The plates were incubated for 7 days and the liquefaction of pectin recorded. The plates were also flooded with 5N Hcl to test for pectin polygalacturinase.

Organisms tested:-

Ps. syringae S5 - S8 - S20 - S36 - W1 - W183 and G2

Ps. mors-prunorum D1 - D2 - D5 - D16 - C9 - C22 - C46 and N5

Ps. phaseolicola P12 - P13 - P14 - P16 - P18 and NZ41

Ps. marginalis 667 - 1558 - 1559 - 1604

Non-pathogenic pseudomonads - 20 strains

Results:-

The results indicated that Ps. marginalis strains as well as 4 non-pathogenic pseudomonads gave identical reactions to that given by the Erwinia carotovora strains used as a control.

None of the other plant pathogenic pseudomonads tested liquefied the pectate gel.

After the plates have been flooded with 5$\bar{N}$ Hcl, a clear area round the mass of the growth of Ps. marginalis, and the other 4 non-pathogenic pseudomonads as well as E. carotovora strain was developed. This clear area indicated the presence of products simpler than pectic acid which is precipitated by Hcl. A very weak reaction was given by some Ps. syringae strains.

Experiment 71. The production of pectin methylestrase

The method used for the detection of pectin methylestrase was the same as described by Dye (1958).

Results:-

Results indicated that only two strains gave a positive reaction, Ps. marginalis strain 1558, and a non-pathogenic pseudomonas strain F300.

Experiment 72. The soft rotting activity of the organisms

This was studied by using the potato tuber slices.

Results:-

Results indicated that Ps. marginalis strains as well as the 4 non-pathogenic pseudomonads strains mentioned before were able to cause the soft rotting of potato slices after 24 hours of incubation in a Petri dish containing damp filter paper at 27°C. None of the other plant pathogenic pseudomonads were able to cause the soft rotting of the potato slices.

Experiment 73. An electrophoretic study of the catalases and esterases of the organisms

The technique of starch gel electrophoresis to study the multiple molecular forms of enzymes, provides additional information on which taxonomic studies may be used (Norris, 1962). Norris (1964) found a close correlation between serological classification and that based on esterase analysis with starch gel electrophoresis in the classification of Bacillus thuringiensis. Robinson (1966) examined cell-free extracts of 24 organisms of the genus Corynebacterium by starch gel electrophoresis for catalase, peroxidase and esterase activity. The results indicated a separation of the members of the genus into distinct sub groups comparable to those obtained by grouping strains according to habitat. Plant pathogenic pseudomonads (32) belonging to different species as well as non-pathogenic strains (5) were examined for the esterase and catalase activity.

The method:-

The preparation of cell-free extract:-

The organisms were grown for 2 days on nutrient agar contained 2% (v/v) glycerol. The bacterial cells were harvested and washed two times with tris citrate buffer, pH 8.7, half the concentration used by Poulik (1957). The bacterial cells were broken by means of an ultrosonic desintegrator for 10 mins. in an ice bath. The preparation was centrifuged at 20,000 g. for 30 mins. at 5°C. The cell-free extract was then concentrated according to the method of Kohn (1959) to a volume of 8 ml. and

then dialysed against tris citrate buffer, as described before, for 24 hours at 5C.

The electrophoretic analysis was conducted in starch gel and the discontinuous buffer system used was the same as described by Lund (1965). The starch gel was made in the tris-citrate buffer as described before, and borate buffer pH 8.8 was used for the electrode vessels.

Electrophoresis was carried out at room temperature and continued until the brown line marking the line of discontinuity between the buffers migrates through the gel 10 cm. past the samples inserts. The "E_f" values of the catalase and esterase bands were measured as described before (experiment 51).

Detection of the enzymes:-

The starch gel was sliced and stained for catalase and esterase as follows

The detection of catalase:- The p-aminophenol and hydrogen peroxide method as described before (experiment 51) was used; the catalase bands stained dark brown where the oxygen was evolved in the gel. The potassium iodide - hydrogen peroxide method described by Baillie & Norris (1963) was also used.

The detection of esterases:- The method used for the detection of esterases was that of Lawrence, Melnick & Weimer (1960) and described by Baillie & Norris (1963). The gel slice was flooded with the following solutions. Tris maleate buffer 0.1 M, pH 6.5, 50 ml., 2 ml. of 1% (w/v) α-naphthylacetate in 50% acetone, and

50 mg., fast blue B salt. The esterase bands developed as red areas and reached maximum intensity in about 2 hrs. at room temperature.

The following organisms tested

Ps. syringae (8):- S5 - S8 - S20 - S36 - W1 - W183 - G2 and NZ7* strain

Ps. mors-prunorum (10):- p6* - P47* - D1 - D2 - D5 - D16 - N5 - C9 - C22 and C46 strain

Ps. syringae f. sp. populea (2):- P38* - P40*

Ps. phaseolicola (6):- P12 - P13 - P14 - P16 - P18 - NZ41

Ps. marginalis (4):- 667 - 1558 - 1559 - 1604

Ps. tabaci (2):- M44 - M45

Non-pathogenic pseudomonads (5):- 5A8 - 4A3 - F300 - Lk7 - CS4

Results:-

The esterase pattern of the organisms

Figure 18 shows the results and indicated the following:-

1. No differences were found between Ps. syringae isolated from pear, citrus, or cherry and Ps. syringae f. sp. populea. All the Ps. syringae strains possessed an esterase based at "E_f"98. A very weak band of esterase was detected occasionally in these organisms at "E_f"83. This is not always reproducable and takes a long time to appear after staining.

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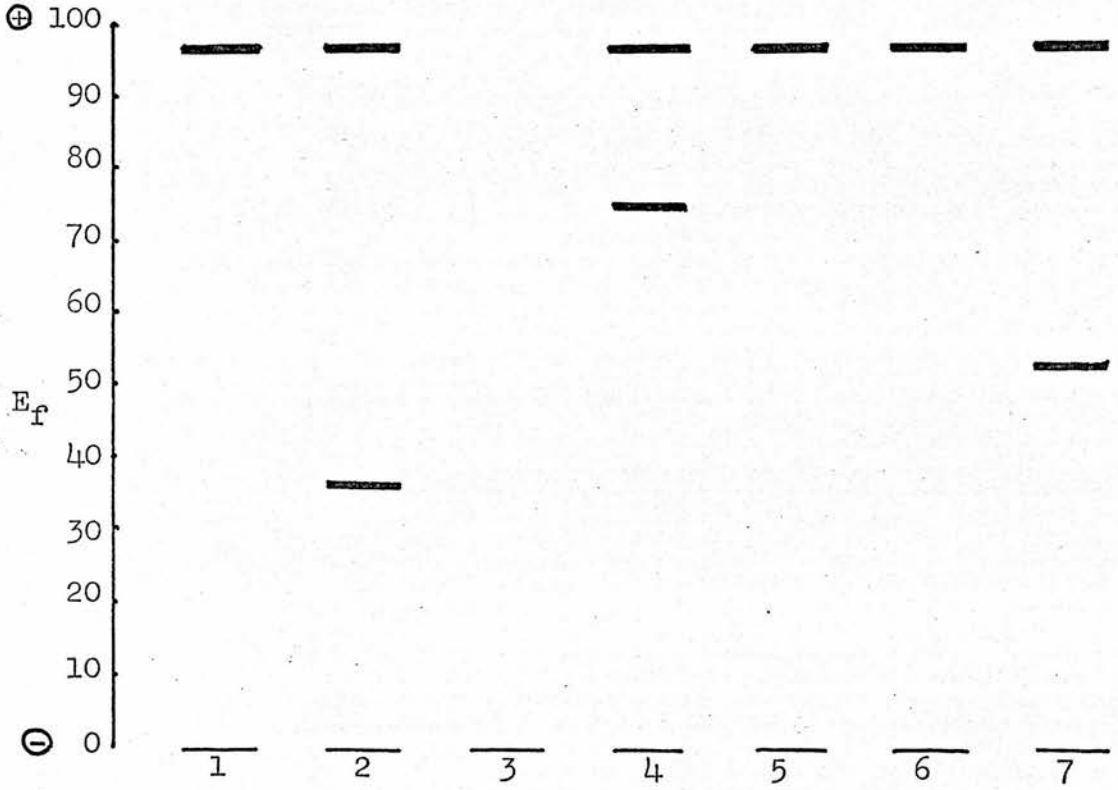
* Strains not tested for catalase activity.

Figure 18. The esterase patterns of different pseudomonads



1 = Ps. syringae strains

2 = Ps. mors-prunorum strains

3 = Ps. phaseolicola strains

4 = Ps. tabaci strains

5 = Ps. marginalis strains

6 = 4A3 - 5A8 - Lk7 - CS4 (Non-pathogenic strains)

7 = F300 Non-pathogenic strain

2. All the Ps. mors-prunorum strains possessed two esterases bands at "E_f"98 and "E_f"37. Only one strain, P47, possessed an esterase identical to Ps. syringae, and this strain could be considered to be Ps. syringae rather than Ps. mors-prunorum because of the similarity of the biochemical characters described in this study and those of Ps. syringae. The species name of that strain was also questioned in the departmental culture list.

3. No esterase bands were detected in Ps. phaseolicola strains.

4. Ps. tabaci strains possessed two esterase bands at "E_f"98 and "E_f"76.

5. Ps. marginalis strains as well as 4 of the non-pathogenic pseudomonads possessed identical esterase band at "E_f"98. One of the non-pathogenic strains, strain F300, possessed another band at "E_f"54. Plate 12 shows the esterase pattern of Ps. syringae and Ps. mors-prunorum strains.

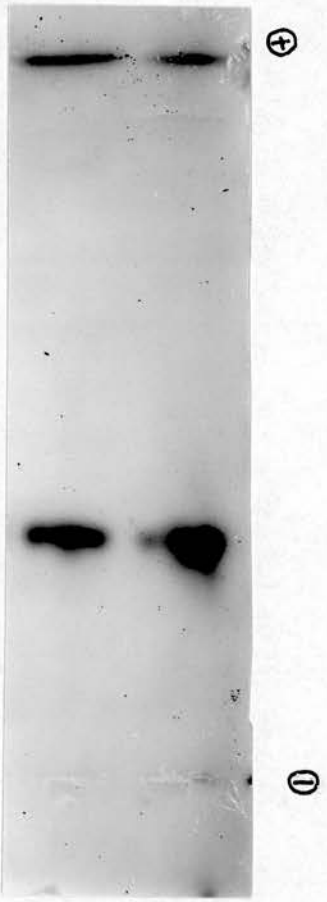
The catalase pattern of the organisms

The p-aminophenol-hydrogen peroxide method gave the best results compared with potassium iodide hydrogen peroxide method, since the colour of the background did not interfere with the colour of catalase bands.

The results indicated that there were no differences between Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola, and Ps. tabaci in their catalases. They all possessed two catalase bands at E_f34 and E_f48.

Plate 12

The esterase patterns of Ps. syringae strains and Ps. mors-prunorum strains



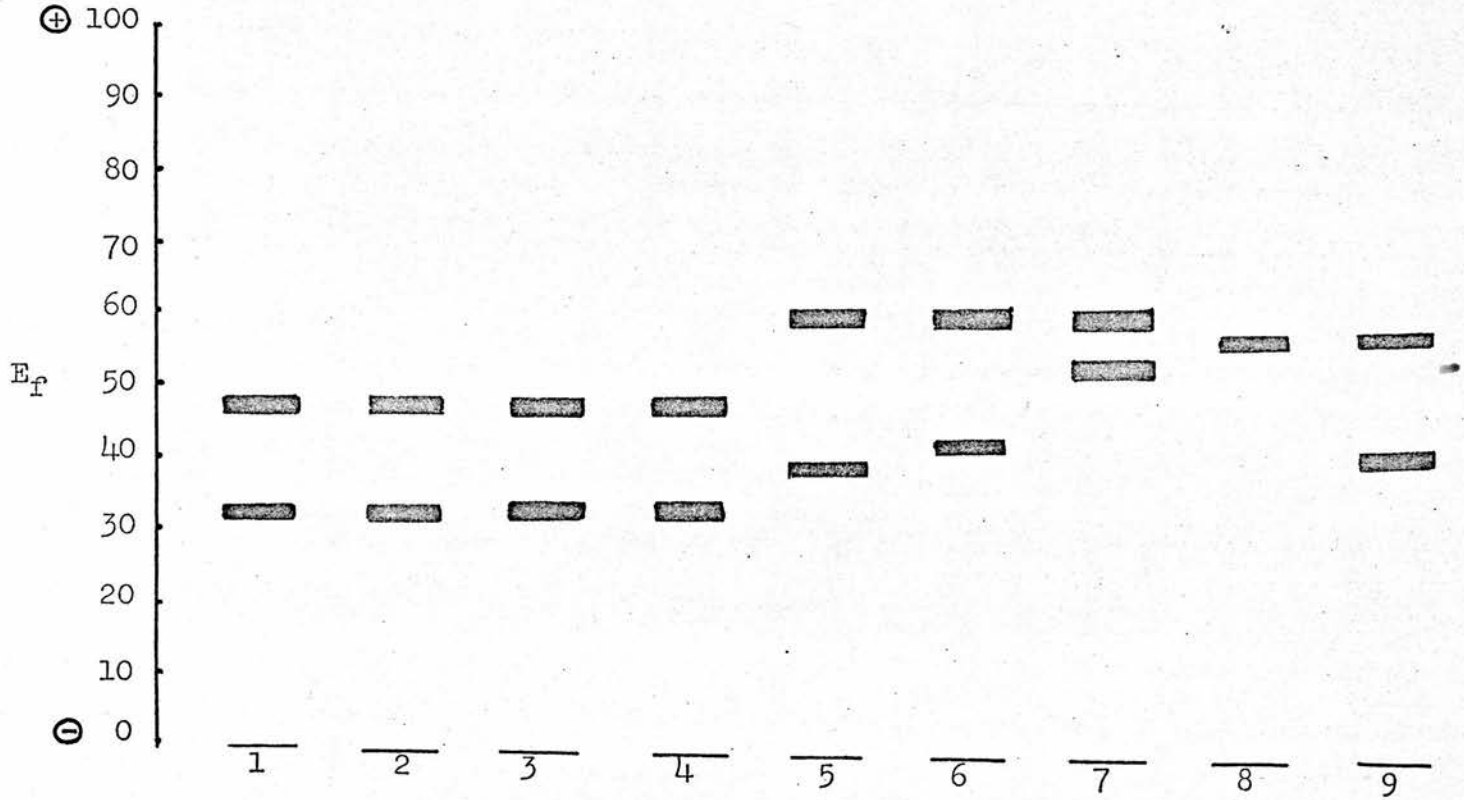
Ps. syringae strains

Ps. mors-prunorum strains

The Ps. marginalis strains, as well as the non-pathogenic pseudomonads, possessed a different pattern of catalase to those described for the other plant pathogenic pseudomonads. Ps. marginalis strains possessed two catalase bands at E_f38 and E_f60. The E_f60 catalase band was also detected in some of the non-pathogenic pseudomonads tested.

Figure 19 summarises the results of the catalase patterns.

Figure 19. The catalase patterns of the organisms



1 = Ps. syringae strains
2 = Ps. mors-prunorum strains
3 = Ps. phaseolicola strains
4 = Ps. tabaci strains

5 = Ps. marginalis strains
6 = F300
7 = Lk7
8 = 4A3
9 = CS4

} Non-pathogenic pseudomonads

The reduction of tetrazolium salts

A connection has been reported between the virulence of phytopathogenic bacteria and their ability to reduce triphenyltetrazolium chloride (T.T.C.). Using nutrient medium containing T.T.C., Kelman (1954) reported that in the case of Ps. solanacearum, cultures derived from red colonies were either weakly pathogenic or non-pathogenic whereas cultures from white colonies with pink centres were highly pathogenic. In contrast, Small & Worley (1956) reported that in the case of Ps. phaseolicola the red colonies were highly pathogenic whereas the white colonies were weakly pathogenic. More recently Friedman (1964) also found that the red colonies of Erwinia carotovora were more virulent than the colonies with small red centres, which were weakly virulent.

It was of interest therefore to study the reduction of tetrazolium salts and the colony appearance on tetrazolium agar in an attempt to discover any differences between the plant pathogenic and non-pathogenic pseudomonads.

Experiment 74. The reduction of 2,3,5-triphenyltetrazolium chloride (T.T.C.)

The ability of the organisms to reduce T.T.C. was tested in the presence of Na succinate as hydrogen donor. The reaction mixture has the following composition:-

0.1% (w/v) Na succinate
0.01% (w/v) T.T.C.

0.3 ml. of the succinate tetrazolium solution were added to fermentative tubes, which were inoculated with a loopfull of the 24 hours old cultures of the organisms grown on glycerol nutrient agar. The tubes were incubated at room temperature.

Organisms tested:-

The following organisms were tested.

Ps. syringae S5 - S8 - S20 - S36 - W1 - W183 - G2

Ps. mors-prunorum D1 - D2 - D5 - D16 - C9 - N5 - C22 - C46

Ps. phaseolicola P12 - P13 - P14 - P16 - P18 - NZ41

Ps. marginalis 667 - 1558 - 1559 - 1604

Non-pathogenic pseudomonads 5A8 - 4A3 - CS4 - Lk7 - F300 - Bt6 - Cb1 - Cb4 - 1A1 - Pt2 - Bn3 - 7A9

Results:-

Results indicated that the tetrazolium salt was reduced to red formazon within 10 mins. No significant differences were found between the plant pathogenic and non-pathogenic pseudomonads. The same results were also obtained when blue tetrazolium and tetrazolium violet were tested with representative strains.

Experiment 75. The colony appearance on tetrazolium agar

The same organisms used in the above experiment were streaked on nutrient agar pH7 containing T.T.C. at 0.005% (w/v) concentration the plates examined after 3 days incubation at 27°C.

Results:-

The colonies of Ps. syringae strains had large red centres

and a narrow colourless border, whereas those of Ps. mors-prunorum had small red centres and wide colourless borders. They were distinguishable from Ps. syringae colonies.

Ps. phaseolicola colonies had pale pink centres and wide colourless borders.

The Ps. marginalis colonies, as well as the non-pathogenic pseudomonads, were similar to those described for Ps. syringae colonies.

Experiment 76. The hydrolysis of nucleic acid

The ability of the organisms to hydrolyse the nucleic acid salt was studied in an attempt to discover any differences between the plant pathogenic and the non-pathogenic pseudomonads. The medium and the method of Jeffries, Holtman & Guse (1957) was used. The agar plates containing the nucleic acid sodium salt was inoculated, 4 organisms in each plate. Plates were incubated at 27°C for 5 days, then flooded with 1N Hcl. A positive reaction is indicated by a clear zone around the mass of growth.

Organisms tested:- The same representative strains as used in experiment 74 were tested.

Results:-

Results indicated that Pt2 - 7A9 - Bn3, atypical pseudomonads strains, gave a very strong reaction. The clear zone was about 15 mm. in diameter. The other organisms gave a weak reaction, 1 - 2 mm. in diameter and no differences were found between the plant pathogenic and non-pathogenic pseudomonads.

Experiment 77. The production of brown pigment from tyrosine

Crosse & Garrett (1963) reported that Ps. mors-prunorum strains are able to produce a brown pigment from tyrosine and in that they are different from Ps. syringae which did not produce such a reaction. It was of interest, therefore, to study the ability of the organisms to produce brown pigment from tyrosine.

The same representative strains as in experiment 74 were used. They were tested on the medium described by Hayward (1964). The plates were incubated at 27°C and examined up to 15 days after the incubation.

Results:-

The results of the test were variable and not reproducible, and in no case was the reaction definite. However, when glycerol nutrient agar containing 0.2% (w/v) L-tyrosine was used no brown pigment was detected in this medium. Hayward (1964) used the same medium as used by Crosse & Garrett (1963). He noted in the examination of Ps. solanacearum strains that "the results varied in intensity between isolates and between different colony form of the same isolate and apparently at different times of testing".

Discussion and Summary

Discussion

In this study an attempt was made to differentiate between the plant pathogenic pseudomonad and the non-pathogenic pseudomonad strains isolated from different sources. Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola, Ps. tabaci and Ps. marginalis were studied to find out why pathogens are able to establish themselves in the plants while the non-pathogenic pseudomonads are not able to do so. The plant pathogens enter the host environment through natural openings or wounds. Lewis (1953) proposed that a balance exists in the host between substances that inhibit and substances that promote parasite growth. The kinds and concentrations of these substances in the host vary as conditions change. These variations can be expected to account for varietal and species resistance and susceptibility of the host. Garber (1956) introduced a nutrition-inhibition hypothesis of pathogenicity. This hypothesis involves the two environments of the host, the nutritional environment and an inhibitory environment. These environments affect the fate of an invading parasite, and of the four possible combinations of the host environments, only the adequate nutrition and ineffective inhibition environments results in virulence.

From the nutritional point of view, the results described in this study indicated no significant differences between the plant pathogenic pseudomonads and the non-pathogenic type; both could satisfy their carbon and energy requirements from a wide range of sugars, organic acids, and amino acids. They were

also able to satisfy their nitrogen requirements from inorganic and organic sources. Therefore the nutritional environment of the host seems to satisfy the nutritional requirements of the organisms, pathogenic and non-pathogenic.

The study of the inhibitory environments (resistance factors) of the host indicated that the phenolic compounds of the hosts are important as a defence mechanism of the plant against fungal infection (Farkas & Kiraly, 1962; Cruickshank & Perrin, 1964). In these reviews there are many reports on the correlation between the phenolic content of the host and the disease resistance, and that the quinones, the oxidation products of these phenolic compounds, are more toxic to the fungi than the phenols. In this study an attempt was made to find differences between the plant pathogenic and the non-pathogenic pseudomonads in relation to their effect on phenolic compounds. The results indicated that the different species of the plant pathogenic pseudomonads could be grouped into two groups. The first group (Group I) consists of Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola, Ps. tabaci and the second group (Group II) consists of Ps. marginalis and related soft rotting organisms.

The first group of pathogens, which is the major group, differs from the second group and from the non-pathogenic pseudomonads, in that they are cytochrome oxidase negative whereas the non-pathogenic pseudomonads, as well as Ps. marginalis strains are cytochrome oxidase positive. In this study a correlation was found between the cytochrome oxidase test and

the presence of cytochrome c which was found in the non-pathogenic pseudomonads as well as in Ps. marginalis strains. Cytochrome c was not detected in organisms of the first group. The cytochrome oxidase test is widely used and has proved to be of great taxonomic value (Steel, 1961 ; Shewan, et al. 1960) and is also useful for the identification of Ps. aeruginosa strains (Kovacs, 1956; Gaby & Hadley, 1957). However, Klinge (1960) reported that the composition of the solid medium had no effect on the results of the cytochrome oxidase test. In this study it has been found that growing the organisms in the presence of excess glucose repressed the cytochrome oxidase test whereas growing the organisms in the presence of Na succinate enhanced the synthesis of the cytochromes. It was further found that the cytochrome c content was greatly increased when the organisms were grown on succinate whereas when they were grown on glucose, cytochrome c was barely detectable or undetectable. The non-pathogenic pseudomonads, as well as Ps. marginalis strains, were found in this study to be able to oxidize phenolic compounds to the highly toxic quinones. The ability of the cytochrome c cytochrome oxidase enzymes to oxidize phenolic compounds was also found by Nagasawa & Guttmann (1959). Smith (1954) also reported the ability of cytochrome c cytochrome oxidase to oxidize phenolic compounds as well as the Nadi reagent. However, the inhibitory effect of Na dieca, a copper chelating compound, on the oxidation of phenol, suggested a participation of a copper enzyme in the oxidation of phenols. A study was

made of the presence of the blue protein "azurin", a copper enzyme which was found in Ps. aeruginosa (Horio, 1958a) and the results indicated its absence from all the plant pathogenic pseudomonads of the first group and its presence in some but not all Ps. marginalis and the non-pathogenic pseudomonads strains.

The inability of the non-pathogenic pseudomonads to become established in the plant could be due to their ability to oxidize the phenolic compounds to quinones whereas the plant pathogenic pseudomonads are able to become established in the plants probably because they do not oxidize the phenolic compounds. The study of the 3 Xanthomonas strains indicated that although they are weakly positive in the cytochrome oxidase test they also did not oxidize phenols, and it was also shown that cytochrome c could not be detected in these strains. The study of the Erwinia species indicated that they did not oxidise the phenolic compounds and they are cytochrome oxidase negative, therefore the ability of erwinias as well as xanthomonads to become established in the plants could be due to their inability to oxidise the phenolic compounds. However, because of the small number of xanthomonads and erwinias examined in this study, more work needs to be done before such conclusions could be drawn. Other aspects relating to phenol-quinone toxicity were investigated and are discussed later on.

Some aspects of the plant defence mechanism against bacterial infection were studied and the results indicated that the phenolic content of the plants, the peroxidase activity, and to a lesser

degree the phenol oxidase activity of the plants were increased when the age of the plants increased, whereas the catalase of the plant decreased on the increase of the age of the plants. These aspects could explain the observation that when the age of bean plants increased, they became more resistant to Ps. phaseolicola infection. This was also found by Patel & Walker (1963) who reported that the youngest leaves of bean plants were more susceptible to Ps. phaseolicola infection than were the oldest leaves. Chand & Walker (1964) also found the same trend in cucumber plants inoculated with Ps. lachrymans. The increase in phenolic content, peroxidase, phenol oxidase are aspects of a defence mechanism of the plant resulting in the formation of the quinones. The results also indicated that the plant peroxidase is more active than the plant phenol oxidase in the oxidation of phenolic compounds to quinones if H_2O_2 is present. This observation led to the examination of the plant pathogenic and the non-pathogenic pseudomonads for their ability to produce H_2O_2. Different amino acids were tested as substrates from which H_2O_2 could be produced, in the presence and absence of other carbon sources, and the results indicated none of the first group of the plant pathogenic pseudomonads produced H_2O_2 from the different amino acids examined whereas the Ps. marginalis strains and the non-pathogenic pseudomonads produced H_2O_2 from these amino acids. The significance of the production of H_2O_2 could be very important and also could explain the inability of the non-pathogenic pseudomonads to be established themselves in

the plant because of their ability to produce H_2O_2, a substrate for the very active peroxidase system of the plant which peroxidized phenols to quinone. The examination of the xanthomonads indicated that they also did not produce H_2O_2. Strains of erwinia (26) also did not produce H_2O_2 (Moustafa, unpublished data). The ability of the plant pathogenic pseudomonads, xanthomonads and erwinias to establish themselves in the plant could also be due to their inability to produce H_2O_2 a substrate of the plant peroxidase. Furthermore, the catalase activity of the plants was found to be increased when the plants were infected. An electrophoretic study also indicated the presence of the bacterial catalase of Ps. phaseolicola in infected bean plants. The bacterial catalase may be of importance in attempts by the organism to destroy any H_2O_2 before the plant peroxidases could use it to peroxidize phenols to quinone.

The more virulent strains of Ps. phaseolicola studied by Rudolph & Stahmann (1964) were found to possess higher catalase activity than less virulent strains. These results suggested that the catalase activity of the pathogenic organisms could be of some importance in an attempt of the pathogen to become established in the plant. Further work on host-pathogen combinations in relation to the catalase activity could throw some light on the importance of such a factor in pathogenicity and on the plant defence mechanisms.

The results described in this study also indicated that all

the plant pathogenic pseudomonads belonging to the major group of pathogens (Group I) were able to infect tobacco plants whereas Ps. marginalis strains and the non-pathogenic pseudomonads did not infect the tobacco plants. Therefore the use of tobacco plants as a test plant for the detection of these pathogens could be of value. Klement (1963) and Klement et al. (1964) found similar results but they did not examine Ps. marginalis. Klement et al. (1964) also reported that Ps. tabaci injected into tobacco leaves started to multiply immediately and after 3-4 days bacterial numbers started to decline and the symptoms appeared only when maximum cell number was reached. They also found that Ps. syringae started to multiply in 24-48 hours in tobacco plants. The multiplication then stopped and the cell number remained constant, or decreased somewhat, and rapid necrosis of host cells could be observed simultaneously with inhibition of bacterial growth. They also found that the saprophytic pseudomonads did not multiply in the plant. Chand & Walker (1964) found the same trend when Ps. lachrymans was inoculated into cucumber leaves. The explanation of such a trend could be due to the plant response to bacterial infection. It has been found in this study that the phenolic content of the plants, the peroxidase activity and the phenol oxidase activity of the pseudomonad-infected plants were greatly increased. These findings on bacterial diseases are in agreement with those reported by many workers for fungal diseases (Farkas & Kiraly, 1962). It would seem, therefore,

that the increase in phenolic content and the phenol oxidizing enzymes is a general response of the plants to infection. An increase of the phenol oxidase and the peroxidase activity of potato plants infected with Erwinia atroseptica was also found (Moustafa, unpublished data).

The mechanism by which the phenolic content of the diseased plants increased was found in this study to be due to the increase of β-glucosidase activity of the infected plants. The activity of this enzyme, which is responsible for releasing the phenols from the glucosides, also increased in potato plants infected with E. atroseptica. However, bacterial β-glucosidase activity, if possessed by the pathogen, could also be responsible for such an increase in the phenolic content of the infected plants. But, as indicated before, Ps. phaseolicola has no B-glucosidase activity although β-glucosidase activity is greatly increased in the diseased plants. The increase in β-glucosidase activity of the infected plants could explain the results obtained by many workers in relation to fungal diseases.

The ability of the organisms to reduce quinone was studied and the results indicated that the plant pathogenic pseudomonads are able to reduce the quinone in the presence of sucrose. This could be due to their ability to produce 2-ketogluconate, a reducing compound, which could reduce the quinone. The non-pathogenic pseudomonads did not reduce the quinone. The ability of the plant pathogenic pseudomonads to reduce the quinone could be another factor by which they are able to establish themselves in

the plants.

It is of interest here to note that E. atroseptica, the causative organism of the black leg disease of potato, was the only species of Erwinia examined which was able to produce a reducing compound from sucrose, and such a property might be a factor for the pathogenicity of E. atroseptica. Graham & Dawson (1960) and Graham (1964) reported that it is not possible to distinguish the erwinias by physiological tests. However, the results described above could be of taxonomic value. The identity of the reducing compound produced from sucrose by E. atroseptica is not known at present but it has an R_f value of 0.31 whereas that of 2-ketogluconate produced by pseudomonads has an R_f value of 0.2 in isopropanol/water solvent. Therefore the compound produced by E. atroseptica is not 2-ketogluconate.

The alteration of plant resistance or susceptibility to pseudomonad infection was studied and the results indicated that bean plants treated with Na dieca, a phenol oxidase inhibitor, and L-ascorbic acid increased the pathogenicity of Ps. phaseolicola. L-ascorbic acid, a reducing agent, was shown in this study to abolish the toxic effect of quinone. Results also indicated that feeding plants with phenols or quinone, reduced or inhibited the disease symptoms and the resistance of the plants to infection was greatly increased.

The mechanism of pathogenicity is clearly not simple in character but is a phenomenon governed by more than one feature. This accepted fact was confirmed by the failure of non-pathogenic

pseudomonads to infect bean plants treated with Na-dieca and L-ascorbic acid.

The results described in this study also indicated that the plant pathogenic pseudomonads, with the exception of Ps. marginalis, did not hydrolyse arginine anaerobically whereas Ps. marginalis and the non-pathogenic pseudomonads were able to do so.

The study of the toxicity of some metals against pseudomonads and other Gram-negative organisms indicated that, in general, the non-pathogenic pseudomonads and Ps. marginalis strains were more sensitive to manganese than the other plant pathogenic pseudomonads. This character was used in conjunction with the phenol oxidase test for the isolation of the plant pathogenic pseudomonads. The analysis of manganese content of the healthy and diseased plants provided no explanation for the manganese results.

Agrobacterium species were found to tolerate a high concentration of manganese. This character was also used for devising a selective medium for the isolation of these organisms.

The results described in this study indicated a positive correlation between cytochrome oxidase, phenol oxidase and the production of H_2O_2 (the cytochrome oxidase positive strains of pseudomonads were found to be able to oxidize phenols and to produce H_2O_2), Baker (1966) reported that Ps. aptata, Ps. delphinii, and Ps. lachrymans were cytochrome oxidase negative. Therefore these organisms also could be grouped with the group I, which included Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola and

Ps. tabaci, and it seems reasonable to suggest that any cytochrome oxidase negative plant pathogenic pseudomonad should also be grouped in the same group.

A second group of plant pathogenic pseudomonads could be Ps. marginalis and related similar plant pathogenic pseudomonads. Baker (1966) studied some of the biochemical characters of Ps. marginalis and Ps. pastinaceae. Her results suggest that the two organisms are identical. This group of plant pathogens, although they are pathogenic, possess all the characters of the non-pathogenic pseudomonads. They are cytochrome oxidase positive, able to oxidize the phenols, and produce H_2O_2, however this group of pathogens are soft rotting organisms and in that they are different from the group I organisms, these soft rotting organisms do not seem to be true pathogens since Ps. marginalis strains failed to infect tobacco plants, and Ps. marginalis seems to be pathogenic only on the salad crops and storage organs of some plants and it could be that their soft rotting activity is the major or only character of their pathogenicity. It is of interest to note that the non-pathogenic pseudomonads which proved to be soft rotting strains, were also able to infect lettuce plants. These non-pathogens were unable to infect tobacco or bean plants.

A third group of plant pathogenic pseudomonads could be Ps. solanacearum strains, which were not included in this study. Hayward (1964) reported that Ps. solanacearum strains are cytochrome oxidase positive and therefore could probably oxidize

phenolic compounds, however this group of organisms causes a general wilt of different host plants. The pathogenicity of these organisms could be quite different from that of organisms causing localized infection, phenol toxicity may not be involved here. However further studies are required to explain the basis of pathogenicity of these organisms.

Table 34 shows the biochemical differences obtained between the plant pathogenic pseudomonads (Ps. syringae, Ps. mors-prunorum Ps. phaseolicola and Ps. tabaci) and the non-pathogenic pseudomonads including Ps. marginalis.

The results described in this study indicated that Ps. syringae, Ps. mors-prunorum, Ps. tabaci, Ps. phaseolicola and Ps. marginalis were different from each other in some biochemical and cultural characters. Many workers have questioned the validity of Ps. mors-prunorum as a species (Erikson, 1945; Paton, 1956; Laviekovich, Klement & Dowson, 1963). However, Crosse & Garrett (1963) reported that Ps. mors-prunorum isolates were biochemically distinct from Ps. syringae isolates and that intermediate forms exist between the two species. Billing (1963) also reported that the majority of isolates of Ps. mors-prunorum and Ps. syringae were distinct. The results described in this study indicated that Ps. syringae strains are different from Ps. mors-prunorum strains in some biochemical characters, and the electrophoretic analysis of the esterases of these organisms confirmed these differences. The analysis of esterase patterns of the different species of pseudomonads seems to be very useful

in separating these species, and it would be of interest to find out what the esterase pattern is of the so-called intermediate forms between Ps. syringae and Ps. mors-prunorum. Table 35 shows the differences between different plant pathogenic Pseudomonas species studied.

Bergey's Manual (1957) cites 90 species of phytopathogenic Pseudomonas, these species names cannot be accepted as true species, on the grounds that they were created according to the name of the host plant from which they have been isolated, or according to the name of the author who first isolated the organism. This is illustrated in the results of Baker (1966) who reported some of the biochemical characters of different plant pathogenic Pseudomonas species. Her results show that Ps. marginalis and Ps. pastinaceae are identical; in this case there is no reason to retain the two organisms as two different species. Undoubtedly, comparative studies and the determination of the host range of these species will cut down their numbers considerably. However, the quantative and qualitative analysis of the phenolic content and other compounds of the hosts as well as the biochemical differences between the different acceptable species of pseudomonads, could be factors which determine the pathogenicity of specific organisms to specific host plants.

The control of bacterial diseases is one of the most important subjects in plant pathology. Antibiotics and Bordeaux mixture have been extensively used for the control of bacterial and

fungus diseases. Dye (1953) found that streptomycin was effective and gave significant control of peach seedlings against Ps. syringae infection. It is of interest to note that it has been reported that treatment of some potato varieties with streptomycin leads to an enhancement polyphenol oxidase activity of the plant, (Farkas & Kiraly, 1962). Also the effect of spraying copper compounds could be in part due to the Cu^{++} stimulating effect on the phenol oxidase activity of the plants. Such suggestions need investigation. This hypothesis does not exclude the direct toxic effect of streptomycin, as Paton (1956) found that strains of Ps. syringae and Ps. morsprunorum, were sensitive to streptomycin. The results described in this study also showed that copper is toxic against the organisms. The resistant varieties of different plants against bacterial diseases could also be achieved through the selection and hybridization of varieties containing high concentrations of phenolic compounds.

Table 34. The biochemical differences between the plant pathogenic pseudomonads and the non-pathogenic pseudomonads and Ps. marginalis strains

	plant pathogenic pseudomonads	Non-pathogenic and Ps. marginalis strains
cytochrome oxidase	-	+
phenol oxidase	-	+
The presence of cytochrome c	-	+
The presence of blue protein	-	d
The production of H_2O_2	-	+
p-benzoquinone reduction	+	-
Pathogenicity on tobacco plants	+	-
Manganese tolerance	+*	-*
Arginine dihydrolase test	-	+

d = Azurin was detected in some but not all the non-pathogenic pseudomonads and Ps. marginalis strains

* = The majority of the strains

Table 35. The biochemical differences between the different plant pathogenic pseudomonas species

	Ps. syringae	Ps. morsprunorum	Ps. phaseolicola	Ps. tabaci	Ps. marginalis
Utilization of tartrate	-	+	-	+	+
Utilization of lactate	+	-	-	+	+
Utilization of ammonium lactate as carbon and nitrogen source	+	-	-	+	+
The hydrolysis of arbutin	+	-	-	+	+
Acid production from sorbitol	+	+	-	+	+
Esterase pattern	See experiment 73.				
Soft rotting activity	-	-	-	-	+

Summary

The aim of this study was first to examine the relationships between some of the plant pathogenic Pseudomonas species (Ps. syringae, Ps. mors-prunorum, Ps. phaseolicola and Ps. marginalis), and secondly to compare the plant pathogenic pseudomonads with the non-pathogenic pseudomonads, isolated from soil and other sources, in an attempt to find the biochemical differences between the two groups of organisms in the hope of discovering why pathogens become established in plant tissue. The nutritional requirements of the organisms and their activity on various carbon and nitrogen sources were studied. The results indicated that both pathogenic and non-pathogenic pseudomonads were able to satisfy their nutritional requirements from a wide variety of sources. The cytochrome oxidase activity of the organisms and its relationship to the presence of cytochrome c and the blue protein (azurin) was studied. Results indicated that the plant pathogenic pseudomonads, with the exception of Ps. marginalis strains, were cytochrome oxidase negative. They possessed neither cytochrome c nor azurin, and in that they differed from the non-pathogenic pseudomonads and Ps. marginalis which are cytochrome oxidase positive and possess cytochrome c. Azurin was detected in some of these organisms.

The action of the organisms on phenolic compounds was studied and the results indicated that the non-pathogenic pseudomonads convert phenolic compounds to quinones oxidatively and possibly peroxidatively by their ability to accumulate

hydrogen peroxide, a substrate for the plant peroxidase. On the other hand, the plant pathogenic pseudomonads, califorms and xanthomonads failed to convert phenolic compounds to quinones. They were also able to reduce quinones back to the phenols. The inability of all the plant pathogens studied, except Ps. marginalis strains, to convert phenols to the highly toxic quinones and their ability to reduce quinones to phenols (a possible counter-action to the plant's defence mechanism) may prove to be a major factor in their successful establishment in the plant. The ability of non-pathogenic pseudomonads to oxidase phenols to quinones, coupled with the possibility of their producing H_2O_2 as a substrate for plant peroxidases which rapidly peroxidises phenols to quinones, (H_2O_2 was not produced by the plant pathogens) may be the significant factor responsible for their failure to establish themselves in plants.

The exception to this general rule is Ps. marginalis, considered by many to be a pathogen, which has proved to be much more closely related to non-pathogenic pseudomonads than to the pathogens. Reasons are given for considering that this organism should not be considered a true pathogen.

The response of the plants to bacterial infection was studied. The phenol oxidase, peroxidase, catalase and β-glucosidase activity of the plants, were found to increase when the plants were infected, leading to an increase in the availability of both phenols and quinones. The catalase found in infected plants appeared to be of bacterial origin and its role in the

establishment of the pathogenic organism is discussed.

The toxicity of some metals against Gram-negative bacteria was studied and the results indicated that the plant pathogenic pseudomonads were able to tolerate a higher concentration of manganese than were the non-pathogenic pseudomonads, and that members of the genus Agrobacterium were exceptional in their ability to tolerate very high concentrations of manganese. These results were useful in the development of selective media for the isolation of plant pathogenic pseudomonads and agrobacteria.

The analysis of catalase and esterase content of the organisms, by starch gel electrophoresis, revealed that the esterase pattern of the organisms was useful in the differentiation of the various plant pathogenic pseudomonads. The results of a study of their biochemical characters, indicated that the named species of the plant pathogenic pseudomonads studied were distinct from one another and were also different from the non-pathogenic pseudomonads isolated from soil and other sources.

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