A COMPARATIVE STUDY ON A YELLOW EPIPHYTIC BACTERIUM OF GRAINS AND CERTAIN SPECIES OF *XANTHOMONAS* DOWSON

By

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INTRODUCTION

Various investigators have found that an appreciable proportion of the epiphytic microflora of cereal seeds and green plants consisted of short rods that formed typical yellow colonies on nutrient agar.

Amos (1) isolated this yellow epiphyte in considerable numbers from wheats and wheat flours and noted its similarity to *Flavobacterium trifolium* (evidently *Flavobacterium trifolii* Mack).

James et al. (15) made counts of bacteria in washings from commercial wheats and found this yellow epiphyte to be present even after repeated washings. They represented 34%, 37% and 30% of the population isolated from wash water, after 12 washings of No. 1, No. 4 and No. 6 grades, respectively, of Manitoba Northern red spring wheat. The fact that these bacteria were present on wheat even after repeated washings offered presumptive evidence of the existence of a bacterial flora that proliferated on wheat. Data presented indicated a relationship between quality, as represented by grades, and total numbers of bacteria. The numbers of bacteria became progressively larger as the grade lowered. Many of the low grades, undoubtedly, were exposed to weathering conditions for long periods of time. This exposure to dampness and warmth would favor the activity of the epiphytic microflora.

Wilson (29) studied 22 isolates and found they had most of the cultural and physiological characteristics of Phytomonas translucens var undulosa Hagborg (now Xanthomonas translucens f. sp. undulosa); a plant pathogen which is the causal agent of "bacterial black-chaff" disease. The author stated that it was conceivable that wheat might harbour a parasitic species with many of the characteristics of a pathogen - a phenomenon that is accepted with respect to parasitic bacteria on the human skin.

Simmonds (23) demonstrated the existence of a bacterial surface flora, on seeds and on other parts of cereal plants, which was antagonistic to Helminthosporium sativum P.K. and B., a common root disease pathogen. Treated cereal seeds were placed in a Petri plate containing nutrient agar spotted with the mold. Retarded growth of the mold was noticeable near the seed. This investigator suggested that the epiphytic flora might, therefore, by their tendency to inhibit growth, play a prominent role in inhibiting the infection of cereals by this fungus. A detailed study of the bacteria concerned was not undertaken. However, when isolations were made, a bacterium of the same gross morphology - a short motile rod - was most frequently obtained. While this description could be applied to the yellow epiphyte, it could also be applied to a species of Pseudomonas which constitutes a considerable portion of the epiphytic microflora of grains (15, 25, 29).

In contrast, the work reported by James et al. (16), appeared to indicate that the phenomenon reported by Simmonds was due probably to competition for nutrients, rather than to the production of any antagonistic substance. These investigators produced evidence that a filtrate of a culture of the yellow epiphyte did not exert the same antagonistic action as did the culture. The same finding was reported on the mixed bacterial flora on grain.

Stark (25) conducted a comparative study on 38 isolates of this yellow epiphyte from wheat, oats, barley and flax. He studied in detail their morphology, cultural characteristics and physiology and compared his findings with those of Duggeli (8), Mack (18), and Huss (14). While there were certain differences among the cultures referred to, especially with regard to physiology, it appeared evident that they were not of sufficient importance to justify considering them to be in different species. Most of the complications arose because different investigators used different methods. According to Stark, the logical place for this species, on the basis of flagellation (one or two polar flagella), appeared to be in the genus Xanthomonas. Stark, however, did not compare his isolates with known representatives of the genus.

In contrast to the findings of Stark, Burkholder (5) stated that while the yellow epiphyte was similar in color to species of Xanthomonas it was distinctly unlike them in other characters. He claimed (4) that isolates of the

yellow epiphyte, encountered in his studies, resembled organisms usually placed in the genus Aerobacter - motile with peritrichic flagella and sometimes producing gas.

According to Burkholder (5) the relationships of the phytopathogenic bacteria in the Eubacteriales are with various soil types, whilst xanthomonads, as far as is known have no counterpart with other bacteria - unless it is with Pseudomonas trifolii.

Goldstein (10) conducted a comparative study on 13 isolates of the yellow epiphyte from wheat and nine known representatives of the genus Xanthomonas. She found, in particular, a close relationship between her isolates and the representatives of Xanthomonas known to produce the "black-chaff" disease on cereals. Goldstein considered the difference between the two groups to be no greater than the differences ordinarily used to distinguish between species. She found no differences in morphology or cultural characteristics. The only physiological difference which could possibly exclude the yellow epiphyte from being considered a member of the genus Xanthomonas was the utilization of asparagine as the sole source of carbon and nitrogen. It should be noted, however, that the use of asparagine as the only source of carbon and nitrogen was not mentioned by Dowson (7), when he first proposed the genus Xanthomonas.

Starr (27) carrying out experiments on the nutritional requirements of the genus Xanthomonas found that certain species, which he referred to as "non-exacting", grew in a

glucose, NH_4Cl, basal salts medium. Further, certain species required glutamic acid and others methionine, while Xanthomonas pruni required nicotinic acid. In a study on several "non-exacting" isolates, Starr found that the addition of glucose to the asparagine medium, resulted in growth at least as good as in the basal medium. He suggested that the inadequacy of asparagine was probably due to its inappropriateness as a carbon or energy source.

The literature reviewed does not refer to the probable pathogenicity of the yellow epiphyte. The genus Xanthomonas contains a number of pathogens which are culturally similar, differing only in the hosts they infect (12). Burkholder (5) suggested that there might be a transition from saprophytism to pathogenicity in certain cases. He did not believe this to be a rapid transition but probably on a par with other evolutionary trends in micro-organisms.

Reid et al. (20) expressed the belief that the tobacco pathogens known to plant pathologists as Pseudomonas tabaci and Pseudomonas angulata were merely the saprophytic soil species Pseudomonas fluorescens, which attacked the tobacco under conditions unfavorable to the plant. To support their contentions they presented data to show that serologically the species were identical.

The members of the genus Xanthomonas and the yellow epiphyte referred to as Pseudomonas trifolii are both characterized by Bergey et al. (3) as producing a water insoluble pigment. According to Burkholder (5), the intracellular yellow pigment occurring in the members of the genus

Xanthomonas has been shown to be identical in all species, but different from the yellow pigment found in species of plant pathogens in other genera.

Starr (26) found that the predominant Xanthomonas pigment was firmly adsorbed on magnesia or alumina and a definite chromatogram was given throughout the genus. A second much less abundant pigment could be demonstrated in some Xanthomonas isolates by chromatography. The pigments reacted similar to carotenoid alcohols.

It is apparent from the above that much more detailed study of this yellow epiphyte is necessary before it can be classified. For example, studies on pathogenicity, on the type of pigment, and on several other aspects, should be carried out simultaneously on several isolates and a number of known representatives of Xanthomonas.

Accordingly, this study was undertaken. It was divided into three parts:

Part 1 Morphology, Cultural Characteristics and Physiology

Part 2 Tests of Pathogenicity

Part 3 Nature of the Pigment

SOURCES OF CULTURES

Samples of wheat, oats, barley, flax and soybeans from various sources were supplied through the courtesy of The Department of Plant Science, The University of Manitoba. The method of James et al. (15) was followed for the isolation of the bacteria. A 10 gm. portion of each sample was transferred aseptically to a six ounce screw top bottle containing 10 gm. sterile gravel and 100 ml. sterile water. This initial dilution was shaken for 30 min. on a to and fro shaking machine. Appropriate dilutions were made and plates were prepared using Difco nutrient agar as a substrate for the bacteria. Incubation was at 25°C for five days.

Typical yellow colonies were selected from plates prepared from each sample of seed. Six of these which conformed with the description of previous investigators (10, 25) with respect to shape of cell, size, clumping arrangement and motility were transferred to nutrient agar slants for further study - thus totaling 30 isolates in all. These were replated and reisolated to make certain that each represented a pure culture. In addition a culture isolated from the coleoptile of Reward wheat which had been surface disinfected, supplied through the courtesy of Dr. R. J. Ledingham of the Dominion Laboratory of Plant Pathology, Saskatoon, Saskatchewan, was transferred to the same medium for comparative study. The code number of each isolate

and its source follows:

w-1 to w-3	macaroni wheat (Triticum durum), grown at Winnipeg, Manitoba, 1949.
w-4	Redman, a hard spring wheat, grown at Saskatoon, Sask., 1949.
w-5	Thatcher, a hard spring wheat, grown at Saskatoon, Sask., 1949.
w-6	Marquis, a hard spring wheat, grown at Saskatoon, Sask., 1949.
O-1 and O-2	Exeter oats grown at Winnipeg, Man., 1949.
O-3 and O-4	Garry oats grown at Winnipeg, Man., 1949.
O-5 and O-6	Fortune oats grown at Winnipeg, Man., 1949.
B-1 to B-6	Montcalm barley grown at Winnipeg, Man., 1949.
F 1 to F-6	Dakota flax grown at Winnipeg, Man., 1949.
S-1 to S-6	Pagoda Soybeans grown at Winnipeg, Man., 1949.
L-2	supplied through the courtesy of Dr. R. J. Ledingham, The Dominion Laboratory of Plant Pathology, Saskatoon, Sask., 1950.

The representatives of the "black chaff" type bacteria were supplied by Dr. W. A. F. Hagborg, Dominion Laboratory of Plant Pathology, Winnipeg. These follow:

H-1	Xanthomonas translucens f. sp. undulosa, 3258, isolated from Thatcher wheat collected at Mariapolis, Man., July 27, 1945.
H-2	Xanthomonas translucens f. sp. undulosa, 1552, isolated from Marquis wheat collected at Morden, Man., July 23, 1943.
H-3	Xanthomonas translucens f.sp. cerealis, 3040, isolated from Thatcher x Regent. R.L. 2040, collected at Winnipeg, Man. July, 1944.
H-4	Xanthomonas translucens f.sp. hordei-avenae, 1907, isolated from barley collected at Winnipeg, Man., July 27, 1940.

Other representatives of Xanthomonas were obtained from The American Type Culture Collection, Washington, D. C. as follows:

T-1 Xanthomonas carotae, 10547.

T-2 Xanthomonas campestris, 7381.

T-3 Xanthomonas pruni, 10017.

EXPERIMENTAL

Part 1 - Morphology, Cultural Characteristics and Physiology

Unless otherwise stated, all studies were carried out according to procedure outlined in The Manual of Methods for Pure Culture Study of Bacteria (6).

Morphology

Vegetative cells of the yellow epiphyte cultured on nutrient agar at 25°C for 24 hr. appeared as rods with rounded ends to coccoid forms, occurring singly and occasionally in pairs and measuring 0.5 to 0.8 by 0.5 to 1.6u. They were gram negative and actively motile. In negative mount preparations there was marked evidence of differentiation in internal structure.

Each pathogen studied showed greater uniformity in length of cells and did not show the same type of differentiation in negative mount preparations.

Stark (25) reported the average size of vegetative cells of isolates of the yellow epiphyte to be 0.8 by 1.6u; Wilson (29), 0.6 to 0.8 by 1.2 to 1.6 u; and Goldstein (10), reporting on 78 cells, 0.7 $\pm$ .03 by 1.7 $\pm$ 1.2 u. The last investigator mentioned measured 54 cells of named species of Xanthomonas. They ranged from 0.7 $\pm$ .07 by 1.7 $\pm$ 1.1 u. While no specific mention of variation in length was made, the figures quoted by the above investigators indicated that the cells measured by them varied as widely as did

those in this study. Amos (1) studied isolates of this yellow epiphyte from wheat and wheat flour and, while he did not quote specific measurements of cell size, remarked on the presence of many short rod forms which he referred to as "cocco - bacilli".

Cultural Characteristics

Nutrient agar colonies of isolates of this epiphyte, incubated at 25°C for five days, were circular, entire, convex, glistening, smooth and yellow. They ranged in size from pin point to 6 m.m. Stark (25) presented evidence which appeared to show that variation in colony size was not due to hereditary transmission but to normal variation.

The intensity of the yellow color varied somewhat, although this characteristic did not prove to be transmissible. An isolate from a colony of one intensity, when plated, produced colonies of varying intensities. Bamberg (2), working with five cultures of Bacterium translucens var undulosa (now Xanthomonas translucens f.sp. undulosa), found similar variations in the color produced.

The cultures of Xanthomonas and the epiphytes studied produced colonies on nutrient agar which were not distinguishable. Nutrient agar streak cultures and nutrient broth cultures of these pathogens likewise were not distinguishable from cultures of the epiphyte.

Reduction of Nitrate

For each culture, duplicate tubes of nitrate broth were examined after incubation at 25°C for three days.

Each culture was tested for nitrites by adding a few drops of sulfanilic acid (8 gm. sulfanilic acid in one litre of 5 N acetic acid) and α naphthylamine reagent (5 gm. α naphthylamine dissolved in one litre of 5 N acetic acid) to 0.5 ml. of culture on a porcelain spot plate. The results are presented in Table 1.

Table 1. Reduction of nitrate to nitrite by isolates of the yellow epiphyte and certain named species of *Xanthomonas*.

Epiphyte	Duplicate 1	Duplicate 2	Epiphyte	Duplicate 1	Duplicate 2
w-1	+	+	B-4	+	+
w-2	+	+	B-5	+	+
w-3	+	+	B-6	+	+
w-4	+	+	F-1	+	+
w-5	+	+	F-2	+	+
w-6	+	+	F-3	+	+
0-1	+	+	F-4	+	+
0-2	+	+	F-5	+	+
0-3	-	-	F-6	+	+
0-4	+	+	S-1	+	+
0-5	+	+	S-2	+	+
0-6	-	-	S-3	+	+
B-1	+	+	S-4	+	+
B-2	+	+	S-5	+	+
B-3	+	+	S-6	+	+
			L-2	+	+
Xanthomonas			*Xanthomonas*		
H-1	-	-	T-1	-	-
H-2	-	-	T-2	-	-
H-3	-	-	T-3	-	-
H-4	-	-	Control	-	-

Table 1 shows that the yellow epiphyte reduced nitrate to nitrite with the exception of isolates 0-3 and 0-6, while none of the species of *Xanthomonas* tested produced nitrite.

Certain additional data on the yellow epiphytes studied by Stark (25) and Goldstein (10) are presented in Table 2 along with similar information on *Pseudomonas trifolii* Huss as presented by Bergey et al (3).

Except in a few instances, which will be discusssed

Table 2. Additional characteristics of the yellow epiphyte based on reports of other investigators.

	Pseudomonas trifolii Huss	Stark	Goldstein
Gram stain	negative	negative	negative
Flagella	Single polar	1-2 polar	1-4 polar
Spores	absent	absent	absent
Nutrient broth	turbid, pellicle	turbid pellicle	turbid, pellicle
Gelatin liquefaction	positive	positive	positive
Litmus milk	alkaline, slow coagulation	alkaline slow peptonization	alkaline, slow peptonization
Nitrate reduction			
to nitrite	positive	positive	positive
to ammonia	negative	negative	negative
Indole production	positive	negative	negative
H_2S production	positive	positive	positive
Fat hydrolysis	- -	Positive	positive
Starch hydrolysis	- -	negative	negative
Monosaccharides			
Pentoses			
Xylose	acid	acid	- -
Arabinose	acid	acid	- -
l-Rhamnose	- -	acid	- -
Hexoses			
Glucose	acid	acid	- -
d. Mannose	- -	acid	- -
d. Galactose	- -	acid	- -
d. Fructose	- -	acid	- -

Table 2. Cont'd.

	Pseudomonas trifolii Huss	Stark	Goldstein
Disaccharides			
Sucrose	acid	acid	- -
Maltose	- -	acid	acid
Lactose	no acid	acid	acid
Trisaccharides			
Raffinose	- -	unchanged	- -
Polysaccharides			
Starch	- -	rarely acid	- -
Dextrin	- -	not acid	- -
Inulin	- -	not acid	- -
Glucosides			
Salicin	- -	acid	acid
Alcohols			
d-Mannitol	acid	- -	- -

-- Not reported.

further, the description on the three types agreed remarkably closely.

Flagella

There is discrepancy with regard to the number of flagella. Huss reported one flagellum; Stark, one or two; and Goldstein one to four. These differences may not be serious, the notable feature being the unanimity with respect to polar attachment.

Fermentation of lactose

Both Stark and Goldstein reported the production of acid from lactose while Huss found no acid produced. However, different media and methods were used in making the determinations. Huss(1) titrated the acid produced in a carbohydrate peptone broth against 0.25 N NaOH. Stark, and Goldstein, used an inorganic basal medium plus lactose and measured the pH electrometrically. Salvin and Lewis (21) pointed out that proteinaceous media may be utilized by certain bacteria with the formation of ammonia which neutralizes the acid produced from the fermentable substance.

Production of indole

Stark tested for indole using Kovac's modification of the Ehrlich Bohme test. Goldstein used the Gnezda oxalic acid technic and confirmed the results using the Goré modification of the Ehrlich Bohme test. These methods are endorsed by The Society of American Bacteriologists (6).

(1) Quoted from Stark (25)

Huss added, among other substances, a few drops of 10% H_2SO_4 when making the test. Sandiford (22) warned that acid-containing reagents might give false positive reactions in cultures tested for indole.

Procedure for testing growth in certain nutrient media

Isolates of the yellow epiphyte and the named species of Xanthomonas studied were tested for growth in certain nutrient media by the following procedure.

All glassware was soaked at least 24 hours in dichromate cleaning solution, rinsed thoroughly in tap water and then in distilled water. The media were sterilized in 5 in. test tubes after the pH had been adjusted with 0.1 N NaOH using a Coleman Model 3D Electrometer.

Cells were transferred from 48 hour nutrient agar slant cultures to 20 ml. sterile water. Inoculations were made by wire loop to duplicate tubes of a test medium from this suspension of cells. This procedure, proposed by Starr and Weiss (28), was followed in order to eliminate carry over of agar or growth accessory factors which might support growth, rather than the nutrients under test. To ensure that failure to grow in the test medium could be attributed to the insufficiency of the medium, rather than to dead inocula, transfers were made simultaneously to duplicate tubes of nutrient broth. Growth in nutrient broth would indicate that the culture was viable and that failure to grow in the test medium could be attributed to the insufficiency of the medium.

Measurements of light transmission were made with a Coleman Model 11 Spectrophotometer standardized at 100% transmission with an uninoculated check. The wavelength was set at 450 mμ. Each duplicate was shaken 10 times and transferred to one of the matched cuvettes. The reading was then made. Relative growth was expressed in terms of density units calculated from the relationship $D = 2 - \log T$, where D is the density and T is percent light transmission.

Asparagine as the sole source of carbon and nitrogen.

According to Bergey et al. (3), the genus Xanthomonas contains organisms for which asparagine is not sufficient as the sole source of carbon and nitrogen. All isolates were tested for growth in the nutrient medium recommended by Starr and Weiss (28). The composition of the medium follows:

0.1% KH_2PO_4
0.02% KCl
0.02% $MgSO_4 \cdot 7H_2O$
0.5% l - asparagine
pH7.4 after sterilization

Incubation was at 25°C for eight days. The results, presented in Table 3, show a difference in growth between yellow epiphytes and the cultures of Xanthomonas in a medium containing asparagine as the sole source of carbon and nitrogen. The epiphytes all grew - with the exception of one isolate 0-3, while the pathogens grew scantily if at all.

Table 3. Relative growth of isolates of the yellow epiphyte and certain named species of Xanthomonas in a medium containing asparagine as the only source of carbon and nitrogen - expressed as density units. (1)

Epiphyte	Duplicate 1	Duplicate 2	Epiphyte	Duplicate 1	Duplicate 2
w-1	0.44	0.35	B-4	0.29	0.31
w-2	0.55	0.57	B-5	0.32	0.27
w-3	0.40	0.36	B-6	0.38	0.35
w-4	0.35	0.39	F-1	0.34	0.35
w-5	0.34	0.30	F-2	0.27	0.36
w-6	0.30	0.38	F-3	0.35	0.31
0-1	0.31	0.27	F-4	0.35	0.32
0-2	0.30	0.29	F-5	0.29	0.30
0-3	0.00	0.00	F-6	0.36	0.34
0-4	0-34	0.33	S-1	0.27	0.34
0-5	0.24	0.30	S-2	0.37	0.37
0-6	0.12	0.11	S-3	0.44	0.46
B-1	0.38	0.34	S-4	0.29	0.30
B-2	0.30	0.25	S-5	0.32	0.38
B-3	0.32	0.25	S-6	0.28	0.30
			L-2	0.39	0.37

Xanthomonas			Xanthomonas		
H-1	0.00	0.00	T-1	0.01	0.02
H-2	0.00	0.00	T-2	0.01	0.00
H-3	0.00	0.01	T-3	0.01	0.00
H-4	0.00	0.01			

(1) Calculated from the relationship $D = 2 - \log T$, where D is density and T is percent light transmission.

The above experiment was repeated except that the medium was adjusted to pH5.0 instead of pH 7.4. All cultures gave the same reading as the uninoculated control, consequently, all density values were zero. An isolate of Pseudomonas aeruginosa grew well in this medium.

To determine whether the density values presented in Table 3 mean much in terms of good growth, six isolates of the yellow epiphyte were cultured in the asparagine medium along with one isolate in the basal medium of Starr to which glutamic acid was added (see page 26). The reaction of each medium was adjusted to pH 6.8. The results are shown graphically in Figure 1.

The density value of the isolate grown in Starr's glutamic acid medium was 0.21 units at one day, which was the same as the average density value of the six isolates cultured in the asparagine medium at three days. At one day the highest density value of any of the six was 0.05 units.

Arginine as the sole source of carbon and nitrogen

The composition of the medium follows:

0.1% KH_2PO_4
0.02% KCl
0.02% $MgSO_4 \cdot 7\ H_2O$
0.1 % 1 - arginine monohydrochloride
pH 7.2 after sterilization.

Incubation was at 25°C. At six days the cultures showed no visible turbidity, therefore spectrophotometer

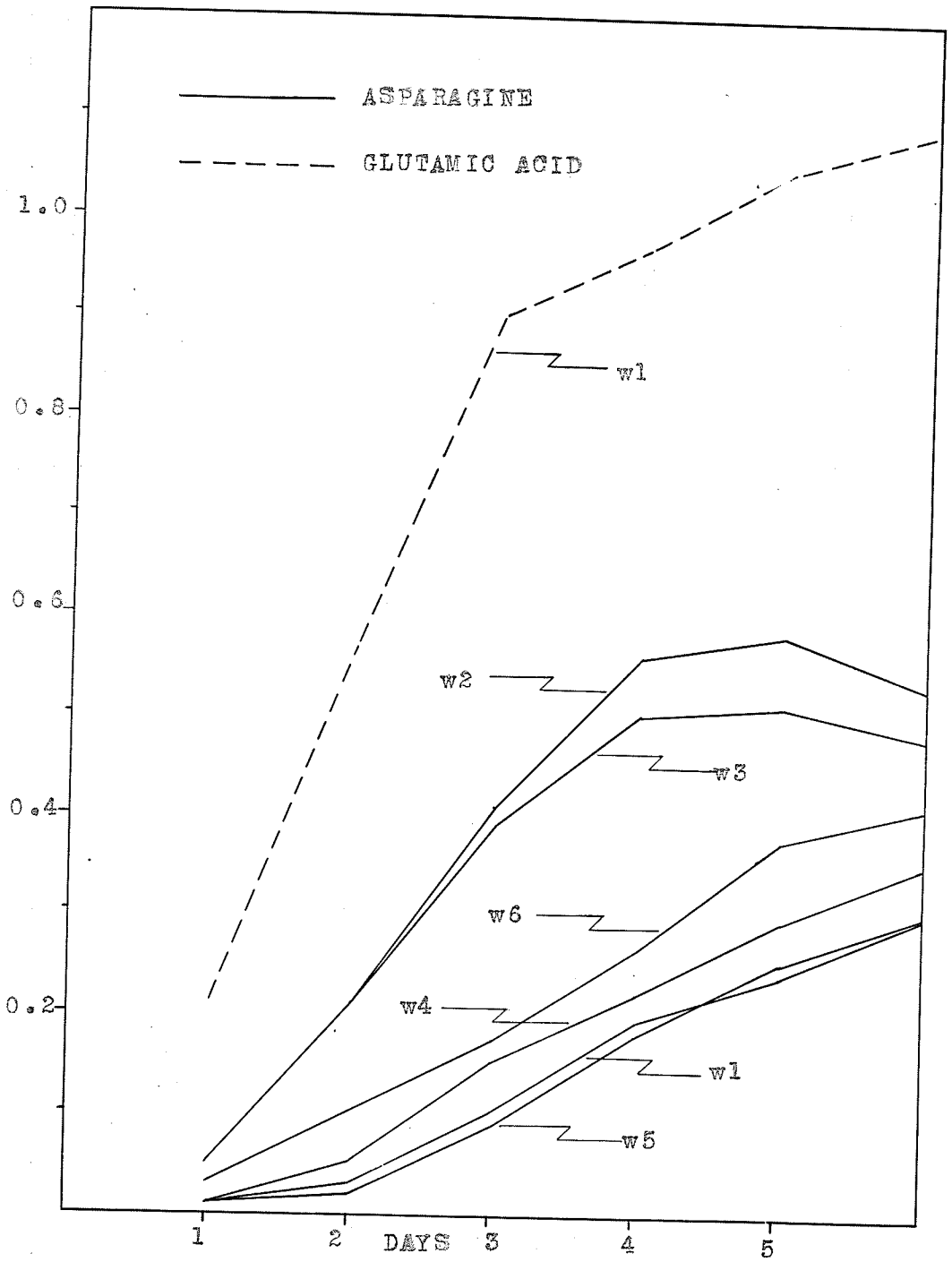

Fig. 1. Growth of six isolates of the yellow epiphyte in a medium containing asparagine as the only source of carbon and nitrogen and of one of the isolates in Starr's glutamic acid medium.

readings were taken at 12 days. The results are presented in Table 4. They show that neither the isolates of the yellow epiphyte nor the named species of Xanthomonas grew well in a medium containing arginine as the sole source of carbon and nitrogen.

Tartaric acid as the sole source of carbon

In an attempt to determine whether the epiphyte would grow in a medium containing tartaric acid as the sole source of carbon, each isolate was tested in the following medium.

0.1% NH_4NO_3
0.1% KH_2PO_4
0.02% $MgSO_4 \cdot 7H_2O$
0.1 % tartaric acid
pH 6.2 after sterilization.

Incubation was at 25°C for six days. The density values ranged from zero to 0.07 units for both the epiphyte and the named species of Xanthomonas, indicating negligible or slight growth. This was in agreement with the finding of Lewis (17) who reported that tartaric acid was not sufficient for the growth of certain plant pathogens. Of course, the failure to grow conceivably might have resulted from some other deficiency in the medium. However, Burkholder (4) reported that members of the Phytomonas campestris group (now in the genus Xanthomonas) grew well in a synthetic medium with inorganic nitrogen as the sole source of this element. This would appear to indicate that the

Table 4. Relative growth of isolates of the yellow epiphyte and certain named species of Xanthomonas in a medium containing arginine as the sole source of carbon and nitrogen - expressed as density units.

Epiphyte	Duplicate 1	2	Epiphyte	Duplicate 1	2
W-1	0.02	0.03	B-4	0.03	0.02
W-2	0.02	0.02	B-5	0.04	0.02
W-3	0.05	0.05	B-6	0.01	0.01
W-4	0.12	0.11	F-1	0.03	0.03
W-5	0.03	0.05	F-2	0.00	0.01
W-6	0.03	0.02	F-3	0.03	0.03
O-1	0.04	0.05	F-4	0.04	0.03
O-2	0.02	0.02	F-5	0.03	0.03
O-3	0.02	0.01	F-6	0.04	0.03
O-4	0.04	0.03	S-1	0.02	0.01
O-5	0.03	0.06	S-2	0.04	0.05
O-6	0.09	0.16	S-3	0.03	0.03
B-1	0.01	0.03	S-4	0.02	0.03
B-2	0.02	0.03	S-5	0.02	0.04
B-3	0.06	0.07	S-6	0.10	0.10
			L-2	0.04	0.05

Xanthomonas			Xanthomonas		
H-1	0.02	0.01	T-1	0.01	0.01
H-2	0.00	0.00	T-2	0.02	0.01
H-3	0.00	0.00	T-3	0.01	0.01
H-4	0.02	0.00			

nitrogen supply probably was adequate in the medium used in this study.

The basal medium of Starr (27)

Starr found that many species of Xanthomonas grew in a glucose, NH_4Cl, basal salts medium at a slower rate than in nonsynthetic media; however, in the same medium with sufficient buffer the final crop was about the same.

The medium (with minor variations) follows:

glucose(1)	0.5 gm.
NH_4Cl	0.1 "
KH_2PO_4	0.2 "
$MgSO_4 \cdot 7H_2O$	0.02 "
B(H_3BO_3)	0.5 μ gm.
Ca($CaCO_3$)	10.0 " "
Cu($CuSO_4 \cdot 5H_2O$)	1.0 " "
Fe($FeNH_4(SO_4)_2 \cdot 12H_2O$)	10.0 " "
I (KI)	0.1 " "
Mn($MnSO_4 \cdot 4H_2O$)	1.0 " "
Mo (powdered Mo)	1.0 " "
Zn ($ZnSO_4 \cdot 7H_2O$)	5.0 " "

distilled water to 100 ml.
pH 6.8 after sterilization.

Incubation was at 25°C for six days. The results, presented in Table 5, show that isolates of the yellow epiphyte, with the exception of 0-3 and 0-6, grew slightly in the basal medium of Starr, while none of the named species of Xanthomonas grew in this medium.

(1) To remove impurities, glucose solutions were treated twice at pH3.0 with charcoal and filtered each time with super cel.

Table 5. Relative growth of isolates of the yellow epiphyte and certain named species of *Xanthomonas* in the basal medium of Starr - expressed as density units.

Epiphyte	Duplicate 1	Duplicate 2	Epiphyte	Duplicate 1	Duplicate 2
w-1	0.16	0.18	B-4	0.21	0.20
w-2	0.31	0.30	B-5	0.22	0.19
w-3	0.29	0.30	B-6	0.15	0.15
w-4	0.16	0.17	F-1	0.22	0.20
w-5	0.23	0.22	F-2	0.16	0.15
w-6	0.28	0.28	F-3	0.23	0.21
0-1	0.31	0.28	F-4	0.12	0.19
0-2	0.10	0.11	F-5	0.27	0.33
0-3	0.00	0.00	F-6	0.19	0.17
0-4	0.22	0.24	S-1	0.29	0.28
0-5	0.15	0.14	S-2	0.23	0.21
0-6	0.01	0.00	S-3	0.20	0.17
B-1	0.30	0.30	S-4	0.16	0.14
B-2	0.23	0.27	S-5	0.16	0.16
B-3	0.27	0.23	S-6	0.27	0.25
			L-2	0.37	0.39

Xanthomonas			*Xanthomonas*		
H-1	0.00	0.00	T-1	0.00	0.00
H-2	0.00	0.00	T-2	0.00	0.00
H-3	0.00	0.00	T-3	0.00	0.00
H-4	0.00	0.01			

The basal medium of Starr plus 0.02% l - glutamic acid

Starr (27) found that certain species of Xanthomonas, which failed to grow in his basal medium, grew well in the same medium to which glutamic acid was added. Accordingly, the isolates used in this study were tested in his medium, with the results presented in Table 6. The epiphyte grew well in this medium while the species of Xanthomonas tested produced variable growth; Xanthomonas carotae (T-1) growing better than any of the others and about as well as certain isolates of the epiphyte.

Acid tolerance

Burkholder (5) reported that the xanthomonads were acid tolerant; therefore, preliminary tests were carried out using two isolates of the epiphyte and four representatives of Xanthomonas to determine whether the epiphyte showed similar acid tolerance.

Nutrient broth was adjusted to four pH reactions ranging from pH 4.5 to pH 6.5 (after sterilization). A 24 hr. nutrient broth culture of each of the six isolates was transferred in duplicate to media at the four pH levels. Incubation was at 25°C and growth was based upon visible turbidity.

Both isolates of the epiphyte produced visible turbidity at all pH levels including pH 4.5 at two days,

Table 6. Relative growth of isolates of the yellow epiphyte and certain named species of Xanthomonas in the basal medium of Starr to which 0.02% l-glutamic acid was added - expressed as density units.

Epiphyte	Duplicate 1	Duplicate 2	Epiphyte	Duplicate 1	Duplicate 2
w-1	0.92	0.88	B-4	0.79	0.82
w-2	0.52	0.52	B-5	0.92	0.95
w-3	0.88	0.88	B-6	0.92	0.88
w-4	0.92	0.95	F-1	0.92	0.72
w-5	0.95	0.92	F-2	0.79	0.82
w-6	0.92	0.88	F-3	0.67	0.74
0-1	0.76	0.85	F-4	0.65	0.69
0-2	0.72	0.85	F-5	0.65	0.63
0-3	0.52	0.60	F-6	0.88	0.74
0-4	0.88	0.95	S-1	0.76	0.85
0-5	0.67	0.60	S-2	0.88	0.85
0-6	0.74	0.63	S-3	0.92	0.92
B-1	0.88	0.85	S-4	0.82	0.76
B-2	0.95	0.82	S-5	0.82	0.74
B-3	0.92	0.95	S-6	0.88	0.85
			L-2	0.92	0.74
Xanthomonas			Xanthomonas		
H-1	0.36	0.44	T-1	0.74	0.60
H-2	0.19	0.18	T-2	0.07	0.05
H-3	0.30	0.22	T-3	0.15	0.14
H-4	0.31	0.36			

while none of the four representatives of Xanthomonas produced growth at pH 4.5 even at six days. Following these preliminary tests all the isolates used in this study, except those from soybeans, were tested for ability to grow in nutrient broth at pH 4.5. The results are presented in Table 7. The results, in general, confirmed the preliminary finding of a difference in acid tolerance between the epiphyte and the pathogens. One isolate of the epiphyte failed to grow and one of the pathogens did grow at this pH. The reason for these two apparent discrepancies is not known; however, it is conceivable that certain isolates in any species might vary from the normal with respect to acid tolerance.

Table 7. Growth of isolates of the yellow epiphyte and certain named species of Xanthomonas in nutrient broth at pH 4.5.

Epiphyte	Duplicate 1	Duplicate 2	Epiphyte	Duplicate 1	Duplicate 2
W-1	≠	≠	B-1	≠	≠
W-2	≠	≠	B-2	≠	≠
W-3	≠	≠	B-3	≠	≠
W-4	≠	≠	B-4	≠	≠
W-5	≠	≠	B-5	≠	≠
W-6	≠	≠	B-6	≠	≠
O-1	≠	≠	F-1	≠	≠
O-2	≠	≠	F-2	≠	≠
O-3	-	-	F-3	≠	≠
O-4	≠	≠	F-4	≠	≠
O-5	≠	≠	F-5	≠	≠
O-6	≠	≠	F-6	≠	≠
			L-2	≠	≠

Xanthomonas			Xanthomonas		
H-1	-	-	T-1	-	-
H-2	≠	≠	T-2	-	-
H-3	-	-	T-3	-	-
H-4	-	-			

Part 2 - Tests of Pathogenicity[1]

Procedure

Following the procedure of Hagborg (11)(13), tests of pathogenicity were carried out using the yellow epiphytes from wheat, oats and barley, the Saskatoon isolate, and the cultures of *Xanthomonas translucens* received from Dr. W. A. F. Hagborg.

Each isolate was tested on Thatcher wheat, Victory oats and Titan barley. Eight to 10 seeds of each host plant were sown per pot and one pot of each host was used for each isolate. When the coleoptiles reached a height of 7 to 15 m.m. the seedlings were inoculated. A flamed sharp nichrome needle was dipped in the inoculum, consisting of a 48 hr. nutrient agar slant culture, and the coleoptile and enclosed primary leaves were pierced three times. Care was taken to keep the wounds well above the surface of the soil to minimize the possible entrance of soil organisms. The inoculated plants were examined for infection at the end of a 10 day period at 25°C.

Results

Not one of approximately 500 tests made with the epiphytes produced characteristic lesions of the disease, whereas in every case the pathogens produced dark water-soaked areas around the site of the infection. The host

(1) Performed under the direction of Dr. W. A. F. Hagborg, Dominion Laboratory of Plant Pathology, Winnipeg, Man.

plants infected by each pathogen follow:

Xanthomonas	
H-1	wheat, barley
H-2	wheat, barley
H-3	wheat, oats, barley
H-4	oats, barley

Although the yellow epiphyte did not produce evidence of infection under the conditions of this investigation, it could conceivably attack these or other hosts under other conditions. Starr (27) speculated on the existence in plants of specific antibiotics which inactivated phytopathogens other than the particular bacterial species which infected that host. Burkholder (5) noted that buckwheat and spinach appeared to be free from bacterial diseases, but, whether these two plants possessed an antibiotic substance which the others lacked was not known.

Host specificity was notably marked with regard to the special forms of Xanthomonas translucens as shown above; yet, as far as is known, these forms are similar in other respects. This is the more remarkable inasmuch as Starr (27) noted that pathogenicity involved a nutritional interrelationship - during its pathogenetic existence the phytopathogen derived its entire nourishment from the host tissue.

Part 3 - Nature of the Pigments

Based upon the reports by Starr (26) and Burkholder (5) on the pigment produced by species of Xanthomonas, a study of the pigments produced by the isolates used in this study was carried out.

Preliminary tests were performed to determine class of pigment. Palmer (19) pointed out that all yellow pigments were not necessarily carotenoids and that carotenoids could be identified by their response to the lipocyan reaction. In this reaction conc. H_2SO_4 or conc. HNO_3 when added to nutrient agar cells on a clean slide produces a bluish color within a few minutes. In addition, the cells treated with H_2SO_4 produce microscopic crystals. Both the isolates of the epiphyte and of the pathogens produced a positive reaction, indicating similarity of pigment on this basis.

Further study required a large crop of cells, therefore, the procedure of Sobin and Stahly (24) was followed. The bacteria were cultured in 12 oz. bottles on a substrate of nutrient agar containing 2% glycerol. Inoculation was made by sterile pipette from a nutrient broth culture. After incubation at room temperature for about seven days, about 10 ml. of 70% aqueous acetone was added and the bacteria removed by scraping the surface with a glass rod bent at a right angle. Water was not used since the bacteria form a hydrophilic suspension. The acetone and

suspended bacteria were removed from the bottles, using suction, and the cells were removed from this solution by centrifugation. A gummy polysaccharide material produced by the bacteria sometimes interfered with centrifuging. The use of more acetone overcame this difficulty. Approximately one gram of moist cells was obtained by this method.

The following tests were applied to all the isolates.

Extracting solvents

Moist cells were transferred to a beaker containing 50 ml. methanol which was placed in hot water that was brought to the boil quickly. The pigments were extracted in a few minutes. It was found that the pigments could be extracted, as well, with other polar organic solvents such as ethanol and acetone, but not with non-polar solvents, such as petroleum ether (skellysolve B), carbon disulfide, carbon tetrachloride, chloroform, ethyl ether or benzene. After having been extracted from the cells and dried in vacuo, the pigments could ~~then~~ be dissolved in non-polar solvents. Neither petroleum ether nor methanol extracted the pigment from dried cells of one of the isolates. This was a chance observation and the study on dried cells was not carried further. According to Starr (26) the failure of a typical non-polar solvent, diethyl ether, to extract the pigment was due to that solvent's lack of wetting action and could be corrected by the addition of a suitable detergent to the diethyl ether.

Partition tests

Separation of types of carotenoid pigments was carried out by the procedure of Sobin and Stahly (24). Water was added to the cell-free methanol extract to give an alcohol concentration of 90%. This was shaken in a separatory funnel with petroleum ether. The pigments remained in the methanol layer, being characteristically hypophasic. This is characteristic of carotenoid acids and carotenoid alcohols.

The methanol layer was drawn off and made alkaline to litmus, diluted with water and shaken in a separatory funnel with diethyl ether. The pigments appeared in the ether layer, being epiphasic in this partition test. This is characteristic of carotenoid alcohols.

Color tests

The methanol extract of moist cells did not show any color reaction on the addition of acids or bases. Petroleum ether solutions of dried pigment gave a light blue color on the addition of conc. H_2SO_4 and no reaction with conc. HCl.

Chromatographic adsorption

The adsorption device was a glass tube 15 cm. long and 15 m.m in diameter sealed at one end to a tube of 5 m.m. bore and approximately 8 cm. in length. The tube was supported in a vertical position by attachment to a vacuum flask. A wad of cotton was placed just above the

constricted portion of the tube and adsorbent was added and packed carefully in small amounts. A portion of the solvent used for dissolving the pigment was poured on the column to test for the presence of cracks in the adsorbent. According to Sobin and Stahly (24) the pigments must be free of water before they are dissolved in the adsorbing solvent. Moisture was removed by adding benzene and evaporating in vacuo at 50°C.

Attempts to chromatograph pigment solutions of ethanol, methanol and acetone extracted from pathogens were not successful even though the following adsorbents were used: $CaCO_3$ (activated by heating at 150°C for at least 5 hr.), activated MgO, Al_2O_3, celite 515 and 535, celite (515) MgO 3:1 and 1:1.

When a dried pigment mixture was dissolved in skelly-solve-B and the solution poured on the adsorption column the pigments formed a narrow yellow band at the top with Al_2O_3 or $CaCO_3$ as the adsorbent. On washing with pure solvent the adsorbed pigments moved slowly through the adsorbent and separated gradually into bands, each representing a different pigment. $CaCO_3$, being a weaker adsorbent, proved superior to Al_2O_3 since the chromatogram developed more quickly.

In most cases three distinct bands formed on the adsorption column. The predominant pigment produced a wide band near the top. This was followed by one and often two narrower bands. The failure of three zones to appear in all trials might be attributed to the fact that one of the

pigments was present in too small an amount. The number of bands obtained from each isolate follow:

Epiphyte	Bands on $CaCO_3$	Xanthomonas	Bands on $CaCO_3$
w-1	3	H-1	3
w-2	2	H-2	3
w-3	3	H-3	2
w-4	3	H-4	3
w-5	3	T-1	2
w-6	3	T-2	3
L-2	2	T-3	3
Sarcina lutea	2		

A mixed chromatogram using pigment extracts from one epiphyte (w-1) and one pathogen (T-2) showed the same three bands in the same relative position as when these extracts were chromatographed separately.

Starr (26) reported only one or sometimes two bands on magnesia or alumina as adsorbents with various species of Xanthomonas.

Spectrometric analysis

Absorption spectrum maxima obtained on a Gaertner spectroscope are presented in Table 8. The original extract in most cases gave only one maximum. In a few extracts a second less definite maximum was produced. The reconstituted extracts on the other hand produced two maxima, with a suggestion of a third from one of the isolates. Except in the case of two isolates the maximum on the original extract was different from either of the maxima on the reconstituted extract. In most cases it fell be-

Table 8. Absorption spectrum data on pigment mixtures in 95% ethanol obtained from isolates of certain bacteria.

Epiphyte	Maxima (m u) Original extract(1)	Reconstituted(2)
w-1	450	440 470
w-2	451	440 470
w-3	450	441 470
w-4	450	440 469
w-5	449	440 470
w-6	450	440 470
L-2	450 483?	440 469
Xanthomonas		
H-1	450	440 470
H-2	450	439 470
H-3	451	440 470
H-4	449	440 470
T-1	451 425?	440 470
T-2	450	441 470 416?
T-3	440 470	440 470
Sarcina lutea	439 469	440 471

? doubtful maxima

(1) Original alcoholic extract of moist cells.

(2) Pigment mixture after elution from the adsorption column with 95% ethanol.

tween the low and the high maximum.

It may be pointed out that Starr(26) found that the maximum on crude ethanol extracts of pigments from various species of Xanthomonas was at 445 m u . However, he did not state the concentration of the alcohol.

The pigments from Sarcina lutea were introduced into this study as a check on technique. The results obtained agreed with the findings of Sobin and Stahly (24), in that 2 pigments were present, each producing a maximum at 440 m u and a second one at 470 m u.

To determine whether the absorption maxima of reconstituted extracts presented in Table 8 were produced by the mixture of pigments or the predominant pigment alone, the predominant pigment of one epiphyte and one pathogen were removed from adsorption columns by removal of the adsorbent and eluted with ethanol. With each isolate, the two maxima on this predominant pigment were the same as for the reconstituted extracts which would appear to indicate that the secondary pigments were in such small quantity as to produce no significant effect on the result obtained with the equipment available at this time.

Reconstituted extracts of two isolates were examined in more detail in a photoelectric spectrophotometer(1). The results are presented in Figure 2. Pigments of the epiphyte (w-3) showed absorption maxima at 423 and 448 m u

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(1) Courtesy of Dr. J. H. Linford, Cancer Relief and Research Institute, Winnipeg.

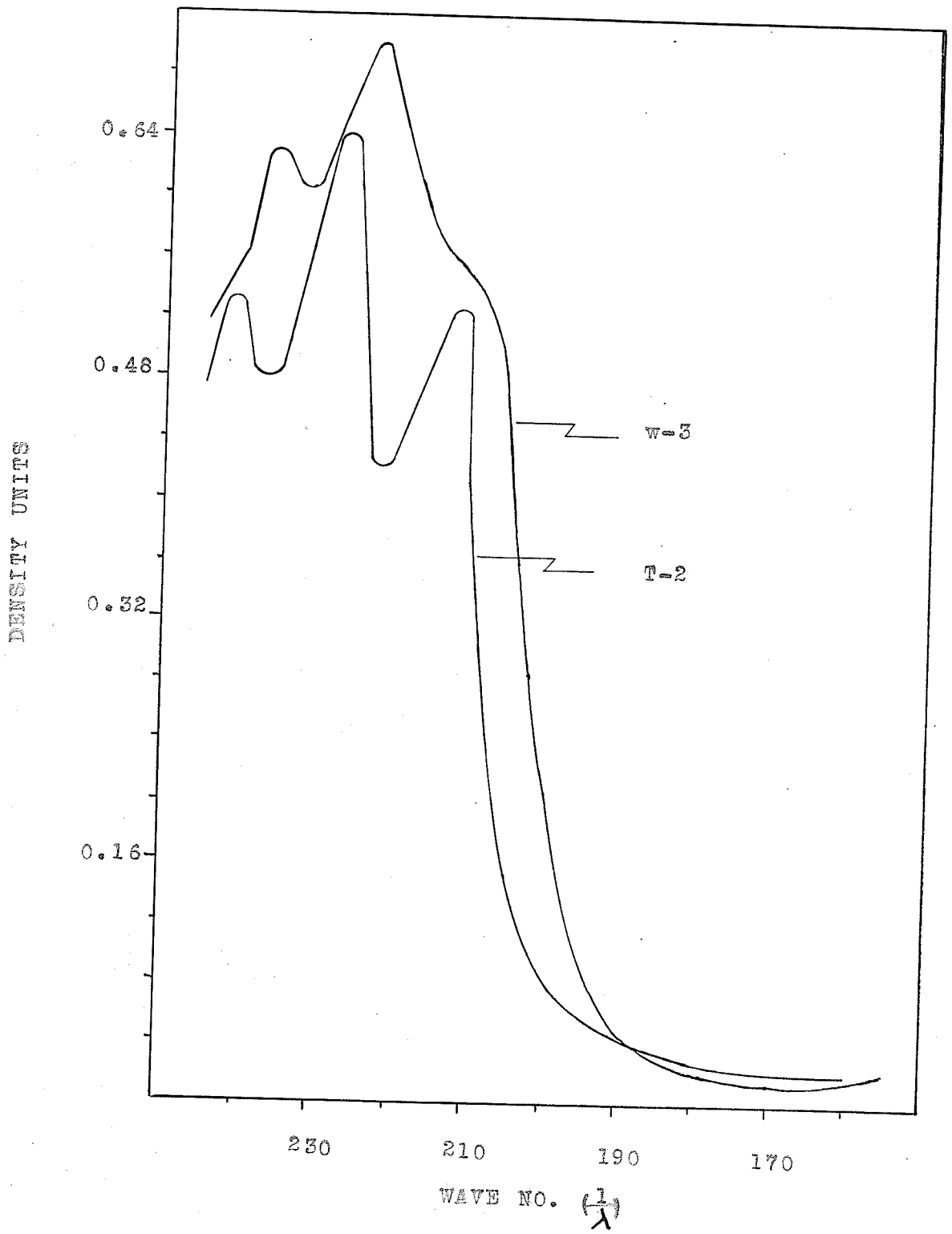

Fig. 2. Light absorption by the reconstituted extract of one epiphyte and one pathogen.

while those of the pathogen (T-2) showed maxima at 413, 440 and 471 m u. In another trial with the same isolates the epiphyte showed only a single maximum at 450 m u and the pathogen three maxima 420, 438 and 469 m u.

On the basis of the limited data available in this study it would appear that the pigment produced by this epiphyte, if not identical with that produced by representatives of the genus Xanthomonas, is similar to it. Probably there is as much variation among replicates of one pathogen as between epiphyte and pathogen. For example, on the pathogen tested with the photoelectric spectrophotometer, the three maxima in the second trial differed from those in the first trial.

The pigments produced by both types were not distinguishable with respect to the extracting solvents, partition tests and color tests used in this study. Chromatographic adsorption showed two pigments common to all isolates both of the epiphyte and of the pathogen. A third pigment was found about as often in the epiphyte as in the pathogens. A chromatogram of a mixture of pigments produced by an epiphyte and a pathogen showed the same three bands as shown by pigments from each type separately. Further, the absorption spectrum maxima of the original alcoholic extracts and the reconstituted extracts obtained on the Gaertner spectroscope were the same on the epiphyte as on the pathogens. The predominant pigment of the epiphyte showed the same maxima as that of Xanthomonas but no finding was made on the maxima of other pigments present.

DISCUSSION

From the foregoing it is apparent that there are many similarities between the yellow epiphyte and the pathogens of Xanthomonas studied. Although this study revealed a morphological difference, inasmuch as there appeared to be a tendency to less uniformity in length of cells in any isolate of the epiphyte than was the case with the pathogens studied, this difference was so slight that any attempt to differentiate them on the basis of this factor would prove hopeless. Furthermore, the variation in size of cells in certain species of Xanthomonas, as presented in Bergey et al. (3), is as great as that found in any isolate of the epiphyte.

The slight growth of the epiphyte in a medium containing asparagine as the sole source of carbon and nitrogen, and the still smaller amount shown by certain pathogens, might indicate a quantitative rather than a qualative difference. The variations to which bacteria are subject, closely related types in particular, might conceivably produce a condition not different in kind but more extreme in degree than that which existed between more complex forms. It should be noted that there is not general agreement among taxonomists respecting what shall constitute a determinative character and any strict dividing-line is chosen arbitrarily - and subject to revision if the need arises. Whether the inability of species of Xanthomonas to use asparagine as the sole carbon and nitrogen

source is of sufficient importance to be considered as a generic characteristic appears questionable. Dowson (7) did not mention this character when he first proposed the genus Xanthomonas.

Probably the epiphyte is merely a more adaptive parasite than the pathogens used in this study since the pathogens failed to show growth in the basal medium of Starr (27) while the epiphyte showed slight growth. Starr divided the species of Xanthomonas into various physiological groups. It would appear that the epiphyte belonged to an intermediate group - more adaptive than some "fastidious" pathogens which did not grow in the basal medium but more specialized than the "non-exacting" species which grew well in it.

Isolates of the epiphyte did not produce evidence of infection on the host plants tested in this study and in this respect appear to differ from Xanthomonas species, inasmuch as this genus, at present, contains only plant pathogens. Elliot (9) pointed out that since the number of bacterial plant pathogens was small compared with the large number of soil and water bacteria, saprophytes, and animal and human parasites, pathogenicity was not a sound basis for generic delineation. Dowson (7) considered pathogenicity unsuitable as a generic character because it did not lead to the arrangement of bacteria into natural groups of related organisms. It would appear that

provision was made by Bergey et al. (3) for including other closely related non-pathogenic species in Xanthomonas since this genus is not limited to plant pathogens alone but is characterized merely as containing "mostly plant pathogens".

This study appeared to show a similarity between pigments of the epiphyte and the species of Xanthomonas studied. Burkholder (5) claimed that the pigment produced by members of Xanthomonas was identical in all species. Accordingly, on this basis, the epiphyte could be considered in the same group as the pathogens. However, too much weight should not be placed on sameness of pigmentation as a basis for genus characterization. In a chance isolate used in this study as a mere check on technique, it was found that the predominant pigment of Sarcina lutea was identical with that of the epiphyte and the pathogens. Sobin and Stahly (24) thought it probable that different strains of a certain species would be found to produce different pigments. They examined 12 strains of Staphylococcus aureus (now Micrococcus pyogenes var. aureus) and found two pigments common to all with some variation in regard to a third. Further, a number of carotenoid alcohols and hydrocarbons have been isolated from different species of Flavobacterium by these investigators.

SUMMARY

1. Thirty-one isolates of the yellow epiphyte from various sources and seven isolates of certain named species of Xanthomonas were plated and reisolated to ensure purity and subjected to comparative study.

2. A slight difference in morphology was noted while the cultural characteristics were identical.

3. Isolates of the epiphyte and the named species of Xanthomonas showed a close agreement in nutritional requirements. Neither group could utilize arginine as the sole source of carbon and nitrogen or tartaric acid as the only carbon supply.

4. A difference was noted between the two groups in a medium containing asparagine as the sole source of carbon and nitrogen, in the basal medium of Starr and in nutrient broth at pH 4.5.

5. Approximately 500 tests of pathogenicity were performed on wheat, oats and barley using 19 isolates of the epiphyte, none of which produced disease, and four representatives of the "black-chaff" bacteria which produced characteristic lesions.

6. Pigments produced by the epiphyte and the named species of Xanthomonas tested were similar with

regard to the lipocyan reaction, extracting solvents, partition tests, color tests and chromatographic adsorption.

7. The predominant pigment of each group showed absorption spectrum maxima at 440 and 470 mμ. on a Gaertner spectroscope, as did the two pigments produced by Sarcina lutea.

8. In each case, one epiphyte and one pathogen showed as much difference in results between duplicate trials as there was between the two cultures, when observed on a photoelectric spectrophotometer.

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