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Culture medium for *Xanthomonas campestris* pv. *oryzae*

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Studies on nutrient requirements of four Chinese strains of *Xanthomonas campestris* pv. *oryzae* in a modified Watanabe's medium led to the development of a new synthetic medium containing sucrose, sodium glutamate, methionine, KH_2PO_4, NH_4Cl and iron chelated with EDTA. The concentration of each ingredient was optimized based on the number of colonies and time required for their appearance. Various concentrations of some nutrients were compared based upon their effects on growth of the pathogen strains and 34 contaminants from rice materials. Tryptone enhanced the growth of *X. c. oryzae* more than that of many contaminants, including *Erwinia herbicola*. Peptone stimulated growth of *X. c. oryzae* without promoting excessive contamination. When compared with other media used for *X. c. oryzae*, the new culture medium enriched with tryptone and peptone gave the highest recovery and earliest appearance of colonies of Chinese strains of this bacterium.

Rice leaf blight caused by *Xanthomonas campestris* pv. *oryzae* (Ishiyama) Dye is one of the most important diseases of rice in Asia and has been spreading through seed over the rice-growing areas of the world. Development of techniques for the detection of this pathogen in rice seeds will assist in controlling spread to disease-free areas.

Seed-borne bacteria can be isolated on semi-selective media and identified by serological techniques (Schaad 1982). At present the key problem in detection of *X. c. oryzae* from rice seeds by this approach is lack of a semi-selective medium, even a good basal culture medium. The organism cannot compete with common contaminants on non-selective laboratory media.

Development of a good culture medium for *X. c. oryzae* should be based on the nutritional physiology of the bacterium. Although Ishiyama (1922) investigated its nutrient requirements, systematic studies did not start until the 1950s (Tagami & Mizukami 1962). In a mineral liquid medium for xanthomonas (Starr 1946), sucrose (5 g/l) and glutamic acid (1 g/l) were the best carbon and nitrogen sources for *X. c. oryzae* (Watanabe 1963a, 1963b). Although inorganic nitrogen sources are not normally utilized, ammonium in the presence of sucrose supports better growth of *X. c. oryzae* (Fang & Hsu 1965). Mineral sulphur or sulphate are not utilized (Watanabe *et al.* 1967) but sulphur-containing amino acids, especially methionine, promote growth (Watanabe *et al.* 1965; Noda & Ohuchi 1983), and ferrous ions (1 ppm) chelated with EDTA are beneficial (Suwa 1962).

Several media for *X. c. oryzae* have been reported (Table 1). Of them, Wakimoto's medium (Wakimoto 1960) and nutrient sucrose agar (NSA; Fang *et al.* 1957) have been commonly used for culture and isolation of the pathogen from freshly infected rice leaves. Based on this previous research, the nutrient requirements of Chinese strains of *X. c. oryzae* were studied in agar media and a basal culture medium for further development of selective medium was developed.

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Table 1. Reported media for *Xanthomonas campestris* pv. *oryzae*

Composition of media (g/l)*

Table 1. Reported media for *Xanthomonas campestris* pv. *oryzae*

Ingredient	Composition of media (g/l)*														
	Goto (Karganilla *et al.* 1973)	MSB (Karganilla *et al.* 1973)	NIAS (Anon. 1959)	Noda & Ohuchi (1983)	NSA (Fang *et al.* 1957)	PRSM (Mizuta 1953)	Suwa (1962)	Tanaka (1964)	Wakimoto (1960)	Watanabe (1963b)	WF-P (Karganilla *et al.* 1973)	YSP (Dye 1962)	Modified 523 (Kado & Heskett 1970)	New synthetic medium	New culture medium
Potato						300			300						
Beef extract					3										
Yeast extract		5										5	10		
Peptone	10	5			5	5	10		5		5	10			2
Tryptone															5
Sucrose	10	10	20	5	10	20	5	15	20	5	20	2	10	10	10
Glutamic acid			2				2	10		1					
Sodium glutamate	1	1		1										5	1
Methionine				0·1										0·1	0·1
Cystine										0·5					
$K_2HPO_4 . 3H_2O$						2	0·1								
KH_2PO_4			2	2				2		2			2	1	1
$Na_2HPO_4 . 12H_2O$									2		2				
$NH_4H_2PO_4$				2											
$(NH_4)_2HPO_4$										3					
NH_4Cl														1	1
$MgSO_4 . 7H_2O$								0·2					0·3		
$MgCl_2 . 6H_2O$			0·05	1			1	1		1				1	1
$FeSO_4 . 7H_2O$			0·1							0·01	0·05				
Fe^{++}- (EDTA)							1ppm							1ppm	1ppm
$MnSO_4 . H_2O$								0·01		0·01					
$Ca(NO_3)_2 . 4H_2O$									0·5		0·5				
NaCl						2									
$NaNO_3$						1									
$CaCO_3$													5		

* They were solidified in this research with 17 g/l of agar.

Materials and Methods

BACTERIA

The six cultures of *X. c. oryzae* included five (OS-14, Ks-1-20, Ks-1-21, Ks-3-8 and Zhe-173) isolated in China and one (PX-86) from the International Rice Research Institute (IRRI) in the Philippines. Strain Zhe-173, which grows poorly on NSA medium, was used mainly to study chemicals and their concentrations for development of the new media. Thirty-four other bacterial strains isolated from rice seeds and leaves, including *Erwinia herbicola* (Lohnis) Dye, a common contaminant in isolation of *X. c. oryzae*, were compared with Zhe-173 in growth on agar media.

BASAL MEDIUM

In this study the medium modified from that of Watanabe (1963b; Table 1) contained (g/l): sucrose, 10; sodium glutamate, 1; KH_2PO_4, 1; $MgCl_2 . 6H_2O$, 1; $FeSO_4 . 7H_2O$, 0·01. Since *X. c. oryzae* does not readily form single colonies on synthetic media, the basal medium was enriched with tryptone (Oxoid) (1 g/l). All tests were carried out on media containing 17 g/l Bacto agar (Difco). Before the media were autoclaved at 100°C for 20 min, their pH was adjusted to 6·7 with 1 mol/l HCl or 1 mol/l NaOH except when medium pH was examined in the experiment.

INOCULATION

The organisms were grown on slopes of the basal medium with tryptone (1 g/l) at 27°C for 24–48 h, and transferred with an inoculation loop (without touching the medium surface) to 1 ml of 0·85% NaCl solution to give an $O.D._{620}$ of 0·4. The bacterial suspension was then diluted in 0·85% NaCl solution to concentrations appropriate for the following inoculations.

For recovery of strain Zhe-173 of *X. c. oryzae*, 0·1 ml of 10^{-6} dilution from the bacterial suspension was spread with a glass rod on a plate containing about 25 ml of medium. Each procedure was replicated five times.

For growth of bacteria on synthetic media or for comparison with the growth of bacteria on the new synthetic medium containing complex nutrients, beef extract, casein hydrolysate, peptone, tryptone or yeast extract, suspensions each at 10^{-3} dilution, were inoculated on individual plates containing about 25 ml of medium, with a 19-point inoculator (each point has a flat bottom 1·2 mm in diameter). Duplicate samples of each medium were inoculated.

INCUBATION, OBSERVATION AND DATA ANALYSIS

Inoculated plates were incubated at 27°C. After spread-inoculation for recovery of bacteria, cultures were examined daily to determine when colonies were first observed. The number of colonies was counted and the colony diameters were measured from the bottom of the plates with a ruler after incubation for 1 week. For measuring colony diameter, 10 single colonies were chosen randomly from each plate (all colonies were measured if less than 10 in the plate). The number of colonies within each experiment (shown in the tables) was examined statistically by analysis of variance and Duncan's multiple range test. Mean colony diameters and standard deviations of the measured data were calculated. After inoculation with the multipoint inoculator, the diameters of the confluent colonies at each inoculated location were also observed and directly recorded.

Results

With tryptone as a carbon and nitrogen source, addition of sucrose (10 g/l) promoted the highest number and increased the size of colonies of *X. c. oryzae* strain Zhe-173 but did not speed their appearance (Table 2). In the presence of tryptone, sodium glutamate (1 g/l) increased number and size of colonies of strain Zhe-173 but, when added at amounts over 1 g/l, started to limit the number and delayed appearance of colonies though their size was not decreased at 5 and 10 g/l; the combination of sodium glutamate (1 g/l) and methionine (0·1 g/l) facilitated formation of more and larger colonies without delaying their appearance (Table 3).

The different phosphate salts added to the basal medium supported different medium pH (6·0–7·2) (Table 4). Potassium dihydrogen phosphate alone gave the medium a pH of 6·4 and 1·0 g/l produced the highest number, the largest size and the most rapid appearance of colonies

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Table 2. Effect of various concentrations of sucrose on the growth of Chinese strain Zhe-173 of *Xanthomonas campestris* pv. *oryzae* on medium containing sucrose in various amounts

Amount of sucrose (g/l)	Mean colony number per plate*	Day first colony visible	Mean colony diameter (mm)†
0	26 a	4	1·5 ± 0·5
5	44 b	4	4·3 ± 0·1
10	55 c	4	4·0 ± 0·3
15	26 a	4	3·5 ± 0·4
20	27 a	4	3·5 ± 0·4
30	26 a	4–5	3·0 ± 0·2

* Colonies were counted 9 d after spread-inoculation. Mean numbers of colonies followed by different letters are significantly different ($P = 0·05$) according to Duncan's multiple range test.

† Mean colony diameters ± S.D. of measured data. Colonies were measured 9 d after spread-inoculation.

Table 3. Effect of sodium glutamate and methionine on the growth of Chinese strain Zhe-173 of *Xanthomonas campestris* pv. *oryzae* on medium containing various amounts of sodium glutamate

Amount of		Mean		Mean
Sodium glutamate (g/l)	Methionine (g/l)	colony number per plate*	Day first colony visible	colony diameter (mm)†
0	0	28 b	3–4	2·6 ± 0·1
1	0	33 c	4	3·4 ± 0·6
5	0	30 bc	4–5	3·6 ± 0·4
10	0	27 b	4–5	3·5 ± 0·5
15	0	26 b	4–5	2·5 ± 0·4
20	0	11 a	5	2·2 ± 0·9
1	0·1	34 c	3–4	3·5 ± 0·4

* Colonies were counted 8 d after spread-inoculation. Mean numbers of colonies followed by different letters are significantly different ($P = 0·05$) according to Duncan's multiple range test.

† Mean colony diameters ± S.D. of measured data. Colonies were measured 8 d after spread-inoculation.

Table 4. Effect of phosphate salts on the growth of Chinese strain Zhe-173 of *Xanthomonas campestris* pv. *oryzae* on medium containing various phosphate salts

Amount of salt (g/l)	pH of medium	Mean colony number per plate*	Day first colony visible	Mean colony diameter (mm)†
K_2HPO_4				
0·2	7·2	13 a	4	4·1 ± 0·4
1·0	7·2	20 b	4	5·0 ± 0·0
2·0	7·2	32 d	4–5	3·9 ± 0·8
3·0	7·2	14 a	6	1·8 ± 0·3
KH_2PO_4				
1·0	6·4	36 e	3	5·5 ± 0·4
2·0	6·4	32 d	3–4	4·2 ± 0·7
3·0	6·4	28 c	4	3·5 ± 0·6
2·0 KH_2PO_4 +				
3·0 $(NH_4)_2HPO_4$	7·0	26 c	3	4·9 ± 1·1
2·0 $NH_4H_2PO_4$	6·0	25 c	3–4	4·6 ± 1·2
2·0 K_2HPO_4	6·7	24 c	4–5	3·4 ± 0·8

* Colonies were counted 9 d after spread-inoculation. Mean numbers of colonies followed by different letters are significantly different ($P = 0·05$) according to Duncan's multiple range test.

† Mean colony diameters ± S.D. of measured data. Colonies were measured 9 d after spread-inoculation.

of strain Zhe-173 when compared with 2·0 or 3·0 g/l and with potassium monohydrogen phosphate, or the combinations used in Watanabe's medium (2·0 g/l KH_2PO_4 and 3·0 g/l $(NH_4)_2HPO_4$) and Noda & Ohuchi's medium (2·0 g/l KH_2PO_4 and 2·0 g/l $NH_4H_2PO_4$) (Table 4).

Ammonium chloride at amounts of 0·5–3·0 g/l tended to facilitate the formation of the colonies and the growth of the bacterium, and at 1·0 g/l promoted the highest number and the largest size of colonies compared with the control without NH_4Cl (Table 5). However, it completely inhibited growth of the bacterium at 5·0 g/l. The ferric salt at 10 mg/l also completely inhibited growth while the ferrous salt at 10 mg/l favoured the development of the colonies (Table 5). The use of 1 ppm ferrous ions chelated with EDTA in the same experiment promoted the highest number and the largest size of colonies without delay in appearance of the colonies. However, when its concentration in the basal medium was increased to 5 ppm, the bacterium did not grow.

A new synthetic medium developed in this research consisted of (g/l): sucrose, 10; sodium glutamate, 5; methionine, 0·1; KH_2PO_4, 1; NH_4Cl, 1; $MgCl_2 . 6H_2O$, 1; Fe^{++} chelated with EDTA, 1 ppm; agar, 17; and pH adjusted to 6·4–6·7. In the comparative study on the growth of *X. c. oryzae* strains on the new medium and four other synthetic media reported for the bacterium (Table 6), growth of all five Chinese strains on the new medium was superior to that on other media. None, except that of Noda & Ohuch (1983), was well suited to growth of these strains although less growth of strain OS-14 was observed on the media of NIAS (Anon. 1959), Tanaka (1964) and Watanabe (1963b). The IRRI strain PXO-86 grew on all the media tested but the colonies did not grow confluent at the inoculated locations.

Five complex composite nutrients (beef extract, casein hydrolysate, peptone, tryptone and yeast extract), each at six different concentrations (0·1, 0·5, 1·0, 5·0, 10 and 20 g/l), were added to the new synthetic medium respectively, and their effect on growth of *X. c. oryzae* strains and 34 bacterial contaminants from rice materials was observed after inoculation. Yeast extract (5 g/l), peptone (5 g/l) or tryptone (5 g/l) allowed the largest size and the most rapid appearance of colonies of *X. c. oryzae*, but yeast extract and peptone facilitated even more rapid growth of contaminants. Tryptone (5 g/l) enhanced the growth of *X. c. oryzae* more than

Table 5. Effect of ammonium, ferric and ferrous ions in different manners on the growth of Chinese strain Zhe-173 of *Xanthomonas campestris* pv. *oryzae* on medium containing NH_4Cl or iron salts in various amounts

Amount of chemical	Mean colony number per plate*	Day first colony visible†	Mean diameter (mm)‡
NH_4Cl (g/l)			
0	22 c	11	4·0 ± 0·5
0·5	24 c	6	5·6 ± 0·4
1·0	33 d	6	5·6 ± 0·3
3·0	30 cd	6	4·2 ± 0·1
5·0	0 a		
$FeSO_4$ (mg/l)			
0	15 b	11	4·2 ± 0·2
10	25 c	6	5·3 ± 0·4
50	29 c	11	4·2 ± 0·1
200	2 a	11	3·7 ± 0·2
Fe^{++}-(EDTA) (ppm)			
1	33 d	6	5·6 ± 0·4
5	0 a		
$FeCl_3$ (mg/l)			
10	0 a		

* Colonies were counted 11 d after spread-inoculation. Mean numbers of colonies followed by different letters are significantly different ($P = 0·05$) according to Duncan's multiple range test.

† Observations were made only 6 d and 11 d after spread-inoculation.

‡ Mean colony diameters ± S.D. of measured data. Colonies were measured 11 d after spread-inoculation.

Table 6. Comparison of the new synthetic medium and several other reported media on the growth of *Xanthomonas campestris* pv. *oryzae* after inoculation by multipoint inoculator

Medium*	Growth of *X. c. oryzae* strain on duplicate plates†					
	Ks-1-20	Ks-1-21	Ks-3-8	Zhe-173	OS-14	PXO-86
New synthetic	6, 7	6, 7	6, 6	7, 7	7, 7	±
NIAS	–	–	–	–	±	±
Noda & Ohuchi	4, 4	3, 4	5, 5	5, 5	8, 8	±
Tanaka	–	–	–	–	2, 3	±
Watanabe	–	–	–	–	±	±

* The recipes of the media are listed in Table 1.

† Growth of the bacterium was observed and measured 6 d after batch inoculation. –, No growth; ±, growth of the bacterium but the colonies did not grow confluent at the inoculated location; the numbers refer to the diameters (mm) of the confluent colonies at the inoculated locations on duplicate plates.

Table 7. Comparison of the effect of the new basal medium and several other reported enriched media on the growth of Chinese strain Zhe-173 of *Xanthomonas campestris* pv. *oryzae* at 27°C

Medium*	Mean colony number per plate†	Day first colony visible	Mean colony diameter (mm)‡
New culture	39 e	2·5	4·5 ± 0·4
Goto	20 c	3	4·0 ± 0·0
MSB	6 a	5	2·4 ± 0·4
PRSM	14 b	2	5·9 ± 0·7
Suwa	19 c	3	3·6 ± 0·4
Wakimoto	12 b	4	4·5 ± 0·4
WF-P	29 d	4	8·1 ± 0·8
YSP	20 c	6	2·0 ± 0·0
Modified 523	28 d	3	4·2 ± 0·3

* The recipes of the media are listed in Table 1.

† Colonies were counted 8 d after spread-inoculation. Mean numbers of colonies followed by different letters are significantly different ($P = 0·05$) according to Duncan's multiple range test.

‡ Mean colony diameters ± S.D. of measured data. Colonies were measured 8 d after spread-inoculation.

that of many contaminants, including *E. herbicola*. Peptone, when its concentration was limited to 2 g/l, stimulated the growth of *X. c. oryzae* without promoting excessive growth of other potential contaminants.

A new culture medium for *X. c. oryzae* was modified from the new synthetic medium by lowering the concentration of sodium glutamate to 1 g/l and adding tryptone (5 g/l) and peptone (2 g/l). It facilitated the growth of *X. c. oryzae* with the highest recovery and the earliest appearance of colonies when compared with other media (Table 7). In the same experiment WF-P medium (Karganilla *et al.* 1973) and PRSM (Mizuta 1953) supported larger sized colonies but either fewer of them or a delay in their growth. The WF-P medium and the modification of the 523 medium of Kado & Heskett (1970) yielded a larger number of colonies than any other except the new medium. PRSM, Goto (Karganilla *et al.* 1973), Suwa (1962) and the modified 523 medium also allowed early appearance of colonies.

Discussion

Since the purpose of this research was to establish a basal culture medium for isolation of *X. c. oryzae* from seeds or other contaminated sources, the studies focused on the development of colonies on an agar medium. The number of colonies and the rate of their appearance are the most important critera for evaluating the

optimum nutrient requirement for isolation of *X. c. oryzae*. The selectivity of complex nutrients added to the medium for enriching culture of the bacterium is also important for the isolation. In this work peptone (2 g/l) promoted the growth of the bacterium more than that of many potential contaminants; tryptone (5 g/l) was shown to be even better for the selective culture of *X. c. oryzae* because the bacterium was better able to compete with many other potential contaminants, including *E. herbicola*, one of the major contaminants in isolation of *X. c. oryzae*.

The growth of *X. c. oryzae* was enhanced by a larger amount of sucrose (10 g/l) in the presence of tryptone in the agar medium compared with the synthetic medium in liquid culture (Watanabe 1963b). Use of neutral glutamate salt instead of glutamic acid facilitated poising the pH. Although cystine was a nitrogen source for growth of the bacterium in shaken liquid culture (Watanabe 1963b), its very low solubility in water may limit its utilization in an agar medium. As found by Noda & Ohuchi (1983), sodium glutamate combined with methionine promoted the colony development of *X. c. oryzae*.

The effect of the different phosphate salts and combinations tested in the experiment on the growth of the bacterium may be partially due to the different pH (6·0–7·2) they gave for media. The favourable pH reported for the growth of different strains of *X. c. oryzae* in liquid culture is between 6·0 and 7·0 (Fang *et al.* 1957; Watanabe 1963a). Dihydrogen phosphate salt (1 g/l) added in the basal agar medium gave a pH of 6·4 and favoured the best colony development. Significant promotion of the recovery of strain Zhe-173 by adding NH_4Cl (1 g/l) into the basal medium verified the results of Watanabe (1963b) and Fang & Hsu (1965) that ammonium could favour the growth of the bacterium. Ferrous ions at suitable concentrations stimulated the colony development of the bacterium, and their efficiency was greatly increased when chelated with EDTA. $MnSO_4 . H_2O$ (1 mg/l) was shown by Watanabe (1963a) to favour the growth of *X. c. oryzae* in liquid culture but, in the present experiments, either no effect or a slight retardation of growth of strain Zhe-173 was found (unpublished results).

The new synthetic and culture media for *X. c. oryzae* were developed by optimizing combination of the ingredients and their amounts based on the above studies. The new culture medium is a suitable basal medium for further studies on the selective culture of *X. c. oryzae* since it promoted the highest recovery and earliest appearance of colonies when compared with other reported media for the bacterium. However, WF-P was also a good medium for enriching culture of the bacterium because it promoted much larger colonies than any other tested media and, at the same time, supported a reasonable number of colonies.

When the five Chinese strains and one IRRI strain of *X. c. oryzae* were tested on various synthetic media, considerable differences in their nutrient requirements were revealed. The new medium and Noda & Ohuchi's medium supported good growth of all five Chinese strains but not the IRRI strain PXO-86. OS-14 differed from the other four Chinese strains since it also grew on the three other media. The new culture medium, which favoured the growth of the Chinese strains, should be tested on strains isolated from areas other than China.

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RI strain PXO-86. OS-14 differed
four Chinese strains since it also
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