STRAINS, VIRULENCE AND CONTROL OF *XANTHOMONAS ORYZAE*

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ABBREVIATIONS

ac	=	acre
a.i	=	active ingredient
Bl	=	Benzimidazole
c	=	about
c.c	=	cubic centimetre
conc	=	concentration
cm	=	centimetre
^{o}c	=	degree centigrade
g	=	gram(s)
G	=	granule
ha	=	hectare
kg	=	kilogram(s)
l	=	litre(s)
LANS	=	Long Ashton nutrient solution
lb	=	pound(s)
max	=	maximum
mg	=	milligram(s)
min	=	minimum
mm	=	millimetre(s)
nm	=	nanometre(s)
no	=	number

p.s.i	=	pounds per square inch
RH	=	Relative Humidity
SIA	=	soft ionagar
WP	=	Wettable powder
wt	=	weight
/	=	per
%	=	percentage

ABSTRACT

The variations in the resistance of rice cultivars in relation to variations in the virulence of isolates of *Xanthomonas oryzae* are characteristic of a vertical host-parasite relationship in which cultivars show differential (vertical) resistance and isolates exist in strains. This testifies gene-for-gene relationships between the hosts and strains of the pathogen.

Strain typing techniques for seedlings, adult plants and for leaves and *in vitro* have been worked out in relation to mass screening for cultivar resistance. Continuous use of one strain under laboratory conditions has been shown to have adverse effect on its virulence due to the increasing proportion of avirulent mutants in the culture. However, for mass screening for cultivar resistant to the disease, either the most virulent strain or one with the widest range of virulence could be used with pure cultures always stored in a deep freeze (-10°c) until used.

Culturally, the disease could be reduced significantly by avoiding the use of naturally contaminated seed and cutting of leaf tips before transplanting in disease endemic areas. Chemically, the disease could be successfully controlled by a new systemic, granular bactericide Rll5799 1% a.i G which is effective, non-phytotoxic, persistent and simple and convenient to apply under flooded conditions.

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INTRODUCTION AND REVIEW OF LITERATURE

The bacterial blight disease of rice (PLATE 1) caused by *Xanthomonas oryzae* (Uyeda & Ishiyama) Dowson is recognised as a destructive or potentially destructive disease in most of the major rice growing countries (OU, 1972; THURSTON, 1973). This is for the following reasons:-

(i) It is a vascular disease which causes wilting or blighting, first of the leaves, sheaths, culms and then of the whole plant (PLATE 2), a symptom popularly known as Kresek (ANON., 1963, GOTO, 1964).

(ii) No effective yet permissible bactericide has yet been developed against the disease (HO, 1977).

(iii) It has been shown to decrease greatly yield in rice (OU, 1972; EXCONDE *et al.*, 1973; HO & LIM, 1977a).

(iv) Increasingly, short-term, nitrogen-responsive, semi-dwarf cultivars are being grown, coupled with the use of large amounts of nitrogen fertilizer. Use of these cultivars and this practice favours infection and spread of the pathogen (WAKIMOTO, 1967; OU, 1972; HO & CHEW, 1974; HO, 1976).

(v) Development of resistant cultivars is slow and difficult.

Objectives

It is proposed to study strains of *Xanthomonas oryzae* in Malaysia in relation to cultivar resistance. This is necessary to:-

(i) identify new genes for resistance.

(ii) develop rice cultivars with stable resistance.

(iii) ensure that cultivar resistance screening programmes are carried out soundly with properly maintained strains.

(iv) facilitate breeding for resistance to specific virulence genes especially in relation to failure of disease resistance.

The study seeks to:-

(i) devise a suitable system of evaluation for strain classification at seedling, adult and flag leaf stages, based on lesion-types.

(ii) devise a rapid and convenient, yet accurate laboratory technique for strain classification.

(iii) improve upon the present multi-prick technique (HO, 1976; HO & LIM, 1977b; HO, 1978) in the mass cultivar resistance screening programme.

(iv) gain some knowledge on the cultural methods of control.

(v) identify effective new bactericide(s) against the disease.



PLATE 1. Typical bacterial blight symptoms produced by *Xanthomonas oryzae*, showing blighting of the leaves of susceptible adult rice plants.



PLATE 2. Kresek symptoms of bacterial blight produced by *Xanthomonas oryzae* on susceptible rice seedlings.

General Justification

Malaysia, a rice growing country and whose staple food is rice, should concentrate on increasing food production through increasing rice production, because, at present it has only achieved 85% self sufficiency despite annual increases of 2.5%. This is because of population increases by 2.7% per annum, which brings annual domestic consumption to 1.6 million tons compared to annual rice production of 1.4 million tons. This shortage of 200,000 tons of rice annually (ANON., 1975) leads to import from countries such as China, Thailand, Pakistan and Australia.

Although the development of more land for rice cultivation seems to be one of the major long-term plans, increase due to cultivation of high yielding rice cultivars and better management techniques has been recognised in the past and is still playing a prominent role in increased rice production in Malaysia (ANON., 1975). This being so, constraints to increased rice production such as those caused by important diseases e.g. bacterial blight should be studied in conjunction with breeding for high yielding cultivars in the light of the reasons given in paragraph 1 above.

Specific Justification

(i) Breeding for resistance is the only effective control at the moment, and this is complicated by the existence of strains of the bacterium.

(ii) The resistance of resistant cultivars often breaks down due to the presence of strains in the natural rice habitat.

(iii) Long-term mass screening for resistance has given variable results because the use of one culture type or strain gives rise to problems inherent in the culture itself.

(iv) Good techniques for storing cultures have to be devised to ensure that strains maintain their virulence for use in mass cultivar resistance screening programmes.

(v) The present multi-prick technique used in the mass cultivar resistance screening programme (HO, 1976; HO & LIM, 1977b; HO, 1978), although efficient, is rather crude, and needs refinement based on the same principles.

(vi) In view of the unpredictable nature of the disease occurrence it is necessary to explore interim short term control measures through cultural and chemical means.

Growth Medium for *Xanthomonas oryzae*

Studies on suitable growth and selective medica for *Xanthomonas oryzae* dates as far back as 1959 when the National Institution of Agricultural Sciences in Japan used a medium specifically for the nutritional studies of the bacterium. From then on, many types of media have been developed mainly by Japanese workers namely WAKIMOTO (1960), SUWA (1962), TANAKA (1964), WATANABE (1966), MURATA (1967), and a few other workers from other countries whose work was not recorded. However, the most widely used of these media is WAKIMOTO's (1960) potato semisynthetic agar (WPSA). KARGANILLA *et al.*, (1973) in their comparative studies of all the culture media came up with a modified Wakimoto's medium (MWPSA) claimed to be a simpler medium which supported very good colony formation. The studies further showed that there was no difference in surface growth on MWPSA when compared with WPSA, cultured by the streaking method.

It is the intention of the study to choose from among the many reported media, a medium which is good for rapid and copious surface growth of *Xanthomonas oryzae* cultured by the streaking method. For this, three media were selected to test for best surface growth in test tube slants used in all the experiments.

The three media selected for the study are:-

(i) Wakimoto's potato semisynthetic agar - WPSA (1960; 1967)

(ii) Modified Wakimoto's potato semisynthetic agar - MWPSA (KARGANILLA *et al.*, 1973).

(iii) Peptone - Sucrose age - PSA.

Strain Differentiation and Virulence in *Xanthomonas oryzae*

Breeding for reistance is probably the most effective means of control, but is complicated by the occurrence of strains of *Xanthomonas oryzae*. Earlier there was some controversy about the significance of the strains reported from several countries (TABLE 1) but work done in Japan and the Philippines has now provided clear evidence of strain differentiation in *Xanthomonas oryzae*.

The first indication that *Xanthomonas oryzae* exists in strains (races, pathotypes, or biotypes) was when a resistant cultivar Asakase became severely affected by the bacterial blight disease which brought about severe epidemic in Japan in 1957 (TAGAMI & MIZUKAMI, 1962; OU & JENNINGS, 1969; OU, 1972). The possibility of the existence of virulent strain of this bacterium was studied by several Japanese workers (KUHARA *et al.*, 1958). Attempts were made to classify bacterial strains on the basis of their virulence on sets of rice cultivars (TAGAMI & MIZUKAMI, 1962). Definite patterns of host-pathogen reactions which identified and classified bacterial strains were proposed by several Japanese workers (KUHARA *et al.*, 1965; KUSABA *et al.*, 1966; WATANABE, 1966; WASHIO *et al.*, 1966; WAKIMOTO, 1967). These studies conducted in Japan (TABLE 1) all show conclusively that "taxonomically different strains" of *Xanthomonas oryzae* exist (TAGAMI & MIZUKAMI, 1962).

Studies in India (TABLE 1) showed variation in the virulence of the bacterium (DEVADATH & PADMANABHAN, 1969; SRIVASTAVA, 1972). Studies conducted in Hawaii (SILVA *et al.*, 1970; BUDDENHAGEN *et al.*, 1972; BUDDENHAGEN & REDDY, 1972) showed only variation in virulence. In fact the variation in the virulence pattern of the isolates on a set of differential cultivars was considered by BUDDENHAGEN & REDDY (1972) to be "continuum differences" which was just an "expression of slight differences, isolate by isolate, across an unending continuum of gradations". He concluded by saying that he did not believe "that such isolate variability represents races in any meaningful sense..." and "that the interaction of rice cultivars with *Xanthomonas oryzae* is an expression of horizontal relationship".

In the Philippines, however, the existence of strains in *Xanthomonas oryzae* (TABLE 1) was realised when resistant cultivars, IR20, IR22, IR26 and Pelita I/I (which have the same dominant gene for resistance) became severely infected by the Isabela strain (PX0) of *Xanthomonas oryzae*

(TIN WIN & KAUFFMAN, 1974; TIN WIN, 1974). This led to further studies by REDDY (1974) which showed that a certain degree of host pathogenic specialization existed in *Xanthomonas oryzae*. In his studies, he found that vertical relationships existed between resitant cultivars and virulent pathotypes while horizontal relationships existed between rice cultivars and weakly virulent strains. Recent report by REDDY & OU (1976) suggested the existence of definite strains of *Xanthomonas oryzae* when their cultivar - isolate interaction studies showed that a "vertical pathotype" existed in *Xanthomonas oryzae*. In more recent studies in the Philippines, MEW (1978 a, b) showed that the host-parasite relationship is vertical between virulence groups and horizontal within virulence groups which in essence suggests that *Xanthomonas oryzae* exists in definite strains in its natural habitat.

TABLE 1. VIRULENCE AND STRAINS OF *XANTHOMONAS ORYZAE*, 1957 - 1978.

Country	Evidence for differences	Year	Source
1. Japan	Strain	1957	TAGAMI & MIZUKAMI, 1962; OU & JENNINGS, 1969; OU, 1972.
2. Japan	Strain	1958	KUHARA *et al.*, 1958.
3. Japan	Strain	1960	WAKIMOTO, 1960.
4. Japan	Strain	1965	KUHARA *et al.*, 1965.
5. Japan	Strain	1966	KUSABA *et al.*, 1966; WATANABE, 1966; WASHIO *et al.*, 1966.
6. Japan	Strain	1967	WAKIMOTO, 1967.
7. India	Virulence	1969	DEVADATH & PADMANABHAN, 1969; SRIVASTAVA, 1972.
8. Hawaii	Virulence	1970	SILVA *et al.*, 1970.
9. Hawaii	Virulence	1972	BUDDENHAGEN *et al.*, 1972.
10. Hawaii	Virulence	1972	BUDDENHAGEN & REDDY, 1972.
11. Philippines	Strain	1974	TIN WIN, 1974; TIN WIN & KAUFFMAN, 1974.
12. Philippines	Strain	1974	REDDY, 1974.
13. Philippines	Strain	1976	REDDY & OU, 1976.
14. Philippines	Strain	1978	MEW, 1978 a, b.

The overall results from studies conducted in Japan, India, Hawaii and the Philippines (TABLE 1) suggest the existence of strains in *Xanthomonas oryzae*, and in the words of TAGAMI & MIZUKAMI (1962) "It is now concluded that taxonomically different strains exist in *Xanthomonas oryzae*". But despite a large volume of work being done on this aspect of the disease, they are still of the opinion that "... the method for classifying bacterial strains on the basis of pathogenicity was not firmly established yet". This is mainly because different workers used different procedures and criteria in strain studies in terms if (i) growth stage (ii) inoculation method and (iii) evaluation system (TABLE 2), which are among the important reasons for the controversy about the existence of strains in *Xanthomonas oryzae* as reported in TABLE 1. The use of weakly virulent isolates may be another reason why definite strains were not shown by the host-pathogen interaction studies of BUDDENHAGEN & REDDY (1972).

TABLE 2. METHODS USED FOR IDENTIFICATION OF STRAINS OF *XANTHOMONAS ORYZAE*, 1967 - 1978.

Growth Stage	Inoculation Method	Evaluation System	Source
1. Seedling Stage (young plant)			
(i) 4-5 leaf stage	Needle Prick	Lesion length-measurement	WAKIMOTO, 1967
(ii) 30-day old	Scissors (clip-inoculation)	Lesion length measurement	REDDY, 1974; REDDY & OU, 1976
(iii) Seedlings	Pin-prick	Lesion size-visual (IRRI's scale, 1965)	BUDDENHAGEN & REDDY, 1972
(iv) Seedlings	Needle prick	Lesion type-visual	KUSABA *et al.*, 1958
2. Adult stage			
(i) 50-60 days old	Scissors (clip-inoculation)	Lesion length measurement	MEW, 1978a, b
3. Reproductive Stage			
(i) Flag Leaf	Needle prick	Lesion size-visual	TAGAMI & MIZUKAMI, 1962

As preliminary study an investigation was done to see if cultivar and strain differentiation existed amoung the rice cultivars and *Xanthomonas oryzae* isolates collected from major rice growing regions of Malaysia. If they existed then laboratory and glasshouse techniques for strain typing would be worked out for Malaysian conditions.

Differential Cultivars and Strains

Many sets of differential cultivars had been identified and used by different workers in Japan, the Philippines and in Hawaii for strain and virulence studies. These sets of differential cultivars vary from country to country as do the distribution of the strains and cultivars.

In Japan, TAGAMI & MIZUKAMI (1962) reported the use of different groups of rice differential cultivars by which strains were classified into three categories based on lesion size formed on the flag leaves and disease interactions in relation to growth stages. The ten commonly used differential cultivars are: Kogyoku, Zensho 26, Asakaze, Norin 27, Morin 18, Oita-mii 120, Beni-sengoku, Jukkoku, Asahi 1 and Takara. WAKIMOTO (1967) listed 19 resistant, intermediate and susceptible differential cultivars used in classifying *Xanthomonas oryzae* strains into 2 main groups with 3 strain types in each group. The differential cultivars are: Koganemaru, Akashinriki, Norin 27, Asakaze, Kogyoku, Shinzeki 1, Norin 12, Tozan 38, Oita-mii 120, Norin 44, Sasashigure, Nakasengoku, Shinsen, Norin 29, Zensyo 17, Taisyo-ako 66, Jukkoku, Aichi-asahi and Kinmaze.

In Hawaii, SILVA *et al.*, (1970) proposed a set of 27 cultivars for use in differentiating virulence in *Xanthomonas oryzae* isolates. BUDDENHAGEN *et al.*, (1972) in their study on the virulence in *Xanthomonas oryzae* isolates from tropical Asia proposed another set of seven differential cultivars namely, Koganemaru, Semora Mangga, TKM 6, Lacrosse-Zenith-Nira, Giza 38, Tsao-Tsuan and Peta. In 1972, BUDDENHAGEN and REDDY proposed yet another set of six differential cultivars which classified *Xanthomonas oryzae* isolates into eight pathogenic groups and "not races" as stated. These differential cultivars are: BJ1, TKM6, Semora Mangga, LZN, Tsao Tsuan and JC 70.

Work in the Philippines (MEW, 1978a) which confirms the existence of strains in *Xanthomonas oryzae* and their classification based on their virulence patterns formed on differential cultivars in relation to specificity of genes to the resistance factor, used five differential

cultivars. They are: IR 8, IR 20, IR 1545-339-2-2, DV 85 and Cempo Selak.

From the studies conducted in Japan, Hawaii and the Philippines, the identification of one set of differential cultivars would seem to be a requisite in any strain classification programme. However, as the virulence of *Xanthomonas oryzae* varies a great deal from region to region (SILVA *et al.*, 1970; OU, 1972), country to country (BUDDENHAGEN *et al.*, 1972; BUDDENHAGEN & REDDY, 1972) and even within countries (MEW, 1978a) the composition of sets of differential varieties also tend to vary from country to country as is evident in paragraphs 2, 3 and 4 above. This maybe necessary to cater for relevant classification of a wide range of local isolates into strains so that local breeding for resistance against the most virulent strains identified in this manner, could be carried out more effectively.

One of the objectives of this study was therefore to identify a good set of differential cultivars from Malaysia and the Philippines.

The use of differential cultivars in strain identification and classification automatically involves some important technical aspects such as the inoculation method and the evaluation system. The review of literature has revealed that different workers used different inoculation methods and evaluation systems (TABLE 2), which explains the discrepancies in the results obtained on the existence of strains in *Xanthomonas oryzae* as shown in TABLE 1. From preliminary studies, it was shown that the strain cultivar interactions could be effectively and accurately expressed in three definite reaction types based on lesion types formed namely: resistant (R), moderate (M), and susceptible (S), which are described in TABLE 5 and shown in PLATE 3 (refer Section 2). It was also shown that the multi-prick method of inoculation (HO & LIM, 1977b; HO, 1978) which is basically similar to the pin prick method (HO, 1972) was an effective method for revealing the expressions of various reaction types. It was also the intention of this study to demonstrate the effective use of the proposed evaluation system (TABLE 5) and the combined use of the pin prick apparatus according to the multi-prick technique, called the mass pin prick method, in the identification and classification of differential cultivars and strains of *Xanthomonas oryzae* respectively.

Strain Typing Techniques for Adult Plant

Accuracy and consistency in strain typing on adult plants should be maintained in order to ensure repeatable and reliable results. This depends on consistent use of factors such as type of leaf used, growth stage of plants, time of evaluation, technique of inoculation and the evaluation scale used for strain identification and classification.

The type of leaf on which inoculation is done for strain study on live plants is important, as different leaves give different results in disease reaction. A study was done to identify the best leaf to be used for inoculation with strains in relation to stages of plant growth.

As strain identification and classification is based on the formation of distinct lesion types giving rise to distinct reaction as indicated by seedling reactions in the previous studies and the newly proposed strain evaluation scale in TABLE 5, it is also important to study methods of inoculation in relation to lesion formation and hence reaction.
In this respect the time of evaluation is also studied in relation to types of leaf used and the growth stage of the plants.

Laboratory Techniques for Strain Typing

Laboratory techniques for strain typing using detached leaf culture has been devised successfully for powdery mildew of wheat caused by *Erysiphe graminis* f.sp. *tritici* (WOLFE, 1969). However, it has not been done for bacterial blight of rice *(Xanthomonas oryzae)*. To date all the strain typing work on *Xanthomonas oryzae* has been done with plants at either the seedling or adult stage (refer TABLE 2). It was conceived to be a good idea to try this technique with bacterial blight of rice for three main reasons:-

(i) It would be very useful and advantageous in places where there are limited facilities for growing rice in standard conditions.

(ii) It provides faster and simpler evaluation of strains.

(iii) It provides better standardization of various factors such as uniformity in growth medium, type and segment of leaves used, place of inoculation and environmental conditions.

PERSON *et al.*, (1957) reported that detached wheat leaves could be maintained for relatively long periods when floated on benzimidazole or kinetin solutions. The metabolic changes in such detached leaves of wheat have been studied by SAMBORSKI *et al.*, (1958), for concentrations of benzimidazole as high as 100 ppm which allow normal development of the wheat disease caused by the rust fungus *Puccinia graminis* (Pers. f.sp. *tritici* (ERIKISS. & HENN). However, this technique of detached leaf culture for studies on host-parasite relationship has since been used and improved upon by several scientists (WOLFE, 1969; RUSSELL *et al.*, 1975), as a result of their work on the rust disease of wheat (*Erysiphe graminis* f.sp. *tritici*) and powdery mildew of barley (*Erysiphe graminis* f.sp. *hordei*).

WOLFE (1969) improved the technique for detached leaf culture by using a soft 0.5 per cent agar medium containing 200 ppm benzimidazole for his work on race identification of *Erysiphe graminis* f.sp. *tritici*. RUSSELL *et al.*, (1975) however, used similar soft agar medium but incorporated with only 150 ppm benzimidazole in clear polystyrene boxes with tight-fitting lids, in his study on the germination of conidia of *Erysiphe graminis* f.sp. *hordei* on detached barley leaves.

In the case of *Xanthomonas oryzae* successful laboratory strain typing using the detached leaf culture technique would depend on several factors which were investigated. The information gathered from work done on detached leaf culture in wheat provides a useful guideline for such investigations.

Culture Maintenance in Laboratory

Routine screening for bacterial blight resistance normally involves repeated use of a virulent isolate over a long period. It is therefore necessary that cultures of such an isolate be well stored and cultured so that its vurlence persists for a long time.

Although many phytopathogenic bacteria especially certain pseudomonads retain their virulence for a long time under laboratory conditions, some especially among the xanthomonads lose their virulence relatively quickly and may even die within one or two weeks on commonly used bacteriological media (KIRALY *et al.*, 1974).

Several methods of preserving bacterial cultures are recommended. These include preservation under mineral oil and lyophilization. *Xanthomonas oryzae* can be preserved in clay suspensions in which a high percentage of viable bacteria can be recovered even after 400 days (GOTO, 1969). It may be kept in phosphate buffer at pH 7.0 and peptone water for a considerable time but could not be kept for a long time in sterile distilled water as could other xanthomonads (OU, 1972). Some of these methods are however, for long term storage. When the same bacterial cultures are frequently used, short term storage methods would be more useful and convenient. However, this involves successive sub-culturing of the bacterium and in the case of *Xanthomonas oryzae* this has been reported to induce formation of avirulent mutant populations which increased gradually (FUJII, 1976). Additions of avirulent mutants to its virulent form inhibited the multiplication of the virulent form in rice leaves and hence lesion enlargement proportional to the amount of avirulent mutants added (FUJII, 1976). Very little difference however, was observed between the two forms in colony type, lysotype and other physiological characteristics (FUJII & UEMATSU, 1975).

KIRALY *et al.*, (1974) further recommended that after growing xanthomonads cutures every one to two weeks they should be kept at 3° to 6°c. On the basis of this recommendation and the fact that the minimum temperature for the growth of *Xanthomonas oryzae* is 5° to 10°c (OU, 1972) and its tendency to change virulence, the present study was planned to investigate variations in virulence under short term storage condition so as to devise a good maintenance technique which would keep the bacterium viable and its original virulence for long periods.

Cultural Control

Increase in nitrogen in the soil and cultivation of nitrogen responsive varieties have been reported to increase considerably the disease in the field in recent years (MIZUKAMI & WAKIMOTO, 1969; HAVE & KAUFFMAN, 1972; SRIVASTAVA, 1972; HO & CHEW, 1974; HO & LIM, 1977c, 1979). It is clear therefore that cultural methods involving the judicious use of nitrogen fertilizers and proper plant density could reduce the disease considerably (HO & LIM, 1979).

Rice seeds, especially those from infected plants, have always been considered one of the important sources of infection in the field. The bacterium had been found to be present in the glumes and occasionally in the endosperm and as such FANG *et al.*, (1956) considered seed to be a source of infection. In India a high percentage of seed infection was found (SRIVASTAVA & RAO, 1964a). The survival of the bacterium in the seeds for various lengths of time up to the next season has been demonstrated (GOTO *et al.*, 1953; WAKIMOTO, 1955; SEKI & MIZUKAMI, 1955; FANG *et al.*, 1956; SRIVASTA & RAO, 1963; YOSHIMURA & TAGAMI, 1967; HSIEH *et al.*, 1974). It has also been reported that infected seeds usually produced infected plants (FANG *et al.*, 1971; SRIVASTAVA & RAO, 1964a, 1964b, 1968; RAO *et al.*, 1971). However, despite all these studies controversy still exists on seed transmission of the pathogen. Although the bacterium has been shown to survive in seeds for a certain period of time, ANON.,(1968).,EAMCHIT *et al.*, (1969), have shown that transmission of the pathogen through seeds was not likely in normal conditions and that no disease symptoms were observed on plants grown from contaminated or infected seeds.

In rice transplanting, seedlings are pulled from the nursery and planted out in the fields. This process often damage the rice root system which according to MIZUKAMI, (1957) attracts swarms of the bacterium towards the roots, and hence facilitates infection of the plants through damaged roots. Also, the leaf tips of the seedlings are often cut before transplanting. This is done to facilitate rapid and easy transplanting and reduces the leaf surface area and hence evapotranspiration. However, according to OU, (1972) these two processes are important causes of infection and one partly responsible for the severe Kresek symptoms in the tropics.

It is because of the controversial nature of seed transmission of the disease and the desire to study this and associated cultural practices under Malaysian conditions that the studies reported below were conducted.

Chemical Control

Bacterial blight is rated as one of the most difficult bacterial diseases to control (TAGAMI & MIZUKAMI, 1962). The problem of effective control still remain. Plant pathologists and plant breeders have been tackling

the problem through breeding for resistance. However, this is a long term solution and hence does not meed immediate needs when epidemics threaten crops.

Chemical control has been an objective of many chemical companies. Yet the number of chemical formulations coming through for field testing is extremely few and declining (EGLI & STRUM, 1980). HO, (1977) reviewed the work on chemical control done in major rice growing countries such as Japan, Philippines, India and Malaysia, and came to the conclusion that at the moment there is no effective yet permissible bactericide for controlling the disease in Malaysia and similar countries. The main reasons include phytotoxicity and mammalian toxicity of the final products in the field. There are however some products which have been tested and found to be effective mainly in Japan but not in Malaysia (HO, 1977). Even then, these reportedly effective chemicals are only partially effective although they may be effective in inhibiting the growth of bacterial pathogen in the laboratory. Because of the controversies in the results of bactericide testings and the slow progress made in the direction of chemical control, the present study was initiated to re-examine some of the ecological factors involved which hopefully might lead to more relevant and effective testings in the field. It is also the intention of this study to identify effective bactericides through stringent screening techniques.

EXPERIMENTAL

1. SELECTION OF GROWTH MEDIUM FOR *XANTHOMONAS ORYZAE*

1.1. Materials and Methods

Media

The three media were prepared with the components stated in TABLE 3. They were boiled for about one hour and pipetted into test tubes (pyrex 150 x 15 mm) at four ml. per tube, and autoclaved for 15 minutes at 15 psi (120°c). After this, they were made into agar slants, the surfaces of which were allowed to dry for a period of two to three weeks at room temperature (25° ± 3°c) before use.

TABLE 3. COMPONENTS OF THREE DIFFERENT MEDIA FOR SURFACE GROWTH OF *XANTHOMONAS ORYZAE* (g/l)

Component[1]	Medium[2]		
	WPSA	MWPSA	PSA
1. Potato decoction	300	-	-
2. Peptone	5.0	5.0	10.0
3. Sucrose	20.0	20.0	10.0
4. Na-glutamate	-	-	1.0
5. $Ca(NO3)_2.4H_2O$	0.5	0.5	-
6. $Na_2HPO_4.12H_2O$	2.0	2.0	-
7. $FeSO_4.7H_2O$	-	0.5	-
8. Agar	17.0	17.0	17.0
9. Distilled Water	1000 ml	1000 ml	1000 ml
10. pH (after autoclaving)	6.8-7.0	6.8-7.0	6.8-7.0

1 : g unless shown as ml

2 : WPSA = Wakimoto's potato semisynthetic agar. MWPSA = Modified WPSA. PSA = Peptone - sucrose agar.

Isolation of Bacterial Isolates

Five isolates obtained from infected rice leaves from two localities in Malaysia were used for the study. They were isolated by the induced-bacterial-ooze method as follows:-

(i) Sections of infected leaves, about 2 cm long were placed on petri-dishes lined with most filter paper.

(ii) The petri-dishes were placed in the super-cool compartment of an ordinary refrigerator with the temperature set at 5° to 7°C for two to three days.

(iii) Bacterial oozes which appeared around fresh lesions especially at the cut ends of the leaf sections were picked up by a sterile loop and streaked on the WPSA slants in a zigzag fashion, and incubated for two to four days at 28° ± 2°C.

(iv) Single colonies of yellow bacteria believed to be *Xanthomonas oryzae* were transferred on to new WPSA slants and incubated for two to three days at 28° ± 2°C.

(v) Bacterial suspensions were made from these re-isolated cultures in sterile distilled water and streaked on to WPSA plates and incubated for two to three days at 28° ± 2°C.

(vi) *Xantomonas oryzae* colonies were picked up from the ends of the final streaks and culture on to WPSA slants. They were incubated for two to three days at 28° ± 2°C.

(vii) These cultures, assumed to be pure, were then tested for pathogenicity according to KOCH's Postulates, using a susceptible cultivar of rice, T(N)1.

(viii) The pathogenic isolates were collected, identified and labelled.

The five isolates used in this study are as follows:-

(i) Bt - 4K8 (obtained from Kedah areas)
(ii) BL - T3 (obtained from Penang areas)
(iii) BL - T2b1 (obtained from Penang areas)
(iv) BL - T2b2 (obtained from Penang areas)
(v) BL - T2b3 (obtained from Penang areas)

Preparation of Bacterial Suspension for Study

One loopful of a two-day old bacterial culture of each of the five isolates was streaked on to the surface of the WPSA slants of each of the different media in a zigzag fashion. Each isolate and each medium was replicated three times. The inoculated WPSA slants were incubated for two days at 28° ± 2°C. Bacterial suspensions were made by pipetting 10 ml of distilled water into each tube culture and dispersed by a rotary mixer (Super-Mixer Cat. No. 1291 Lab-Line instrument Inc Melrose Park III). Bacterial suspension prepared in this way gave a concentration of about 10^9 cells/ml (HO, 1976).

Methods of Assessment

Two methods of assessment were used to measure surface bacterial growth after two days' incubation. They are:-

(i) Visual Growth Assessment method (VGA)
(ii) Optical Density method (OD)

The VGA method was used for the surface growth of cultures before suspensions were made, to observe and score on their nature and extent of their growth. This method is much aided by experience in dealing with *Xanthomonas oryzae* cultures. Generally the nature and extent of surface growth could be scored visually as:-

(i) Good growth
(ii) Fair growth
(iii) Poor growth

The OD measurements were done by means of the Spectronic 20 (Bausch & Lomb) spectrophotometer at a wavelength of 620 nm using standard bacterial suspensions prepared as described above. Their populations were estimated using the calibration graph in FIG 1.

All three replicates of all the treatments were scored for VGA and OD after two days' incubation and recorded.

1.2 Results

Results for the three media on the surface growth of five isolates of

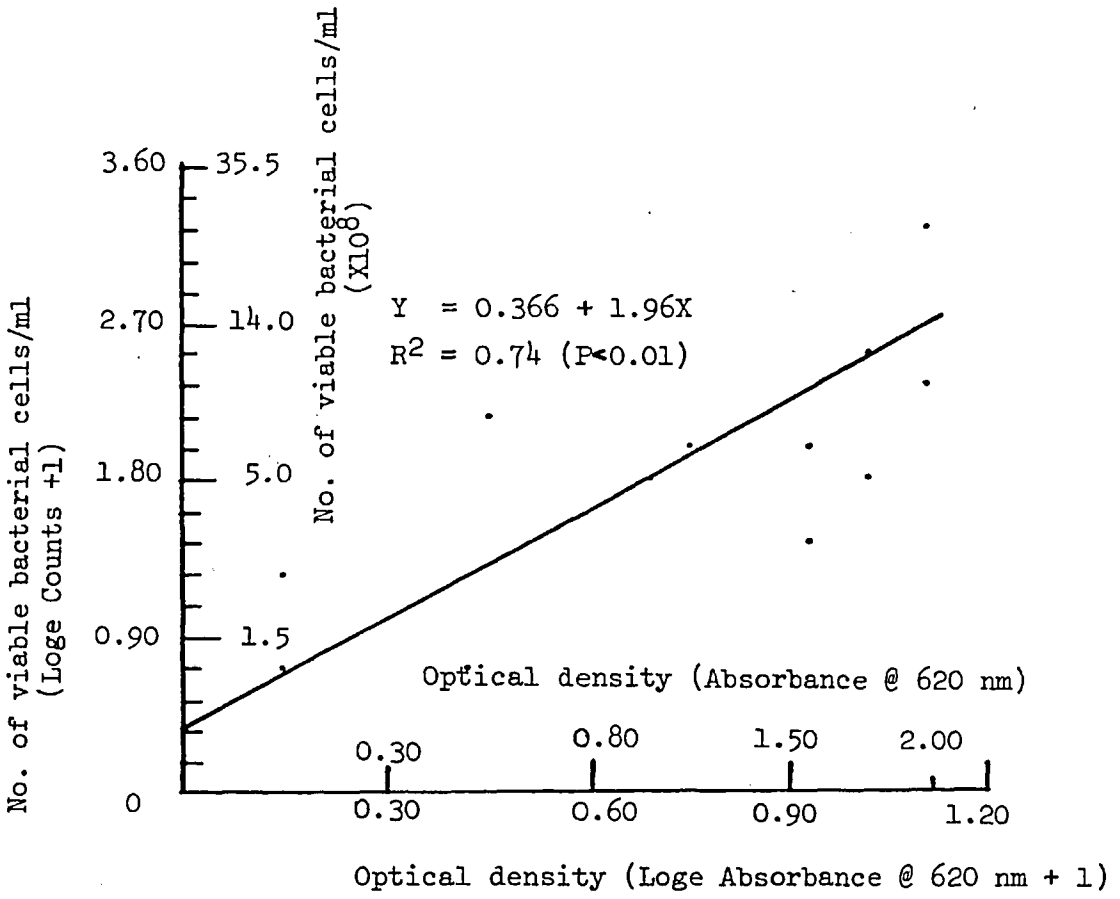


FIG.1. Calibration of the density of *Xanthomonas oryzae* suspension obtained from 48-hour old surface cultures (HO, 1981).

Xanthomonas oryzae in terms of visual growth and optical density, are presented in TABLE 4.

TABLE 4. EFFECTS OF THREE AGAR MEDIA ON SURFACE GROWTH OF 5 *XANTHOMONAS ORYZAE* ISOLATES.

Medium[1] / Isolate	WPSA		MWPSA		PSA	
	VGA	OD	VGA	OD	VGA	OD
Bt - 4K8	Good	1.6	Fair	1.4	Poor	1.1
BL - T3	Good	1.5	Fair	1.4	Poor	1.0
BL - T2b1	Good	1.6	Good	1.5	Poor	1.0
BL - T2b2	Good	1.6	Fair	1.4	Poor	1.0
BL - T2b3	Good	1.5	Good	1.5	Poor	1.1
Average	Good	1.6	Fair	1.4	Poor	1.0

1 : WPSA = Wakimoto's potato semisynthetic agar.
MWPSA = Modified WPSA.
PSA = Peptone - sucrose agar.
VGA = Visual growth assessment (mean of 3 replicates).
OD = Optical density measured in absorbance at 620 nm (mean of 3 replicates).

The results of visual growth assessments show that all the five isolates gave "good growth" on WPSA, "good" to "fair growth" on the MWPSA and "poor growth" on PSA (TABLE 4). The optical density measurements confirm this trend with average measurements of 1.6, 1.4 and 1.0 respectively indicating that the WPSA is the best medium for surface growth of *Xanthomonas oryzae*.

1.3 Discussion

Although the WPSA was shown to be the same as MWPSA in term of surface growth of *Xanthomonas oryzae* by the streaking method (KARGANILLA *et al.*, 1973), the study shows that in terms of visual growth assessment and optical density measurements the WPSA is superior to its modified form and the PSA. This is probably because the WPSA has potato decoction as one of its ingredients which MWPSA and PSA did not have and also had essential elements as provided by calcium nitrate and di-sodium phosphate which PSA did not have. However, in terms of number of viable bacterial cells, it can be seen from FIG.1., that both the WPSA and MWPSA (with mean optical densities of 1.6 and 1.4 respecitvely) gave more than 10^9 cells/ml while that of PSA (with mean optical density of 1.0 gave only about 6 x 10^8 cells/ml).

2. PRELIMINARY STUDY ON CULTIVAR AND STRAIN DIFFERENTIATION

2.1. Materials and Methods

Bacterial Isolates

Twenty bacterial isolates obtained from seven localities in the western and north-western regions of peninsular Malaysia, were purified and cultured on WPSA. The isolates and the areas where they were collected were as follows:-

		Locality	
	Isolate	State	Area
(i)	PTIC	Penang	Pinang Tunggal
(ii)	PR5i	"	Permatang Rambai
(iii)	PR11a	"	" "
(iv)	BL - T1b	"	Bumbong Lima
(v)	BL - T3	"	" "
(vi)	BL - T6	"	" "
(vii)	BL - T7	"	" "
(viii)	BL - T8	"	" "
(ix)	BL - T2b	"	" "
(x)	BL - F4a	"	" "
(xi)	BL - F5a	"	" "
(xii)	PK1	"	Permatang Kuang
(xiii)	Bt. 18a-12e	Kedah	Batu Lapanbelas
(xiv)	Bt. 4K2	Perak	Batu Empat, Krian
(xv)	Bt. 4K3	"	" " "
(xvi)	Bt. 4K4	"	" " "
(xvii)	Bt. 4K5	"	" " "
(xviii)	Bt. 4K7	"	" " "
(xix)	Bt. 4K8	"	" " "
(xx)	SK3	"	Sungai Kota, Parit

Preparation of Bacterial Suspension for Inoculation

Sufficient bacterial suspensions of the twenty isolates were prepared according to the procedures in Section 1.1.

Test Cultivars

Three rice cultivars of three generally different reactions to the bacterial isolates were used for the study. They are as follows:-

(i) IR22 - generally resistant (R)
(ii) Jaya - generally moderately resistant (M)
(iii) T(N)1 - generally susceptible (S)

Sufficient seedlings of these three cultivars were raised on wet nursery beds in the field for 25 days, after which they were transplanted at the rate of two seedlings per hill and ten hills per cultivar per isolate. The plants were fertilized with three types of fertilizers at the rate of 90 kg N, 45 Kg P_2O_5 and 35 Kg K_2O/ha, in the forms of urea, superphosphate and muriate of potash respectively. Urea was split equally for two applications - half was applied at transplanting and the other half at maximum tillering stage, while superphosphate and muriate of potash were applied only at transplanting. All fertilizers were broadcast manually.

Inoculation

The plants were inoculated at maximum tillering stage (50-60 days after sowing) at the rate of ten hills per cultivar per isolate by means of the multi-prick inoculation technique described by HO (1976), HO & LIM (1977b) and HO (1978).

Disease Assessment

Disease reactions were evaluated 20-21 days after inoculation using the assessment scale based on lesion types proposed in TABLE 5. Five hills were randomly selected for assessment. Two tillers were assessed per hill giving a total of ten tillers assessed per cultivar per isolare. As only one leaf was assessed per tiller, the total number of leaves assessed was also ten leaves per cultivar per isolate.

Only the second fully expanded top leaves which had been inoculated around the mid leaf blade region were assessed. This was done to standardise all sampling and disease assessment procedures as much as possible.

TABLE 5. SCALE FOR ASSESSING CULTIVAR RESISTANCE AND STRAIN REACTIONS OF *XANTHOMONAS ORYZAE* BASED ON LESION TYPES FORMED ON RICE CULTIVARS

Disease scale	Description of infection based on lesion type	Reaction	Approximate leaf area infected
0	No lesion	Resistant (R)	25% or less
1	Negligible lesion		
2	Lesion limited, rounded or elongated with well-defined margins.		
3	Lesion covering about $\frac{1}{4}$ to $\frac{1}{2}$ leaf blade with defined margins.	Moderately resistant (M)	50% more or less
4	Lesion covering about $\frac{3}{4}$ leaf blade	Susceptible (S)	75% or more
5	Lesion covering entire leaf blade downwards.		

2.2. Results and Discussion

Evaluation of isolate reactions on the three rice cultivars based on lesion type (TABLE 5) proved to be convenient in that three definite reaction types (R, M, S) could be easily and broadly catergorised (TABLE 6) as shown in PLATE 3. As such all the ten scores per cultivar per isolate gave similar reaction types. This was also possible through the standardization of all sampling and disease assessment procedures as mentioned in Section 2.1 above. Results in TABLE 6 show that certain isolates gave completely different reactions. A generally resistant cultivar, IR22, was resistant to most of the isolates but moderately resistant to some, and a generally moderately resistant cultivar, Jaya had reactions ranging from resistant to susceptible, whereas a generally

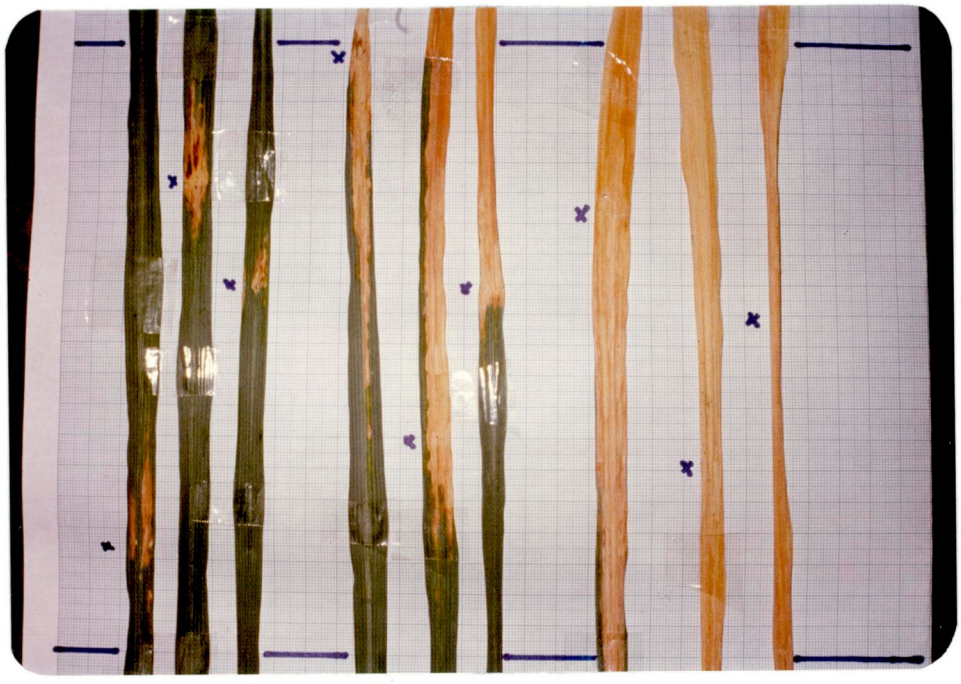
susceptible cultivar was moderately resistant to some and susceptible to others. These reactions suggest a vertical relationship between *Xanthomonas oryzae* isolates and rice cultivars as defined by VAN DER PLANK (1963, 1968) and TARR (1972). This confirms results of studies conducted by several workers in Japan and the Philippines (TABLE 1).

TABLE 6. DISEASE REACTIONS[1] OF 3 RICE CULTIVARS OF DIFFERENT RESISTANCE TO 20 *XANTHOMONAS ORYZAE* ISOLATES.

Isolate \ Cultivar	IR22 (R)[2]	Jaya (M)[2]	T(N)1 (S)[2]
1. PT1C	R	M	M
2. PR5i	R	M	M
3. Bt. 18a-12e	R	R	M
4. BL - T1b	M	M	S
5. BL - T2b	M	M	S
6. BL - T3	M	M	S
7. BL - T6	M	M	S
8. BL - T7	M	S	S
9. BL - T8	R	M	S
10. PR 11a	R	R	M
11. BL - F4a	R	R	M
12. BL - F5a	R	R	M
13. Bt. 4K2	R	M	M
14. Bt. 4K3	R	R	M
15. Bt. 4K4	R	R	M
16. Bt. 4K5	R	R	M
17. Bt. 4K7	R	R	M
18. Bt. 4K8	R	M	M
19. PK1	R	M	M
20. SK3	R	R	M

1 : Each score was the same on each of the 10 leaves scored.
R = Resistant. M = Moderately resistant. S = Susceptible.

2 : General reaction types.



R M S

PLATE 3. Classification of disease reaction based on lesions types produced by *Xanthomonas oryzae* isolates on rice cultivars. R = Resistant, M = Moderately resistant, S = Susceptible. Note that these reactions were produced irrespective of the points of inoculation (x) on similar leaves.

As certain isolates exerted similar reaction types on the host cultivars it is possible to group these isolates according to their disease reaction patterns on host cultivars. Based on this, the twenty isolates tested could then be placed into five groups as shown in TABLE 7.

TABLE 7. GROUPING OF 20 *XANTHOMONAS ORYZAE* BASED ON REACTION TYPES PRODUCED ON 3 RICE CULTIVARS OF DIFFERENT RESISTANCE

Group \ Cultivar	IR22	Jaya	T(N)1	Isolates
I	R	R	M	Bt. 18a-2e, PR11a, BL - F4a BL - F5a, Bt. 4K3, Bt. 4K4, Bt. 4k5, Bt. 4K3.
II	R	M	M	PT1C, PR5i, Bt. 4K2, Bt. 4K8, PK1.
III	R	M	S	BL - T8.
IV	M	M	S	BL - T1b, BL - T2b, BL - T3, BL - T6.
V	M	S	S	BL - T7.

3. CULTIVAR AND STRAIN DIFFERENTIATION

3.1. Seedling Reactions and Inoculation Technique

3.1.1. Materials and Methods

Bacterial Isolates

Ten of a total of 56 isolates of *Xanthomonas oryzae*, collected from major rice growing regions of Malaysia were randomly selected for study and were as follows:-

		Locality	
Isolate code	Isolate	State	Area
A	BL - T8	Penang	Bumbong Lima
B	PM(A)12a	Penang	Permatang Merah
C	BL - T2b	Penang	Bumbong Lima
E	PM(K)1	Penang	Permatang Manggis
H	PK3	Penang	Permatang Kuang
I	Bt.18a-12e	Kedah	Batu Lapanbelas
K	Bt.4K2	Perak	Batu Empat, Krian
L	Bt.4K8	Perak	Batu Empat, Krian
M	PM(MC)1	Penang	Penaga
S	PM6	Penang	Penaga

Preparation of Bacterial Suspensions for Inoculation

Sufficient bacterial suspension of the 10 isolates were prepared according to the procedures in Section 1.1

Test Cultivars

Seven cultivars from Malaysia (SMII, Jaya, Bahagia, Mahsuri, MRI, MR33, MR35), six from Philippines (IR8, IR20, IR22, DV85 Cempo Selak, IR1545-339-2-2) and one from Taiwan (T(N)1) were used. Seeds of the 14 cultivars were selected to give near 100% germination.

Growing of Plants

Plastic trays (34 x 21 x 5 cm) were filled with a loam soil (TABLE 8) up to 4 cm and well soaked for 2 days before sowing each tray with 2 rows of 7 cultivars separated by a 3 cm space.

Each row contained 20 seeds which were covered with a thin layer of the same soil and watered twice a day to keep them wet and the trays were placed in bigger water-containing trays (PLATE 4) in a glasshouse where temperature (23°-36°C) and relative humidity (62-91%) were kept suitable for growth of rice seedlings (TABLE 9). Artificial light for 16 hours daily was such that plants grew well with good green healthy foliage They were used only at the seedling stage. No fertilizer was applied and soil was kept water-logged.

Inoculation

Seedlings were inoculated 27 days after sowing with bacterial suspension in 2 ways, with the pin prick apparatus (HO, 1972; PLATE 5) around the mid-foliage region of the seedlings such that each leaf blade received at least 2 pricks; and the scissors method (= shearing method, HO, 1973; clip-inoculation

TABLE 8. DETAILS[1] OF THE SOIL USED FOR GROWING RICE SEEDLINGS FOR STRAIN TYPING OF *XANTHOMONAS ORYZAE* AND CULTIVAR RESISTANCE EVALUATION IN THE GLASSHOUSE.

Sample Indentification	Greenhouse soil[2]
Texture	Silt loam
pH	5.4
Phosphorus	
(i) Amount (mg/l)	18
(ii) Index	2
Potassium	
(i) Amount (mg/l)	100
(ii) Index	1
Magnesium	
(i) Amount (mg/l)	87
(ii) Index	2
Conductivity	
(i) Micro mhos	2121
(ii) Index	0
(iii) Lab. Density (g/cc)	1.0
Nitrate Nitrogen (mg/l)	21

1 : Soil analysis was carried out by the Agricultural Development and Advisory Service (ADAS), Reading, England, 19 February 1979.

2 : Locally known as "Surrey loam" (DAVIES, 1979).



PLATE 4. Planting method used for strain typing of *Xanthomonas oryzae* and screening for cultivar reistance at seedling stage in the glasshouse.

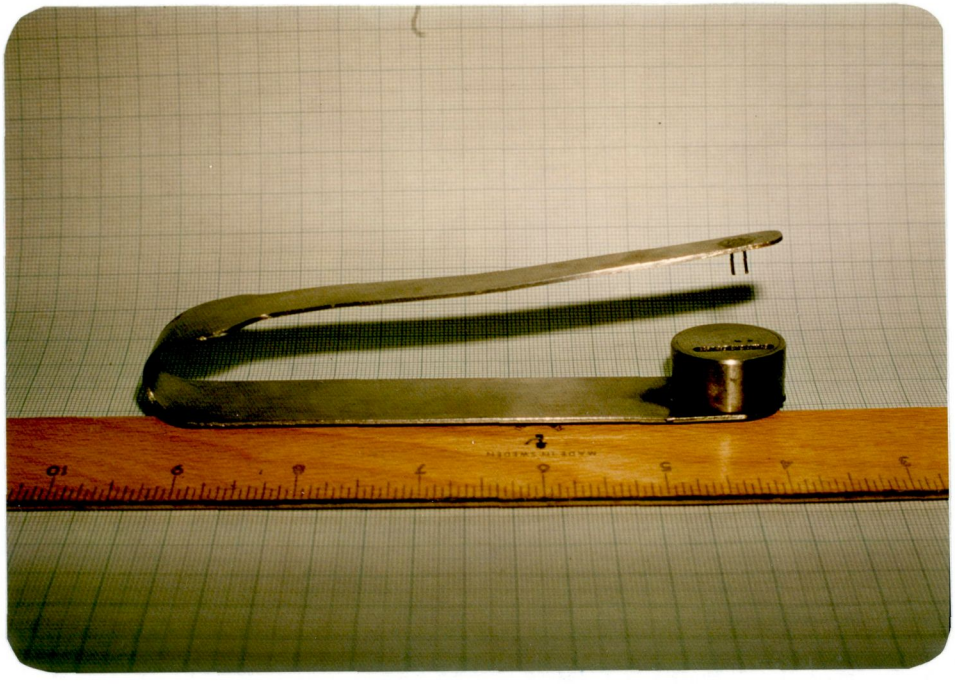


PLATE 5. The pin prick apparatus used for mass or individual inoculation of seedling leaves for strain typing of *Xanthomonas oryzae* and cultivar resistance evaluation.

TABLE 9. MEAN MONTHLY MAXIMUM AND MINIMUM TEMPERATURE (°C) AND RELATIVE HUMIDITY (%) FROM NOVEMBER 1978 TO JULY 1979 IN THE GLASSHOUSE AT SILWOOD PARK

Year	1978		1979							
Month	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jly	Mean
Temperature (°C)										
Max.	28	33	35	35	39	38	40	38	38	36
Min	15	26	27	27	26	23	20	22	22	23
Relative Humidity (%)										
Max.	94	86	90	98	93	93	88	91	86	91
Min.	67	69	71	75	62	55	52	56	50	62

method, KAUFFMAN *et al.*, 1973), where a pair of scissors, dipped in a bacterial suspension before each cutting, inoculated the seedlings when shearing the top 7-10 cm of the leaf blades.

Disease Assessment

Disease reactions were evaluated 14 days after inoculation on the 0-5 scale given in TABLE 5. Ten topmost inoculated leaves per cultivar per isolate which had been inoculated around the mid-leaf blade region were randomly selected for the assessments. Where the "lesion type" criterion was difficult to apply, a criterion based on the "approximate leaf area infected" was used as a guide. This was particularly needed for leaves inoculated by the scissors method.

3.1.2. Results and Discussion

The mean disease scores for the mass pin prick and the scissors inoculation methods (TABLES 10 & 11) indicate that isolate reations varied widely across the set of 14 cultivars with average disease scores ranging from minimum (1) to maximum (5). This was also true for cultivar resistance across the set of 10 isolates. Such cultivar and isolate differences were significant at the 0.1% level (TABLE 12) by the non-parametric Friedman two-way analysis of variance by ranks (SIEGEL, 1956) and at the 1% level by the parametric F-test (TABLE 13). Similar results were given by the scissors inoculation method

TABLE 10. DISEASE SCORES[1] OF 10 *XANTHOMONAS ORYZAE* ISOLATES ON SEEDLINGS OF 14 RICE CULTIVARS INOCULATED BY THE MASS PIN PRICK METHOD

Cultivar \ Isolate	A	B	C	F	H	I	K	L	M	S	Cultivar mean (Rank)
IR20	1.5	3.0	3.5	3.0	4.0	2.5	1.5	3.0	4.0	3.0	2.9 (8)
IR22	1.0	3.0	3.5	3.0	4.0	1.5	1.0	2.5	3.3	3.0	2.5 (11)
IR1545-339-2-2	1.0	1.5	3.0	1.0	2.0	1.5	1.0	2.0	3.0	1.0	1.7 (14)
MR33	1.5	3.0	4.5	3.0	4.0	3.0	1.4	3.0	4.7	4.0	3.1 (6)
MR1	1.0	3.5	3.0	1.0	4.7	1.0	1.5	1.5	4.0	4.0	2.5 (11)
DV85	1.0	1.5	2.0	3.0	3.0	2.5	3.0	3.0	3.0	2.0	2.5 (11)
Jaya	2.0	4.0	4.5	3.0	4.7	3.0	3.0	3.5	4.5	4.0	3.7 (4)
Mahsuri	1.5	3.0	3.5	2.0	4.0	1.0	3.0	1.5	4.0	3.0	2.7 (10)
IR8	4.0	5.0	4.5	4.0	5.0	4.5	1.8	3.0	4.7	4.0	4.1 (1)
Cempo Selak	1.0	4.0	1.0	3.0	3.0	2.0	3.0	4.0	4.0	3.0	2.9 (8)
SMII	3.0	4.2	4.5	3.5	4.8	3.5	4.0	3.5	4.7	4.0	4.0 (2)
T(N)1	3.0	4.3	3.7	3.5	4.3	3.5	3.0	4.5	4.3	3.5	3.8 (3)
MR35	1.0	4.5	3.7	3.0	4.3	1.5	1.5	3.5	3.7	4.0	3.1 (6)
Bahagia	2.5	4.1	3.8	2.0	4.7	3.0	3.0	3.5	4.5	4.0	3.5 (5)
Isolate mean (Rank)	1.8 (10)	3.5 (3)	3.5 (3)	2.7 (7)	4.0 (1)	2.4 (8)	2.3 (9)	3.0 (6)	4.0 (1)	3.3 (5)	

1 : Mean of 10 disease scores from 2 replicates, based on a 0-5 scale of increasing disease (see TABLE 5).

TABLE 11. DISEASE SCORES[1] OF 10 *XANTHOMONAS ORYZAE* ISOLATES ON SEEDLINGS OF 14 RICE CULTIVARS INOCULATED BY THE SCISSORS METHOD.

Cultivar \ Isolate	A	B	C	F	H	I	K	L	M	S	Cultivar mean (Rank)
IR20	1.0	1.5	2.5	2.0	4.5	1.5	1.5	2.0	4.0	2.2	2.3 (10)
IR22	2.0	2.5	4.1	2.0	4.1	2.0	1.5	2.0	3.9	2.3	2.6 (6)
IR1545-339-2-2	1.0	1.0	2.5	1.0	2.0	1.0	1.0	2.0	3.0	1.0	1.6 (13)
MR33	2.0	3.5	4.1	2.0	4.2	2.0	2.0	2.0	4.3	3.3	2.9 (3)
MR1	2.0	3.5	3.5	2.0	4.1	2.0	2.0	1.5	3.9	2.0	2.7 (5)
DV85	1.0	2.0	1.5	1.5	2.0	1.5	1.5	1.0	2.0	1.2	1.5 (14)
Jaya	1.0	2.0	3.4	2.0	4.0	2.0	1.5	1.5	4.5	2.0	2.4 (9)
Mahsuri	1.5	2.0	2.3	1.5	2.0	1.5	1.5	1.5	3.5	1.8	1.9 (11)
IR8	2.0	3.9	3.9	2.0	4.8	3.0	1.5	2.0	4.7	3.0	3.1
Cempo Selak	1.5	2.0	1.5	1.5	2.0	1.5	2.0	2.0	2.0	1.5	1.8 (12)
SMII	2.0	3.0	3.7	2.0	4.2	2.0	2.0	2.3	4.8	2.7	2.9 (3)
T(N)1	2.0	2.5	4.5	2.5	4.7	2.0	2.0	3.5	4.2	4.6	3.3 (1)
MR35	1.5	2.5	2.9	2.0	4.0	1.5	2.0	2.0	4.8	2.0	2.6 (6)
Bahagia	1.5	2.5	2.5	2.0	4.7	2.0	2.0	1.5	3.5	1.9	2.5 (8)
Isolate mean (Rank)	1.6 (10)	2.5 (4)	3.1 (3)	1.9 (6)	3.7 (2)	1.8 (8)	1.7 (9)	1.9 (6)	3.8 (1)	2.3 (5)	

1 : Mean of 10 disease scores from 2 replicates, based on a 0-5 scale of increasing disease (see TABLE 5).

TABLE 12. NON-PARAMETRIC TEST[1] FOR VARIATIONS IN DISEASE SCORES OF 10 *XANTHOMONAS ORYZAE* ISOLATES ON SEEDLINGS OF 14 RICE CULTIVARS INOCULATED BY THE MASS PIN PRICK AND THE SCISSORS METHODS

Source of variation	Degree of freedom	Friedman's chi-square statistical value (Xr^2)	
		Mass pin prick method	Scissors method
Cultivar	13	59.9 ***	55.0 ***
Isolate	9	87.3 ***	57.1 ***

1 : Friedman two-way analysis of variance by ranks (SIEGEL, 1956)
*** = Significant at the 0.1% level.

TABLE 13. ANALYSIS OF VARIANCE FOR DISEASE SCORES OF 10 *XANTHOMONAS ORYZAE* ISOLATES ON SEEDLINGS OF 14 RICE CULTIVARS INOCULATED BY THE MASS PIN PRICK METHOD (DATA IN TABLE 10).

Source of variation	Degree of freedom	Sum of squares	Mean square	Observed F-values	Tabular F-values	
					5%	1%
Replicate	1	0	0			
Cultivar[1]	13	118.09	9.08	227.0**	2.58	3.91
Error (a)	13	0.48	0.04			
Isolate[2]	9	142.28	15.81	1581.0**	3.18	5.35
Error (b)	9	0.09	0.01			
Cultivar X Isolate[3]	117	104.73	0.90	26.47**	1.35	1.55
Error (c)	117	3.99	0.034			
Total	279					

1 : Coefficient of variation a (CVa) = 6.5%
2 : CVb = 3.2%
3 : CVc = 6.0%

(TABLES 12 & 14), indicating that both inoculation methods differentiated cultivar resistance and isolate reactions to similar degrees. Both methods gave a highly significant interaction (at the 1% level) between cultivars and isolates (TABLE 13 & 14). The results according to VAN DER PLANK (1963, 1968) strongly suggest a vertical host-parasite relationship with cultivars showing differential (vertical) resistance and *Xanthomonas oryzae* isolates existing in strains which differed in virulence and not aggressiveness. The results confirm those of preliminary studies (in Section 2), REDDY & OU, (1976) and most Japanese reports (TAGAMI & MIZUKAMI, 1962). By ranking isolate mean disease scores in order of decreasing virulence and cultivar mean disease scores in order of decreasing susceptibility (TABLES 10 & 11), the 2 methods of inoculation showed good agreement for the isolates but differed in 4 cultivars viz. IR22, MRI, Jaya and Cempo Selak.

For the purpose of strain typing, the mass pin prick method of inoculation was better comparatively because it produced lesions easily assessed by the proposed disease scale (TABLE 5) which proved to be realistic, convenient and rapid for large scale assessment. This was because the disease reaction types, resistant (R), moderately resistant (M) and susceptible (S) were clearly produced on similar leaves irrespective of the points of inoculation (PLATE 4). Also, the effects of cultivar, isolates and interactions between them, were more precisely measured by the mass pin prick method as their F-values were much greater than those of the scissors method (TABLES 13 & 14).

TABLE 14. ANALYSIS OF VARIANCE FOR DISEASE SCORES OF 10 *XANTHOMONAS ORYZAE* ISOLATES ON SEEDLINGS OF 14 RICE CULTIVARS INOCULATED BY THE SCISSORS METHOD (DATA IN TABLE 11)

Source of variation	Degree of freedom	Sum of squares	Mean square	Observed F-values	Tabular F-values	
					5%	1%
Replicate	1	0.36	0.36			
Cultivar[1]	13	80.14	6.16	102.67**	2.58	3.91
Error (a)	13	0.79	0.06			
Isolate[2]	9	168.74	18.75	937.5 **	3.18	5.35
Error (b)	9	0.18	0.02			
Cultivar X Isolate[3]	117	63.15	0.54	15.43**	1.35	1.55
Error (c)	117	4.07	0.035			
Total	279					

1 : Coefficient of variation a (CVa) = 10.2%
2 : CVb = 5.9%
3 : CVc = 7.8%

The differential resistance to isolates of *Xanthomonas oryzae* in rice cultivars was probably due to specificity based on the gene-for-gene relationship between cultivars and isolates. Hence strain typing to characterise isolates of *Xanthomonas oryzae* in term of virulence could probably be most efficiently and realistically done following the techniques described and based on differential disease reaction patterns on 2 differential cultivars of widely differing reaction patterns (IR1545-339-2-2 and IR8) and 1 of intermediate reaction pattern (Cempo Selak) where the proportions of postulated resistant, moderately resistant and susceptible genes were approximately equal (30 : 40 : 30%) and well distributed across the isolates irrespective of their general virulence (TABLE 15). For strain typing purposes, the moderately resistant reaction could be safely lumped together with the susceptible reaction because it is a typical lesion which tends to become susceptible with changes in ecological factors. On the basis of differential disease reactions formed on the 3 differential cultivars a total of 9 strains (Xo_1 - Xo_9) comprising of 6 virulence classes could be used to characterise the isolates (TABLE 16) into weak (isolates A, I) moderately weak (isolates K, F, L, S, B, H), moderately virulent (isolate M). None was found to be either very weak or very virulent. However, certain cultivars do exhibit either non-differential, very little or part differential reactions against the isolates. These are IR1545-339-2-2, MRI, IR20, MR35, Jaya, T(N)1, SMII and IR8 (TABLE 15). Using 6 selected cultivars, isolates of *Xanthomonas oryzae* could also be characterised in term of range of virulence as shown in TABLE 17 into 6 virulence groups. Although there are obvious differences between the two systems of strain classification, however, both systems could identify the most virulent strain or the one with the widest range of virulence which could then be used in mass screening for cultivars resistant to the disease. However, it must be pointed out that different disease reaction patterns would be obtained when different differential cultivars were used as different sets of genes for resistance would be involved. It is believed that virulence of the pathogen is often inhereited oligogenically following the gene-for-gene system of host-parasite relationships, which strengthens belief that for disease to occur the genes of the pathogen must match those of the host (VAN DER PLANK, 1968). This therefore provides an alternative basis whereby cultivars could be characterised in terms of genes for susceptibility matching genes for virulence in different isolates.

TABLE 15. REACTION TYPES[1] OF 10 *XANTHOMONAS ORYZAE* ISOLATES ON SEEDLINGS OF 14 RICE CULTIVARS INOCULATED BY THE MASS PIN PRICK METHOD

Isolate[2] / Cultivar[3]	A	K	I	F	L	S	B	C	H	M	% R	% M	% S
IR1545-339-2-2	R	R	R	R	R	R	R	M	R	M	80	20	0
DV85	R	M	M	M	M	R	R	R	M	M	40	60	0
MRI	R	R	R	R	R	S	S	M	S	S	50	10	40
IR22	R	R	R	M	M	M	M	S	S	M	30	50	20
Mahsuri	R	M	R	R	R	M	M	S	S	S	40	30	30
Cempo Selak	R	M	R	M	S	M	S	R	M	S	30	40	30
IR20	R	R	M	M	M	M	M	S	S	S	20	50	30
MR35	R	R	R	M	S	S	S	S	S	S	30	10	60
MR33	R	R	M	M	M	S	M	S	S	S	20	40	40
Bahagia	M	M	M	R	S	S	S	S	S	S	10	30	60
Jaya	R	M	M	M	S	S	S	S	S	S	10	30	60
T(N)1	M	M	S	S	S	S	S	S	S	S	0	20	80
SMII	M	S	S	S	S	S	S	S	S	S	0	10	90
IR8	S	R	S	S	M	S	S	S	S	S	10	10	80

1 : R = Resitant. M = Moderately resistant. S = Susceptible

2 : In order of increasing virulence.

3 : In order of increasing susceptibility.

TABLE 16. TYPING AND CLASSIFICATION OF *XANTHOMONAS ORYZAE* ISOLATES BASED ON DIFFERENTIAL DISEASE REACTION[1] PATTERNS ON 3 RICE DIFFERENTIAL CULTIVARS

Strain	Differential cultivar			Virulence	Classification of isolates
	IR1545-339-2-2	Cempo Selak	IR8		
Xo_1	R	R	R	Very weak	
Xo_2	R	R	M-S	Weak	A, I
Xo_3	R	M-S	R	Moderately	K
Xo_4	R	M-S	M-S	weak	F, L, S, B, H
Xo_5	M-S	R	R	Moderately	
Xo_6	M-S	R	M-S	virulent	C
Xo_7	M-S	M-S	R		
Xo_8	M-S	M-S	M-S	Virulent	M
Xo_9	S	S	S	Very virulent	

1 : R = Resistant. M = Moderately resistant. S = Susceptible

TABLE 17. CLASSIFICATION OF *XANTHOMONAS ORYZAE* ISOLATES BASED ON RANGE OF VIRULENCE[1] ON 6 RICE DIFFERENTIAL CULTIVARS

Differential cultivar / Virulence group	IR1545-339-2-2	MRI	MR35	IR20	Jaya	T(N)1	Range of Virulence	Classification of isolates
I	R	R	R	R	R	R	Very limited	
II	R	R	R	R	R	M	Limited	A
III	R	R	R	R	M	S	Moderately	K
	R	R	R	M	S	S	limited	I
IV	R	R	M	S	S	S	Moderately	F, L
	R	M	S	S	S	S	wide	S, B, H
V	M	S	S	S	S	S	Wide	C, M
VI	S	S	S	S	S	S	Very wide	

1 : R = Resistant. M = Moderately resistant. S = Susceptibile

4. STRAIN TYPING TECHNIQUES FOR ADULT PLANTS

4.1. Comparison of Two Inoculation Methods at Maximum Tillering Stage in the Glasshouse

4.1.1 Materials and Methods

The experiment was carried out in glasshouse in a randomised complete block design with three replicates. Glazed and holeless circular-clay pots of 30 x 28 cm (diameter) were used for the experiment. Typical rice soil, the Kundor-Tualang association was filled into each pot used in the experiment, leaving about 5 cm at the top to hold water.

Five pots were used for each treatment. The pots were transplanted with 25-day old seedlings of a susceptible rice cultivar T(N)1 obtained from the nursery, at the rate of two seedlings per pot. The treatments were as follows:-

T_1 = Plants inoculated by the "incisor" inoculation method (PLATE 6). It is so called because the two perforated inoculation plates (each of 5 x 3 mm) resemble the upper incisors of the teeth.

T_2 = Plants inoculated by the pin prick inoculation method (PLATE 5).

T_3 = Plants inoculated with water by the incisor method.

T_4 = Plants inoculated with water by the pin prick method.

The plants were fertilized with urea, super-phosphate and muriate of potash at the rate of 90 kg N/ha, 45 Kg P_2O_5/ha and 35 Kg/ha respectively throughout the growth period. Urea was applied in two equally split applications as basal dressing before transplanting and at maximum tillering stage one week before inoculation, whereas the super-phosphate and the muriate of potash were applied entirely as basal dressings. The plants were treated with treatments T_1 to T_4 at the maximum tillering stage (50 days after sowing) using a virulent strain of *Xanthomonas oryzae* (Isolate M) for treatments T_1 and T_2 and water for the two controls T_3 and T_4. Bacterial suspension for inoculation was prepared according to procedures in Section 1.1.

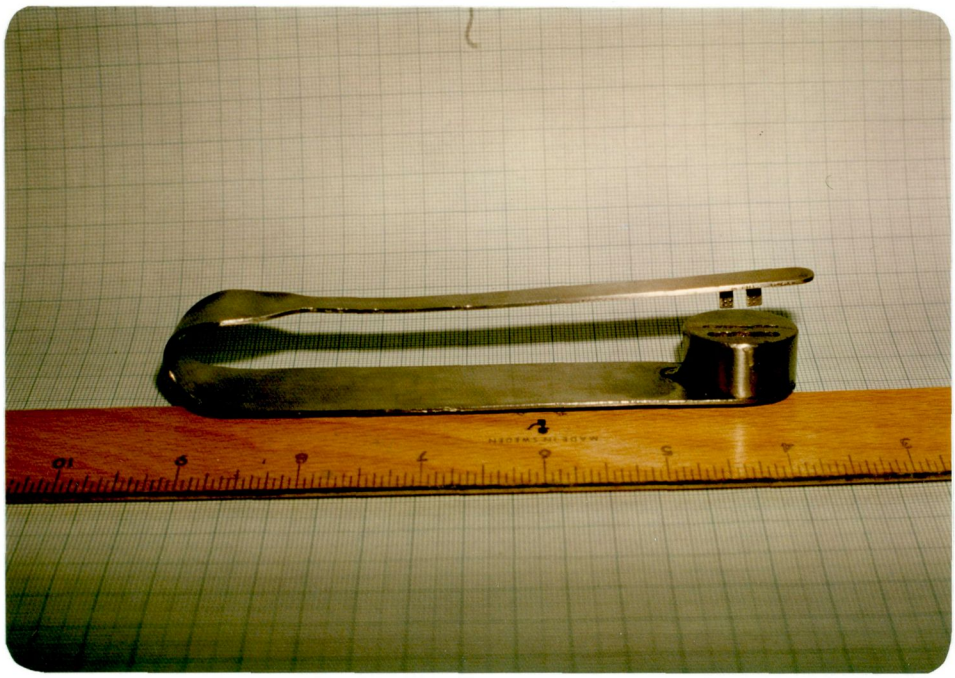


PLATE 6. The "incisor" inoculation apparatus used for inoculating rice leaves with *Xanthomonas oryzae* for strain and cultivar resistance evaluation in adult plants.

The plants of the control plots were inoculated first before using the inoculation apparatus for treatments T_1 and T_2. The 1st and 2nd fully opened top leaves of two tillers were inoculated per pot giving a total of ten 1st and ten 2nd fully opened top leaves inoculated per treatment per replicate. The leaves were inoculated individually in the mid-leaf blade region on either side of the mib-rib. The extend of disease was scored at 14 days after inoculation using the disease assessment scale in TABLE 5.

4.1.2. Results

With reference to TABLE 18, it can be seen that the two controls using the incisor and the pin prick methods of inoculation did not give any disease. Between the two methods of inoculation, the incisor method caused disease earlier as the initial symptom was produced 4 days after inoculation (DAI) and advanced symptom at 5 DAI compared to the pin prick method which produced these effects only at 6 DAI and 7 DAI respectively.

TABLE 18. MEAN DISEASE SCORES[1] OF 2 INOCULATION METHODS ON 1ST AND 2ND FULLY OPENED TOP LEAVES OF A SUSCEPTIBLE CULTIVAR T(N)1 14 DAYS AFTER INOCULATION WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* IN THE GLASSHOUSE, WITH INITIAL AND ADVANCED LESION DEVELOPMENT TIMES

Type of leaf / Treatment	1st leaf			2nd leaf			Lesion development (DAI)[2]	
	Replicate 1	2	3	Replicate 1	2	3	Initial	Advanced
T_1 (Incisor)	4.2	4.0	3.6	4.4	4.3	3.8	4	5
T_2 (Pin prick)	3.1	3.2	3.1	3.7	3.7	3.6	6	7
T_3 (Incisor-water)	0	0	0	0	0	0	-	-
T_4 (Pin prick-water)	0	0	0	0	0	0	-	-

1 : Mean of 10 disease scores, based on a 0-5 scale of increasing disease (see TABLE 5)

2 : DAI = Days after inoculation.

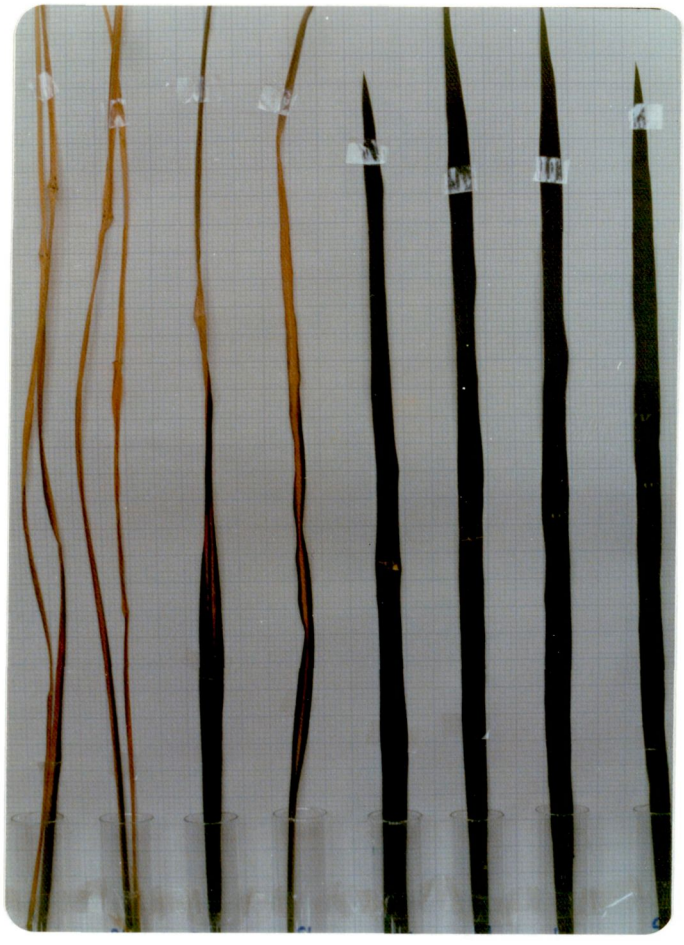
TABLE 19. MEAN DISEASE SCORES[1] OF TWO INOCULATION METHODS ON 1ST AND 2ND FULLY OPENED TOP LEAVES OF A SUSCEPTIBLE RICE CULTIVAR T(N)1 14 DAYS AFTER INOCULATION WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* IN THE GLASSHOUSE AND RESULTS OF CHI-SQUARE TEST

Type of leaf / Treatment	1st	2nd	Chi-square test[2]
T_1 (Incisor)	3.9	4.2	NS
T_2 (Pin prick)	3.1	3.7	**
Chi-square test[2]	**	*	

1 : Mean of 30 disease scores from 3 replicates based on a 0-5 scale of increasing disease (see TABLE 5).

2 : NS = Not significant at the 5% level. * = Significant at the 5% level. ** = Significant at the 1% level.

The average disease scores produced by both methods of inoculation on the 1st and 2nd fully opened top leaves (TABLE 19) show that the latter were more susceptible to the disease than the former. Chi-square tests (SIEGEL, 1956) indicate that the incisor method did not produce significant difference in the mean disease scores on the 1st and 2nd leaves although the latter did give a slightly higher disease score. However, the pin prick method did differentiate (PLATE 7) the significantly higher disease score (at 1% level) on the 2nd leaf which was however, significantly lower (at 5% level) than that produced on similar leaf by the incisor method but gave similar susceptible reactions (TABLES 19 & 5). This indicates that the pin prick method was more sensitive to effects caused by changes in plant . It emphazises the importance of using the same type of leaf for strain evaluation in identification and classification and in the evaluation of cultivar resistance. Therefore where lesion types and disease reaction types are the main criteria it is more suitable for strain indentification and classification, to use the pin prick method of inoculation on the 2nd fully opened top leaves at maximum tillering stage under glasshouse conditions, and assessed at 14 days after inoculation.



1 2 3 4 5 6 7 8

PLATE 7. Lesions produced by a virulent strain of *Xanthomonas oryzae* on the 1st and 2nd fully opened top leaves of a susceptible rice cultivar T(N)1 inoculated by the pin prick (pp) and the incisor (Inc) methods. (1, 2 = Inc. method on 1st and 2nd leaf respectively. 3, 4 = pp. method on 1st and 2nd leaf respectively. 5, 6 = Inc. method - control on 1st and 2nd leaf respectively. 7, 8 = pp. method - control on 1st and 2nd leaf respectively).

4.2. Reactions of Four *Xanthomonas oryzae* isolates on Six Differential Cultivars at Maximum Tillering Stage in the Glasshouse

4.2.1. Materials and Methods

25-day old seedlings of 6 differential cultivars (see TABLE 17) were transplanted at the rate of 2 seedlings per pot. The pots (15 cm diameter) had been lined with polythene bags to make them water-proof and filled with loamy clay soil (TABLE 8) leaving sufficient space at the top to hold water. Three pots were used for each cultivar. Types and rates of fertilizers applied and their time of application were similar to those in Section 4.1.1. The mass pin prick method of inoculation as described in Section 3.1.1 was used to inoculate the differential cultivars at maximum tillering stage (50 days after sowing) using standard bacterial suspensions (refer Section 1.1) of 4 isolates (A, S, C, M) respresenting 3 virulence groups (TABLE 17). The experiment was carried out in flooded conditions and disease reactions were evaluated on the 2nd fully opened top leaves of all tillers 14 days after inoculation using the disease scale in TABLE 5.

4.2.2. Results

The reaction patterns of the 4 isolates on the differential cultivars (TABLE 20) suggested the classification of the isolates into 3 virulence groups, limited (isolate A), moderately wide (isolate S) and wide (isolates C, M) according to established reaction patterns on seedlings in TABLE 17. The results were similar to those obtained on seedlings (TABLE 17), indicating the suitability of the system of classification of *Xanthomonas oryzae* isolates based on range of virulence on differential cultivars at maximum tillering stage.

4.3. Comparison of Two Inoculation Methods at the Flag Stage in the Glasshouse

4.3.1. Materials and Methods

Two inoculation methods, the incisor and pin prick methods as in Section 4.1 were tested. The procedures, treatments and the rest of the materials and methods were similar to those in Section 4.1 except that only the flag leaves were inoculated. Five flag leaves were inoculated per pot and 5 pots were used for each treatment.

TABLE 20. REACTIONS[1] OF 4 *XANTHOMONAS ORYZAE* ISOLATES INOCULATED BY THE MASS PIN PRICK METHOD ON 6 RICE DIFFERENTIAL CULTIVARS AT MAXIMUM TILLERING STAGE AND THEIR CLASSIFICATION INTO RANGE OF VIRULENCE[2]

Isolate \ Differential cultivar	IR1545-339-2-2	MR1	MR35	IR20	Jaya	T(N)1	Range of Virulence
A	R	R	R	R	R	M	Limited
S	R	R	M	M	S	M	Moderately wide
C	M	M	M	M	S	S	Wide
M	M	M	M	S	S	S	Wide

1 : Each reaction was assessed from 6 plants in 3 pots.
R = Resistant. M = Moderately resistant. S = Susceptible.

2 : Classification was based on established pattern in TABLE 17.

Disease assessments were carried out 14, 20 and 25 days after inoculation using the scale in TABLE 5, on 3 randomly picked flag leaves per pot giving a total of 15 flag leaves scored per treatment per replicate.

4.3.2. Results

The incisor method gave initial and advanced disease symptoms 2 days earlier (TABLE 21) than those produced by the pin prick method. Although Chi-square tests (TABLE 22) show that the disease scores between inoculation treatments and those between scoring times within inoculation treatments were significantly different (at the 5% - 1% levels) it can be seen that scoring done at 20 days after inoculation was sufficient to give a susceptible score of 3.7 by the pin prick method (TABLES 22 & 5). As the incisor method gave an equally susceptible score, at 20 days after inoculation, which was significantly higher (at the 5% level) it is felt that the method tended to give an unnecessarily higher disease score and hence would not be sufficiently sensitive in differentiating various disease reaction types based on lesion types compared to the pin prick method,

TABLE 21. MEAN DISEASE SCORES[1] OF 2 INOCULATION METHODS AT 3 SCORING TIMES AND THEIR INITIAL AND ADVANCED SYMPTOM DEVELOPMENT ON FLAG LEAVES OF A SUSCEPTIBLE CULTIVAR T(N)1 AFTER INOCULATION WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* IN THE GLASSHOUSE.

Scoring time / Treatment	14 DAI[2]			20 DAI			25 DAI			Symptom development (DAI)	
	Replicate			Replicate			Replicate			Initial	Advanced
	1	2	3	1	2	3	1	2	3		
T_1 (Incisor)	3.7	3.5	3.5	4.1	4.1	4.1	4.4	4.4	4.1	5	7
T_2 (Pin prick)	2.1	3.1	3.1	3.6	3.7	3.8	4.0	4.0	4.3	7	9
T_3 (Incisor-water)	0	0	0	0	0	0	0	0	0	-	-
T_4 (Pin prick-water)	0	0	0	0	0	0	0	0	0	-	-

1 : Mean of 15 disease scores, based on a 0-5 scale of increasing disease (see TABLE 5).

2 : DAI = Days after inoculation.

TABLE 22. MEAN DISEASE SCORES[1] OF 2 INOCULATION METHODS AT 3 SCORING TIMES AFTER INOCULATION ON FLAG LEAVES WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* IN THE GLASSHOUSE AND RESULTS OF CHI-SQUARE TESTS[2]

Scoring time / Treatment	14 DAI[3]	20 DAI	25 DAI	Chi-square test	
				14 & 20 DAI	20 & 25 DAI
T_1 (Incisor)	3.6	4.1	4.3	**	*
T_2 (Pin prick)	2.8	3.7	4.1	**	**
Chi-square test	**	*	*		

1 : Mean of 45 disease scores from 3 replicates based on a 0-5 scale of increasing disease (see TABLE 5).

2 : * = Significant at the 5% level. ** = Significant at the 1% level.

3 : DAI = Days after inoculation.

hence confirming similar results on maximum tillering stage in Section 4.1. However, the scoring time of between 20-25 days after inoculation would be best recommended for the pin prick method on flag leaves.

4.4. Strain Evaluation and Mass Screening for Cultivar Resistance at the Flag Leaf Stage in the Glasshouse

4.4.1. Materials and Methods

The experiment was carried out in a glasshouse in routine screening manner using plastic water-tight rectangular trays (60 x 37 x 12 cm). The trays were filled with tropical rice soil (Kundor-tualang association) leaving about 3 cm to hold water above the soil surface. Seedlings of the 8 cultivars (MR1, MR35 IR20, IR22, Bahagia, Jaya, T(N)1 and SMII) were tranplanted from 25-day old nurseries at 2 seedlings per hill. Each tray was planted with 6 hills of one cultivar. Normal types and rates of fertilizer applications were applied to the plants (refer Section 4.1.1). The pin prick and the incisor method of inoculation tested and the isolate used were as described in Section 4.1.1. The disease was assessed using the scale in TABLE 5, 14, 20 and 25 days after inoculation.

4.4.2. Results

The results (TABLE 23) confirm that the incisor method was not sufficiently sensitive in differentiating various disease reactions hence cultivar resistance. This was evident for all 3 scoring times in which the disease produced by the incisor method was always more and hence the comparatively higher disease reactions in most cultivars (PLATE 8). However, when comparing with seedling reactions done 5 months earlier using similar isolate and method of inoculation (TABLE 23), it can be seen that even at 25 days after inoculation, a number of resistant and moderately resistant instead of susceptible reactions were recorded on some cultivars (MR1, MR35, IR20, IR22 and Bahagia). This suggests that the virulence of the strain used may have decreased because of constant sub-culturing and temporary storage in the laboratory over a period of time. However, the study

TABLE 23. MEAN DISEASE SCORES[1] AND REACTIONS[2] OF 8 RICE CULTIVARS CAUSED BY A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE*[3] ON FLAG LEAVES USING 2 INOCULATION METHODS[4] 14, 20 and 25 DAYS AFTER INOCULATION IN THE GLASSHOUSE.

Cultivar	Scoring Time						Seedling reaction[6] (14 DAI)
	14 DAI[5]		20 DAI		25 DAI		
	Inc	pp	Inc	pp	Inc	pp	
MR1	2.0 (R)	0 (R)	2.1 (R)	1.0 (R)	2.3 (R)	1.3 (R)	S
MR35	2.1 (R)	0 (R)	2.2 (R)	1.3 (R)	3.5 (S)	1.5 (R)	S
IR20	2.9 (M)	1.0 (R)	3.6 (S)	1.3 (R)	4.0 (S)	2.7 (M)	S
IR22	3.7 (S)	1.1 (R)	4.0 (S)	2.0 (R)	4.3 (S)	2.6 (M)	M
Bahagia	3.0 (M)	1.6 (R)	3.7 (S)	2.5 (M)	4.0 (S)	3.0 (M)	S
Jaya	3.0 (M)	3.2 (M)	3.7 (S)	3.8 (S)	4.4 (S)	4.0 (S)	S
T(N)1	3.2 (M)	2.4 (R)	4.0 (S)	4.0 (S)	4.2 (S)	4.0 (S)	S
SMII	3.6 (S)	3.4 (M)	3.8 (S)	3.6 (S)	4.1 (S)	4.9 (S)	S

1 : Mean of 6 diseas scores, based on a 0-5 scale of increasing disease (see TABLE 5).

2 : R = Resistant. M = Moderately resistant. S = Susceptible.

3 : Isolate M.

4 : Inc = Incisor method. pp = pin prick.

5 : DAI = Days after inoculation.

6 : Reactions of Isolate M inoculated by the pin prick method in the glasshouse 5 months earlier (see TABLE 15 in Section 3).



PLATE 8. Different disease reactions caused by a virulent strain of *Xanthomonas oryzae* on 3 rice cultivars (T(N)1, IR22, IR20) of different resistance, inoculated by the incisor method (left of each cultivar) and the pin prick method (right of each cultivar) and assessed at 20 days after inoculation.

shows that the best time for assessing host-parasite reactions at flag leaf stage using the pin prick method of inoculation in the glasshouse would be 25 days after inoculation as the reactions produced were most reflective of seedling reactions.

4.5. Comparison of Two Inoculation Methods at Maximum Tillering Stage in the Field

4.5.1. Materials and Methods

The experiment was carried out in the main season 1979/80 in Tanjong Karang. 25-day old seedlings of a susceptible cultivar SMII were transplanted into the field at 2 seedlings per hill and a planting density of 10 x 10 cm. Types and rates of fertilizers applied and their timing of application were similar to those in Section 4.1.1. The experiment was conducted in a randomised complete block design with 3 replicates in plot sizes of 3 x 3m, 0.5 m between plots and 1 m between replicates. The 4 treatments were as follows:-

T_1 = Guntacker inoculation method (PLATE 9).

T_2 = Scissors inoculation method (= shearing method, clip-inoculation (HO, 1973; KAUFFMAN *et al.*, 1973).

T_3 = $Control_1$ - Guntacker inoculation method.

T_4 = $Control_2$ - Scissors inoculation method.

The plants were treated with the 4 treatments at maximum tillering stage (50 to 60 days after sowing). The guntacker inoculation method was a modification of the multiprick inoculation method which has been used successfully by HO (1976), HO & LIM (1977) and HO (1978) for evaluating cultivar resistance in mass screening and yield loss studies. It involves similar multineedle pad with 15 hypodermic needles in 2 rows mounted on to the guntacker (PLATE 9) which gives it the semi-automatic jabbing mechanism once the trigger is pressed and is used for inoculating the thick mid-foliage region of the plants. The machine has an inoculation reservior at the top which contains the bacterial inoculum fed in through a small hole on the top cover which could be opened and closed by rotating the cover.

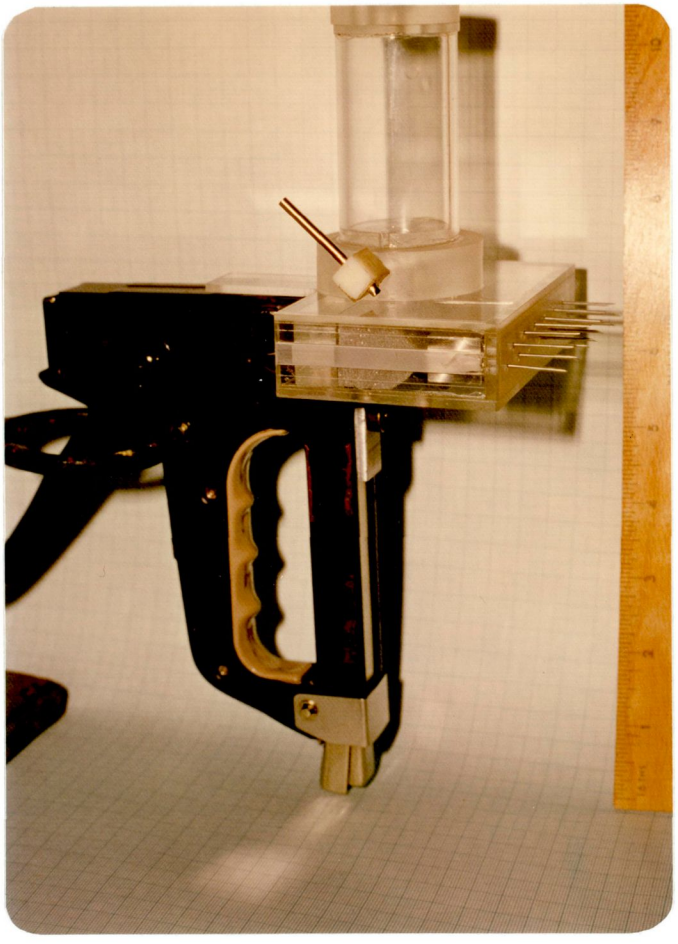


PLATE 9. The guntacker inoculation apparatus used for mass field inoculation of adult rice cultivars in strain and cultivar resistance evaluations.

The inoculum is then fed into the multineedle chamber through a lever at the side which opens the valve at the bottom of the inoculum reservior. As the machine is quite powerful, only 1 jab is necessary to inflict sufficient wounds on to most of the leaves. It is used with the aid of the inoculation support similar to that of the multiprick inoculation method (HO & LIM, 1977).

The scissor method is operated in the same way as indicated by HO (1973) and KAUFFMAN *et al.* (1973), in which every inoculation was done after dipping the pair of scissors into the inoculum. The 2 controls (T_3 & T_4) were treated with distilled water before the apparatus were used to inoculate the bacterium into the plants of the treated plots (T_1 & T_2). The isolate used and the preparation of the bacterial suspension for inoculation were similar to Section 4.1.1.

Eight fully opened top 2nd leaves per treatment per replicate were scored using the disease evaluation scale in TABLE 5 at 14, 20 and 25 days after inoculation.

4.5.2. Results

The controls (T_3 & T_4) did not give any disease (TABLE 24). The guntacker method gave overall superiority in terms of higher disease scores for all the 3 stages of disease assessments at 14, 20 and 25 days after inoculation. Although the mean disease scores of both treatments at the various scoring times were significantly different (at the 5% level) as shown by the Chi-square tests (TABLE 24), the time which gave a susceptible reaction type for both methods (mean disease scores of 3.6-4.3) was 20-25 days after inoculation. However, between these scoring times the guntacker method gave slightly higher disease scores than those given by the scissors method although they were not significantly different (TABLE 24). As the guntacker method had the further advantage of giving a definite lesion type development easily assessed by the proposed disease evaluation scale (TABLE 5), it is best recommended for use in strain evaluation study and cultivar resistance evaluations at a scoring time between 20-25 days after inoculation on the 2nd fully opened top leaves of the tillers.

TABLE 24. MEAN DISEASE SCORES[1] OF 2 INOCULATION METHODS ON THE 2ND FULLY OPENED TOP LEAVES OF A SUSCEPTIBLE RICE CULTIVAR SMII AT 14, 20 AND 25 DAYS AFTER INOCULATION WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* IN THE FIELD AND RESULTS OF CHI-SQUARE TEST

Scoring time / Treatment	Days after inoculation (DAI)			Chi-square test[2]	
	14	20	25	14 & 20 DAI	20 & 25 DAI
T_1 (Guntacker)	3.3	3.8	4.8	*	*
T_2 (Scissors)	1.9	3.6	3.8	**	*
T_3 (Guntacker-water)	0	0	0		
T_4 (Scissors-water)	0	0	0		
Chi-square test[2] (T_1 & T_2)	**	NS	NS		

1 : Mean of 24 disease scores from 3 replicates, based on a 0-5 scale of increasing disease (see TABLE 5)

2 : NS = Not significant at the 5% level. * = Significant at the 5% level. * = Significant at the 1% level.

4.6. Evaluation of Guntacker Inoculation Method for Strain Typing and Mass Screening for Cultivar Resistance at Maximum Tillering Stage in Glasshouse and Field

4.6.1. Materials and Methods

4.6.1.1. Glasshouse

The guntacker method of inoculation as described in Section 4.5.1 was used to inoculate 11 rice cultivars in a glasshouse. The isolate used, preparation of the bacterial suspension, soil type, pots, experimental design, types and rates of fertilizers applied were similar to those in Section 4.1.1. The inoculation procedure was similar to that in Section 4.5.1. The disease was assessed 14 days after inoculation using the scale in TABLE 5.

4.6.1.2. Field

The method was further tested in the field following similar techniques on 7 rice cultivars. 25-day old seedlings of each cultivar were transplanted at 2 seedlings per hill with a planting distance of 10 x 10 cm and 20 cm between cultivars. A total of 48 hills were planted for each cultivar in rectangular of 8 x 6 hills. With 2 rows of each cultivar used as guard rows all round, only the centre 8 hills (4 x 2 hills) were inoculated at maximum tillering stage (50 days after sowing). The isolate used, preparation of bacterial suspension for inoculation, types and rates of fertilizers applied were similar to those in Section 4.1.1. The disease was assessed on the 2nd fully opened top leaves of 4 randomly picked tillers per hill 20 days after inoculation, using the scale in TABLE 5, giving a total of 32 scores per cultivar.

4.6.2. Results

Preliminary observations show that susceptible cultivars developed disease earlier than resistant cultivars. Based on the mean disease scores and reactions obtained (TABLE 25), the various groups of cultivars of differing resistance could be accordingly catergorised into their respectively disease reactions 14 days after inoculation in glasshouse conditions (PLATE 10) and 20 days after inoculation in field conditions, indicating the suitability of the guntacker method in strain typing and mass screening for cultivar resistance at maximum tillering stage in glasshouse and field conditions. However, when compared to the disease reactions of seedlings inoculated by the mass pin prick method with the same strain in glasshouse conditions 12 months earlier, there is a clear indication of a drop in the virulence of the strain (TABLE 25). This is because the disease reactions of cultivars IR1545-339-2-2, MR1, MR35, IR20 and IR22 which were previously (12 months earlier) rated as moderately resistant to susceptible became resistant in the present tests.

TABLE 25. MEAN DISEASE SCORES[1] AND REACTIONS[2] OF A STRAIN OF *XANTHOMONAS ORYZAE*[3] ON SOME RICE CULTIVARS INOCULATED BY THE GUNTACKER METHOD AT MAXIMUM TILLERING STAGE IN THE GLASSHOUSE AND FIELD.

Cultivar	Glasshouse[4] (Jan. 1980)	Field[5] (Jan. 1980)	Seedling reaction (14 DAI)[6] (Jan. 1979)
IR1545-339-2-2	1.0 (R)	-	M
MR1	1.6 (R)	1.5 (R)	S
MR35	1.6 (R)	2.3 (R)	S
IR20	1.4 (R)	1.8 (R)	S
IR22	2.0 (R)	-	M
Mahsuri	3.0 (M)	-	S
Bahagia	3.3 (M)	3.3 (M)	S
Jaya	3.9 (S)	4.3 (S)	S
SMII	4.2 (S)	4.2 (S)	S
T(N)1	3.9 (S)	4.1 (S)	S
IR8	4.1 (S)	-	S

1 : Mean of 30 disease scores from 3 replicates for glasshouse text and mean of 32 scores for field test, based on a 0-5 scale of increasing disease (see TABLE 5).

2 : R = Resistant. M = Moderately resistant. S = Susceptible.

3 : Isolate M.

4 : Assessed at 14 DAI on a 0-5 scale of increasing disease (see TABLE 5)

5 : Assessed at 20 DAI on a 0-5 scale of increasing disease (see TABLE 5)

6 : Reactions of Isolate M inoculated by the mass pin prick method in the glasshouse 12 months earlier (see TABLE 15 in Section 3). DAI = Days after inoculation.



PLATE 10. Characteristic reactions of resistant (IR1545-339-2-2, MR1, IR20, MR35), moderately resistant (Bahagia) and susceptible (SMII, T(N)1, IR8) cultivars to a strain of *Xanthomonas oryzae* on the 2nd fully opened top leaves inoculated by the guntacker method at maximum tillering stage in the glasshouse.

4.7. Discussion

The significant difference in the amount of disease in the 1st and 2nd fully opened top leaves of the same tiller is of considerable interest. It emphasizes the importance of constance use of and assessment on the same type of leaves for disease experiments on rice if individual leaves were used in the assessment. The fact that the 1st leaf produced significantly less diease then the 2nd probably illustrates the interesting phenomenon of young actively growing protoplasts (as in the 1st leaf) reacting more actively against the invading bacterium than those of the older leaf (2nd leaf) as implied by WOOD (1978) which suggests the production of comparatively more phytoalexin or other inhibitors by the young actively growing protoplasts of the 1st leaf. This in essence may bring about discrepancies in cultivar resistance evaluation if different leaves of different cultivars were used in disease assessments.

In this study the incisor method of inoculation was taken to be the glasshouse equivalent of the scissors method used in routine field screening trials, and the pin prick method as the glasshouse equivalent of the guntacker method (= multi-prick method) recommended for use in field mass testings. The main advantage of field methods is that they can be used rapidly on larger number of cultivars in field conditions.

The incisor method gave more disease under glasshouse conditions probably because many more vascular bundles of the leaf blades were severed hence permitting more bacteria to enter the leaves. Under glasshouse conditions where the total amount of sunlight was certainly reduced, it produced excessive disease. The scissors method however, produced less disease than the guntacker method under field conditions although more vascular bundles had also been severed as in the incisor method.

The assessment of disease reactions in these inoculation studies has been based on types of lesions formed, hence the incisor and the scissors methods of inoculation would not be suitable

as they tend to exacerbate the disease in glasshouse conditions. On the other hand, the study showed conclusively that the pin prick or mass pin prick and the guntacker methods of inoculation could be used effectively under this system of evaluation, at seedling, maximum tillering and flag leaf stages of plant growth in glasshouse and field conditions. The results were consistent with those of most Japanese workers' (KUSABA *et al.*, 1958; TAGAMI & MIZUKAMI, 1962).

It is noted that the same strain rendered the resistant and moderately resistant cultivars either moderately resistant or susceptible when tested about 12 months earlier on seedlings (TABLES 23 & 25). It would appear therefore that the virulence of the strain used had decreased considerably probably due to repeated sub-culturing (FUJII, 1976) followed by temporary storage in a refrigerator over a period of time.

4.8. Summary

The results of the studies and inference from the details of the techniques of strain typing and screening for cultivar resistant against *Xanthomonas oryzae* at seedling, maximum tillering and flag leaf stages of rice growth in both glasshouse and field conditions, can be summarised as shown in TABLE 26.

TABLE 26. SUMMARY OF TECHNIQUES IN STRAIN TYPING AND SCREENING FOR CULTIVAR RESISTANCE AGAINST *XANTHOMONAS ORYZAE* AT VARIOUS STAGES OF RICE GROWTH.

Growth stage	Inoculation method	Inoculum level[1]	Assessment time	Assessment scale	Suitable for:
1. Seedling[2] (25-28 DAS)[3]					
1.1 Glasshouse	Mass pin prick	10^9cells/ml	12-14 DAI[5]	Lesion type[6]	ST[7] & MSCR[8]
1.2 Field[4]	Mass pin prick	10^9cells/ml	14 DAI	Lesion type	ST & MSCR
2. Maximum tillering[2] (50-60 days					
2.1 Glasshouse	Mass pin prick guntacker or multi-prick	10^9cells/ml	14 DAI	Lesion type	ST & MSCR
2.2 Field	Guntacker or multi-prick	10^9cells/ml	20-25 DAI	Lesion type	ST & MSCR
3. Flag leaf					
3.1 Glasshouse	Pin prick	10^9cells/ml	20-25 DAI	Lesion type	ST & SCR[9]
3.2 Field[4]	Pin prick	10^9cells/ml	20-25 DAI	Lesion type	ST & SCR

1 : Prepared from 48-hour old pure cultures grown on Wakimoto's (1960) potato semisynthetic agar slants.
2 : Only the 2nd fully opened top leaves are assessed.
3 : DAS = Days after sowing.
4 : Recommended through inference from available results.
5 : DAI = Days after inoculation.
6 : Assessed on a 0-5 scale of increasing disease in TABLE 5.
7 : ST = Strain typing.
8 : MSCR = Mass Screening for cultivar resistance.
9 : SCR = Screening for cultivar resistance.

5. LABORATORY TECHNIQUES FOR STRAIN TYPING

5.1. Determination of Best Medium, Type and Segment of Detached Leaf for Detached Leaf Culture

5.1.1. Detached Whole Leaf

5.1.1.1. Materials and Methods

Cultivar

In an attempt to determine the type of leaf and whether detached whole leaves could be used in detached leaf culture, the first and second fully opened leaves of 28-day old seedlings of 6 cultivars were used for the study. The cultivars were: SMII, MR1, Mahsuri, IR20, IR8, and Cempo Selak. The first 3 are typical Malaysian cultivars whereas the last 3 are representative cultivars from the Philippines. Before use, seedlings of these cultivars were maintained for 28 days under flooded conditions in the glasshouse under conditions suitable for rice (refer TABLE 9 in Section 3).

Medium

A soft agar medium containing 100 ppm benzimidazole and 0.5% Ionagar was used. The 2 components were boiled in a water bath until the agar was dissolved and the molten agar mixture was poured into transparent plastic trays with tight-fitting lids (36 x 23 x 10 cm) at 500 ml/tray. Two such soft agar trays were prepared for the experiment. The soft agar was allowed to cool and set horizontally before use.

Procedure

Twenty-eight-day old seedlings of the 6 cultivars were taken into the laboratory where the first and send fully opened top leaves of each seedling were removed 1 cm above the axis of leaf blade and sheath with a pair of scissors, and the lower ends were immediately stuck into the soft agar trays for as short a length as practicable. The experiment was done in 4 replicates. The trays were tightly closed with lids and left under ordinary fluorescent light for 7 hours daily. Results were recorded first day, then every other day for about 2 weeks.

Assessment

The main criterion in the assessment of the suitability of either the first or the second fully opened detached whole top leaves for detached leaf culture, is the extent to which they remained green. This was measured according to the scale in TABLE 27.

5.1.1.2. Results

There was a clear difference in the colour reactions of the first and second fully opened top leaves (TABLE 28). The first leaves of all cultivars except Cempo Selak stayed greener than the second leaves. The first leaves of Mahsuri, SMII and IR8 stayed green until the 5th day which was 2 days longer then those of IR20, MR1 and Cempo Selak. However, on the 7th day onwards both types of leaf turned yellow-green to yellow then to brown (scores of 3-6) progressively, indicating that whole leaves were not suitable for detached leaf culture because of early senescence. However, it was noticed that the yellowing started from the tip downwards spreading gradually towards the base and that much of the first leaves tended to curl up from the sides much more than the second leaves. This suggests that the second fully opened top leaves would be more suitable than the first and that while the whole leaf would not be suitable as such, the basal or the lower half of the leaf of c 5 cm would be suitable for detached leaf culture.

5.1.2. Medium and Segment of Detached Leaf

5.1.2.1. Materials and Methods

Experiment 1

Cultivar

A susceptible Malaysian cultivar SMII was used. Seedlings were maintained under flooded conditions in the glasshouse (refer Section 3.1.1) for 28 days before use. As the results of previous experiment (Section 5.1.1) indicated that the second fully opened top leaves were slightly better than the first, only these were used. It was the intention to determine which segment (each of c 5 cm) of the leaf blade namely (i) basal (ii) middle or (iii) end segment was most suitable.

TABLE 27. SCALE FOR ASSESSING THE DEGREE OF GREENESS ON DETACHED LEAVES IN DETACHED LEAF CULTURE.

Scale	Visual description of degree of greeness
1	Green (= original)
2	Light green
3	Yellow-green
4	Yellow
5	Orange
6	Brown

TABLE 28. COLOUR REACTIONS[1] OF FIRST AND SECOND WHOLE LEAVES OF 6 RICE CULTIVARS IN DETACHED LEAF CULTURE WITH 100ppm BENZIMIDAZOLE IN SOFT AGAR

Cultivar	Type of leaf	Day after detachment					
		1st	3rd	5th	7th	9th	11th
1. IR20	1st	1	1	3	4	5	6
	2nd	1	2	3	4	5	6
2. MR1	1st	1	1	3	4	5	6
	2nd	1	3	3	4	5	6
3. Mahsuri	1st	1	1	1	4	5	6
	2nd	1	2	2	4	5	6
4. SMII	1st	1	1	1	3	4	6
	2nd	1	2	3	4	5	6
5. IR8	1st	1	1	1	4	5	6
	2nd	1	2	2	4	5	6
5. Cempo Selak	1st	1	1	2	4	5	6
	2nd	1	1	2	4	5	6

1 : Obtained from all 4 replicates based on a 1-6 scale of decreasing greeness (see TABLE 27).

Medium

It was also the intention to determine the best medium of the ones used by WOLFE (1969) and RUSSELL *et al.*, (1975) and modifications as shown in TABLE 29. The various concentrations of benzimidazole solution (B_1) were prepared from benzimidazole (molecular wt. = 118.14) from BDH Chemicals Ltd., Poole, England. The various concentrations of Long Ashton nutrient solution (LANS) were prepared from the stock solutions recommended by HEWITT (1952), as shown in TABLE 30.

The Ionagar was used as recommended by WOLFE (1969) and RUSSELL *et al.*, (1975) at 0.5 per cent in all treatments involving SIA. The Ionagar used was No. 2 obtained from Oxoid Limited, London S.E. 1, England. All solutions and dilutions involved in all the above treatments were made with distilled water and boiled in a water bath until the Ionagar was fully dissolved. About 8 ml of each of the solutions and agar media were pipetted into transparent polystyrene boxes (5.7 x 3.7 x 2.3 cm) with tight-fitting lids. Sufficient quantities were prepared for the experiment to be carried out in duplicate. The media were left to cool and set at a gradient of $\underline{c}$ 10°.

Procedure

Other than what has been stated above, the rest of the procedures were similar to those in Section 5.1.1.1 above. It must be stated that the lower ends were stucked into the thicker part of the soft Ionagar and covered tightly with the lid. For liquid media, the leaf segments were floated immediately on the surface according to SAMBORSKI *et al.*, (1958).

Assessment

The criteria and method of assessment was similar to those of Section 5.1.1.1 (TABLE 27).

TABLE 29. TWENTY FOUR TREATMENTS (INDICATED BY NUMBERS) FORMED BY COMBINING EACH OF THE 6 BASE TREATMENTS (TREATMENTS 1-6) WITH THE VARIOUS FACTORS IN EACH COLUMN.

Base treatments[1]	+ 0.5% SIA[2]	- B_1	- B_1 + 0.5% SIA
1. Distilled water + 50 ppm B_1	7	13	19
2. 0.25 conc. LANS + 50 ppm B_1	8	14	20
3. 0.5 conc. LANS + 50 ppm B_1	9	15	21
4. 0.5 conc. LANS + 100 ppm B_1	10	16	22
5. 0.5 conc. LANS + 200 ppm B_1	11	17	23
6. 1.0 conc. LANS + 50 ppm B_1	12	18	24

1 : B_1 = Benzimidazole. LANS = Long Ashton nutrient solution

2 : SIA = Soft Ionagar

TABLE 30. MACRO AND MICRO NUTRIENT COMPOSITION OF HEWITT'S (1952) LONG ASHTON NUTRIENT SOLUTION[1] (HEWITT, 1966).

Macro and micro nutrient solution	g/2 litres
1. KNO_3	404
2. $Ca(NO_3)_2$ or $Ca(NO_3)_2.4H_2O$	656 or 944
3. $NaH_2PO_4.2H_2O$ or $NaH_2PO_4.H_2O$	416 or 368
4. $MgSO_4.7H_2O$	368
5. Iron EDTA (sodium salt)	77.02
6. $MnSO_4.4H_2O$	4.46
7. $CuSO_4.5H_2O$	0.50
8. $ZnSO_4.7H_2O$	0.58
9. H_3BO_3	3.72
10. $(NH_4)6\ mo_7O_{24}.4\ H_2O$	0.18

1 : To make 1000 ml of full concentration of standard Long Ashton nutrient solution referred to in the text, add 2 ml of each of the solution 1,2,3,4 and 1 ml of each of the solutions 5,6,7, 8,9, 10 to 986 ml of distilled water.

Experiment 2

The aim of this experiment was to detemine the concentration of benzimidazole in liquid and semi-solid media best for detached leaf culture.

The cultivar, type of leaf, procedure and the assessment scale used were similar to that in Experiment 1. The differences lie in the segment of leaf blade used and the treatments investigated. Only the basal segment of the second fully opened top leaves were used.

The treatments included different concentrations of benzimidazole in liquid and semi-solid forms and involved one set in liquid form, one set in semi-solid form, one set of semi-solid form being covered with 8 ml distilled water in each box and another set of semi-solid form being covered with 8 ml of 50 ppm benzimidazole in each box. The different treatments are as shown in TABLE 31.

Experiment 3

The aim of this experiment was to study the combined effect of the most suitable concentration of benzimidazole (B_1) and 0.25 concentration of Long Ashton nutrient solution (LANS) in delaying the senescence of detached leaf segments.

The cultivar, type of leaf, procedure and the evaluation scale used were similar to those in Experiment 1. However, as in Experiment 2 only the basal segments (5 cm) of the 2nd fully opened top leaves were used.

The treatments consisted of different concentractions of B_1 in combination with 0.25 concentration of LANS in liquid and semi-solid forms as shown in TABLE 32.

TABLE 31. TWENTY FOUR TREATMENTS (INDICATED BY NUMBERS) FORMED BY COMBINING EACH OF THE 6 BENZIMIDAZOLE (B_1) CONCENTRATION BASE TREATMENTS WITH THE VARIOUS FACTORS IN EACH COLUMN.

Conc. of B_1	+ 0.5% SIA[1]	± 0.5% SIA c̄ dist.H_2O[2]	± 0.5% SIA c̄ 50 ppm B_1
1. 0 ppm	7	13	19
2. 50 ppm	8	14	20
3. 100 ppm	9	15	21
4. 150 ppm	10	16	22
5. 200 ppm	11	17	23
6. 250 ppm	12	18	24

1 : SIA = Soft Ionagar.

2 : c̄ = covered with. dist. = distilled.

TABLE 32. TWENTY FOUR TREATMENTS (INDICATED BY NUMBERS) FORMED BY COMBINING EACH OF THE 6 BENZIMIDAZOLE (B_1) CONCENTRATION BASE TREATMENTS WITH THE VARIOUS FACTORS IN EACH COLUMN.

Conc. of B_1	+ 0.25 conc. LANS[1]	+ 0.5% SIA[2]	+ 0.25 conc. LANS + 0.5% SIA
1. 0 ppm	7	13	19
2. 50 ppm	8	14	20
3. 100 ppm	9	15	21
4. 150 ppm	10	16	22
5. 200 ppm	11	17	23
6. 250 ppm	12	18	24

1 : LANS = Long Ashton nutrient solution.

2 : SIA = Soft Ionagar.

5.1.2.2. Results

Experiment 1 (see Section 5.1.2.1)

Results obtained from experiment 1 (TABLE 33) indicate that Long Ashton nutrient solution (LANS) in liquid media (treatment number (14) - (18)), soft Ionagar media (treatments (20) - (24)), and distilled water (treatments (13) & (19)) did not prolong greeness in leaf segments beyond the 5th day of detachment. However, when used in combination with benzimidazole senescence was delayed to a considerable extent in both liquid media (treatments (2) - (6)) and soft Ionagar media (treatments (8) - (12)). The various concentrations of LANS did not seem to affect the persistence of greeness in detached leaf segments either in liquid or soft Ionagar media 9 days after detachment (FIG.2). However a minimal amount of 0.25 concentration of LANS seemed to suffice.

The degree of greeness in detached leaf segments varied with the concentration of benzimidazole in liquid media (treatments (3), (4) & (5)) and soft Ionagar media (treatments (9), (10) & (11)). On the 11th day, liquid media seemed to be better than soft Ionagar media at 100 to 150 ppm benzimidazole (FIG.3). However, at the 9th day segments of the leaves kept green equally well in both liquid and soft Ionagar media at concentrations between 100 to 200 ppm (FIG. 3, PLATES 11 & 12). There was a clear difference among the different types of leaf segments used especially in the presence of benzimidazole. This was especially pronounced for treatments with 0.5 concentration Long Ashton nutrient solution together with 100 ppm benzimidazole in liquid medium (treatment (4)) and in soft Ionagar medium (treatment (10)) where the basal segment maintained its original green colour (score 1) to the 9th day, while the middle segment (in liquid medium only) and end segment did not (FIG.4). The same trend was seen up to the 11th day. However, the same treatment in soft Ionagar medium maintained the original green colour (Score 1) in the basal and middle segments and delayed senescence for both segments up to the 9th day. On the 11th day however,

TABLE 33. COLOUR REACTIONS[1] OF BASAL (B_2) MIDDLE (M) AND END (E) SEGMENTS OF THE 2ND FULLY OPENED TOP LEAVES OF RICE VARIETY SMII TO VARIOUS CONCENTRATIONS OF LONG ASHTON NUTRIENT SOLUTION (LANS), BENZIMIDAZOLE (B_1) AND THEIR COMBINATIONS IN SOFT IONAGAR (SIA) AND LIQUID MEDIA.

Day after detachment	Medium	(1) Dist. H_2O + 50 ppm B_1			(2) 0.25 Conc.LANS + 50 ppm B_1			(3) 0.5 Conc. LANS + 50 ppm B_1			(4) 0.5 Conc. LANS + 100 ppm B_1			(5) 0.5 Conc. LANS + 200 ppm B_1			(6) 1.0 Conc. LANS + 50 ppm B_1		
		B_2	M	E	B_2	M	E	B_2	M	E	B_2	M	E	B_2	M	E	B_2	M	E
1st	B_1 + LANS	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5th		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7th		3	3	3	2	2	2	2	2	2	1	1	1	1	1	1	2	2	2
9th		4	5	6	3	4	5	3	4	5	1	2	4	1	3	4	3	4	5
11th		4	5	6	4	5	6	4	5	6	3	4	5	3	4	5	4	5	6
		(7) (1) in SIA			(8) (2) in SIA			(9) (3) in SIA			(10) (4) in SIA			(11) (5) in SIA			(12) (6) in SIA		
1st	B_1 + LANS in SIA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5th		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7th		2	3	3	2	2	2	2	3	3	1	1	1	1	1	1	2	2	2
9th		4	6	6	3	5	5	3	4	6	1	1	2	1	1	2	3	4	5
11th		5	6	6	5	6	6	4	5	6	4	5	5	3	4	5	4	5	6
		(13) (1)-50 ppm B_1			(14) (2)-50 ppm B_1			(15) (3)-50 ppm B_1			(16) (4)-50 ppm B_1			(17) (5)-200 ppm B_1			(18) (6)-50 ppm B_1		
1st	LANS only	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5th		2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
7th		4	4	5	4	4	5	4	4	5	4	4	5	4	4	5	4	4	5
9th		4	5	6	4	5	6	4	5	6	4	5	6	4	5	6	4	5	6
11th		5	6	6	5	6	6	5	6	6	5	6	6	5	6	6	5	6	6
		(19) (7)-50 ppm B_1			(20) (8)-50 ppm B_1			(21) (9)-50 ppm B_1			(22) (10)-100ppm B_1			(23) (11)-200ppm B_1			(24) (12)-50 ppm B_1		
1st	LANS in SIA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5th		2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
7th		4	4	5	4	4	5	4	4	5	4	4	5	4	4	5	4	4	5
9th		4	5	6	4	5	6	4	5	6	4	5	6	4	5	6	4	5	6
11th		5	6	6	5	6	6	5	6	6	5	6	6	5	6	6	5	6	6

[1] : Mean of 2 replicates based on a 1-6 scale of decreasing greeness (see TABLE 27).

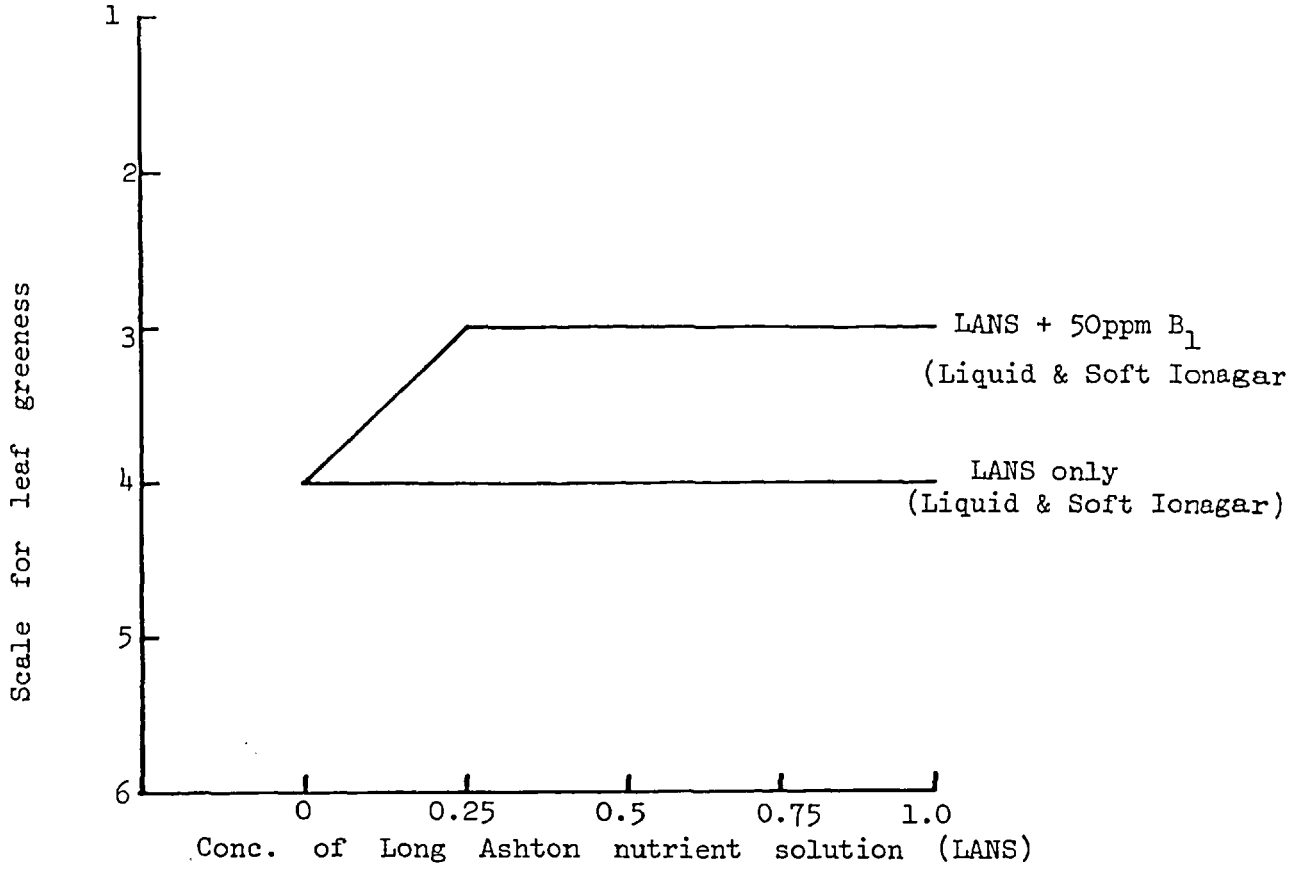


FIG.2. Effect of various concentrations of Long Ashton nutrient solution and in combinations with 50 ppm benzimidazole on the degree of greeness on the basal segment of rice leaf blade in liquid and soft Ionagar media 9 days after detachement. (1 to 6 - decreasing greeness).

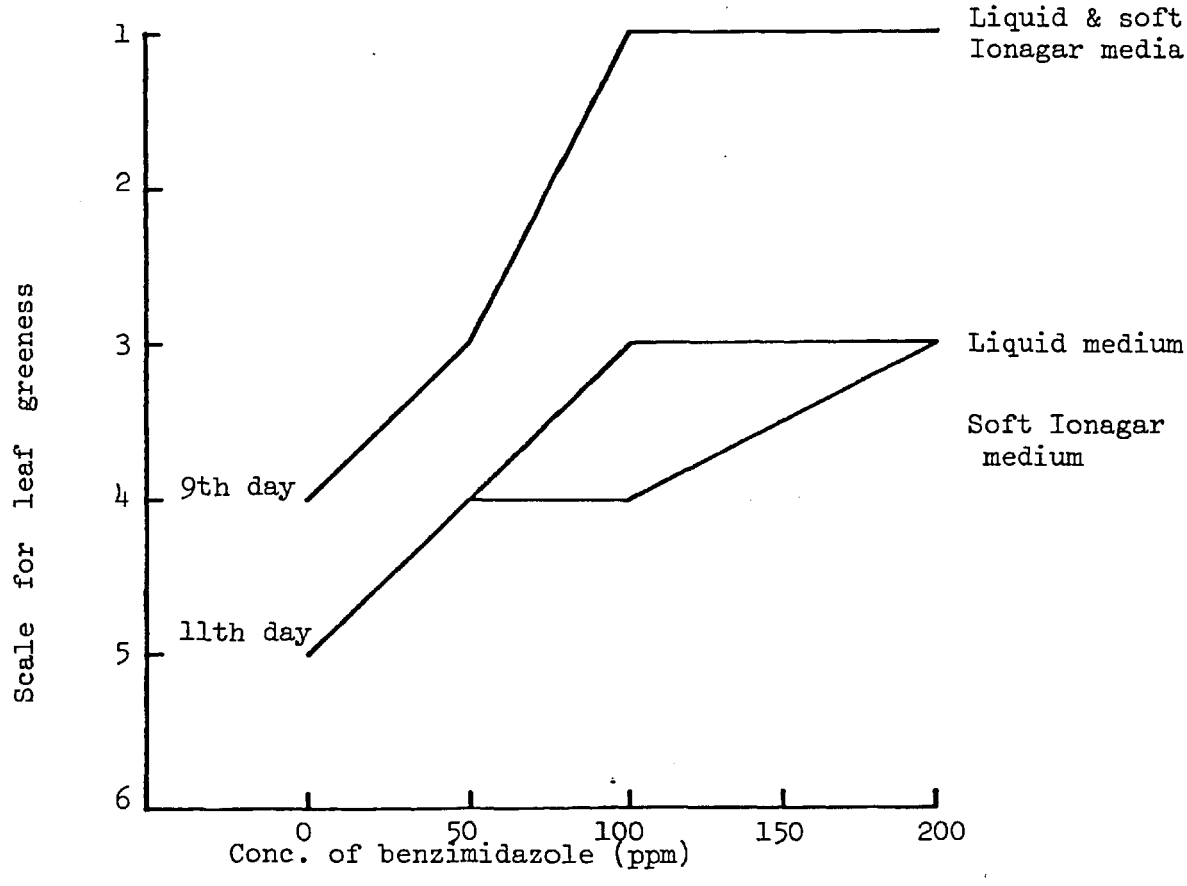


FIG.3. Effect of various concentrations of benzimidazole on the degree of greeness on the basal segment of rice leaf blade in liquid and soft Ionagar media 9 and 11 days after detachment. (1 to 6 - decreasing greeness).

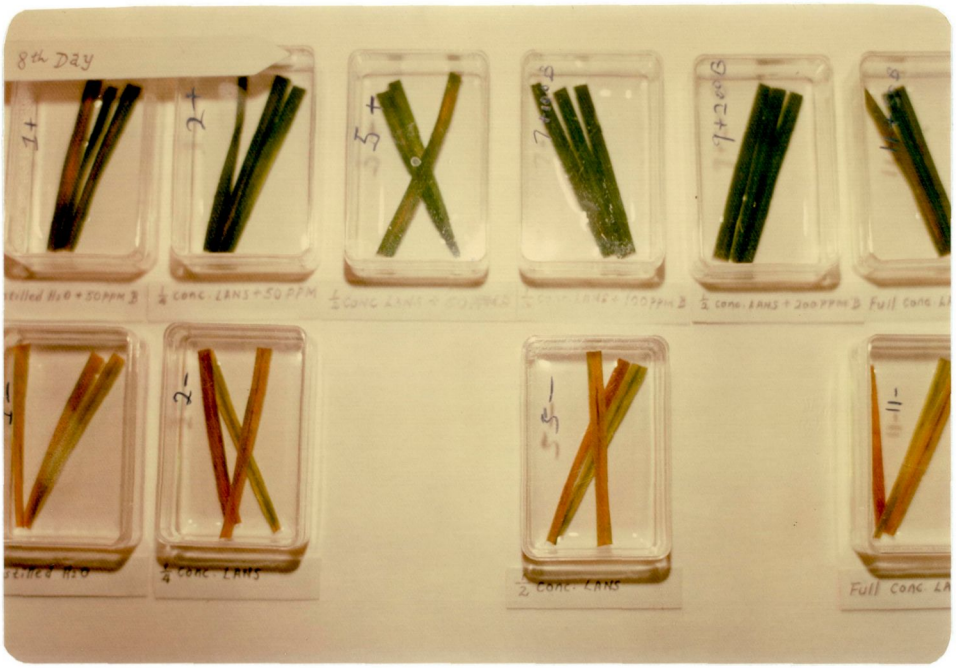


PLATE 11. Colour reactions of basal, middle and end segments of the 2nd fully opened top leaves (all in each box) of rice cultivar SMII 9 days after detachment in liquid media of benzimidazole (B) and Long Ashton nutrient solution (LANS) and their combinations of different concentrations.

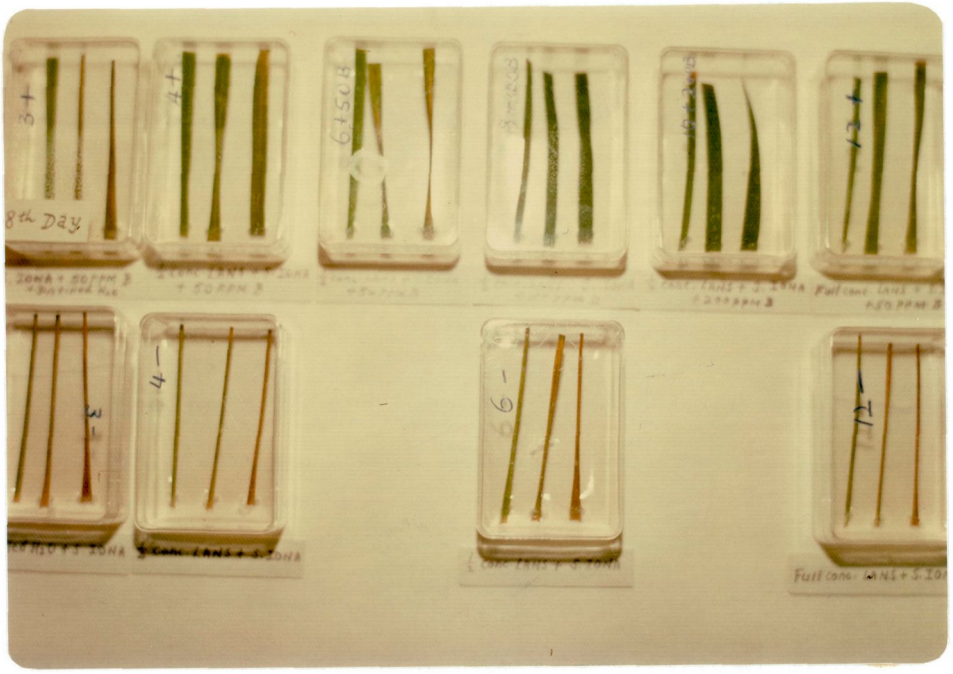


PLATE 12. Colour reactions of basal, middle and end segments of 2nd fully opened top leaves (arranged left to right in each box) of rice cultivar SMII, 9 days after detachment in soft agar media of benzimidazole (B) and Long Ashton nutrient solution (LANS) and their combinations of different concentrations in 0.5% soft Ionagar (S.IONA).

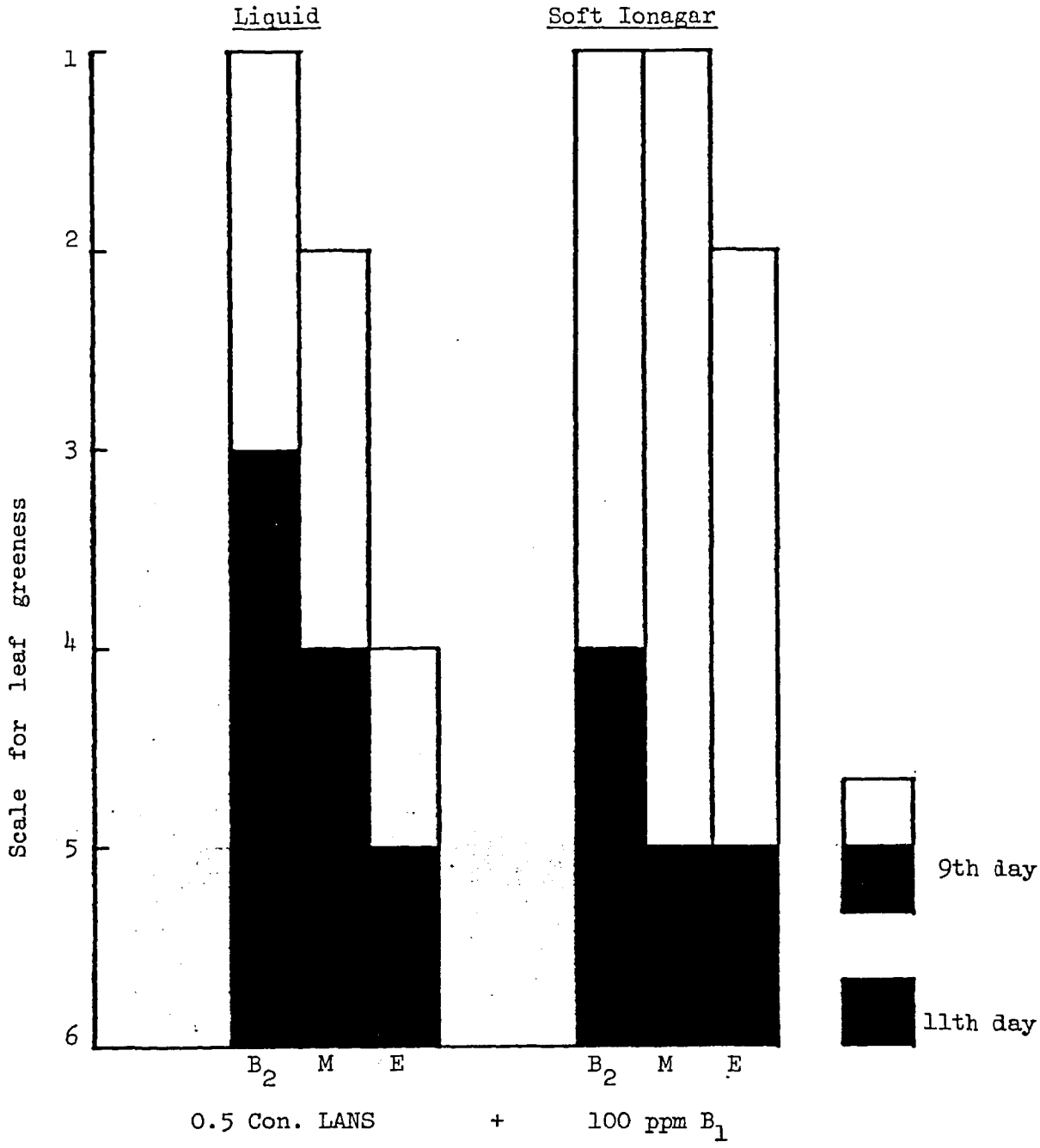


FIG.4. Effect of 0.5 concentration of Long Ashton nutrient solution (LANS) and 100 ppm benzimidazole (B_1) on the degree of greeness on the basal (B_2), Middle (M) and End (E) segments of rice leaf blade in liquid and soft Ionagar media on the 9th and 11th day after detachment. (1 = most green; 6 = least green).

senescence set in for all segments in liquid and soft Ionagar media. Hence benzimidazole and Long Ashton nutrient solution in soft Ionagar medium had an additional advantage over the liquid media in that both the basal and middle segments remained green even up to the 9th day (FIG. 4), indicating that infact the lower half of the leaf blade could be used in detached leaf culture.

Experiment 2 (see Section 5.1.2.1)

Results from Experiment 2 (TABLE 34) show the effects of variations in the concentrations of benzimidazole. Greeness in leaf segments was maintained up to the 7th day at concentrations of benzimidazole between 100 to 200 ppm in liquid and soft Ionagar media (treatments (3), (4), (5)) and treatments ((9), (10), (11) respectively). But with benzimidazole at 200 ppm and above, scorching occurred at the top and top edges of the leaf segments in liquid and in soft Ionagar media (treatments (5), (6) and treatments (11), (12) respectively). However, at 150 ppm and above in soft Ionagar, benzimidazole caused scorching at the top of the leaf segments from the 5th day (treatments (10), (11), (12)). At 0 ppm benzimidazole in soft Ionagar medium, the top half of the leaf segments curled inwards from the sides from the 5th day (treatment 7), suggesting dehydration. However, flooding of soft Ionagar media with equal volume of water and 50 ppm benzimidazole separately across the set of treatments did decrease these effects (treatments (13) - (18) and treatments (19) - (24) respectively). It was evident that benzimidazole not only delayed leaf senescence but also prevented dehydration even at 50 ppm (treatments (7) and (8)). This effect was obtained only when benzimidazole was added directly into the soft Ionagar media because flooding the soft Ionagar medium with 50 ppm benzimidazole (treatment (19)) did not delay senescence or prevent dehydration from the 3rd day after detachment.

TABLE 34. COLOUR REACTIONS[5] OF THE BASAL SEGMENTS OF 2nd FULLY OPENED TOP LEAVES OF RICE CULTIVAR SMII TO VARIOUS CONCENTRATIONS OF BENZIMIDAZOLE (B_1) IN LIQUID AND SOFT IONAGAR (SIA) MEDIA

Day after detachment	Medium	(1) 0 ppm B_1 R1	R2	(2) 50 ppm B_1 R1	R2	(3) 100 ppm B_1 R1	R2	(4) 150 ppm B_1 R1	R2	(5) 200 ppm B_1 R1	R2	(6) 250 ppm B_1 R1	R2
1st	Benzimidazole (B_1)	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		2	2	1	1	1	1	1	1	1^1	1^1	1^1	1^1
7th		3	3	2	2	1	1	1	1	1^1	1^1	2^1	2^1
9th		5	5	4	4	2	3	2	2	3^1	3^1	3^1	3^1
11th		6	6	5	5	4	4	3	3	4^1	4^1	4^1	4^1
13th		6	6	5	5	5	5	4	4	5^1	5^1	5^1	5^1
		(7) (1) in SIA		(8) (2) in SIA		(9) (3) in SIA		(10) (4) in SIA		(11) (5) in SIA		(12) (6) in SIA	
1st	B_1 in soft Ionagar (SIA)	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		2^4	2^4	2	2	1	1	1^2	1^2	1^2	1^2	2^2	2^2
7th		3^4	3^4	3	3	1	1	2^2	2^2	2^2	2^2	3^2	3^2
9th		5^4	5^4	4	4	2	2	3^2	3^2	3^2	3^2	4^2	4^2
11th		6^4	6^4	5	5	3	3	4^2	4^2	4^2	4^2	5^2	5^2
13th		6^4	6^4	5	5	5	5	5^2	5^2	5^2	5^2	6^2	6^2
		(13) (7) c̄ H_2O		(14) (8) c̄ H_2O		(15) (9) c̄ H_2O		(16) (10) c̄ H_2O		(17) (11) c̄ H_2O		(18) (12) c̄ H_2O	
1st	B_1 in SIA covered with (c̄) water	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		2^2	2^2	2	2	2	2	1^3	1^3	1^2	1^2	1^2	1^2
7th		3^2	3^2	3	3	3	3	1^3	1^3	2^2	2^2	2^2	2^2
9th		5^2	5^2	4	4	3	3	2^3	2^3	3^2	3^2	3^2	3^2
11th		6^2	6^2	5	5	5	5	3^3	3^3	4^2	4^2	5^2	5^2
13th		6^2	6^2	5	5	5	5	4^3	4^3	5^2	5^2	5^2	5^2
		(19) (7) c̄ 50ppm B_1		(20) (8) c̄ 50ppm B_1		(21) (9) c̄ 50ppm B_1		(22) (10) c̄ 50ppm B_1		(23) (11) c̄ 50ppm B_1		(24) (12) c̄ 50ppm B_1	
1st	B_1 in SIA covered with (c̄) 50 ppm B_1	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		2^4	2^4	1^3	1^3	1	1	1	1	1^3	2^3	1^2	2^2
7th		3^4	3^4	1^3	1^3	2	2	2	2	2^3	3^3	2^2	3^2
9th		5^4	5^4	3^3	4^3	3	4	4	4	4^3	4^3	4^2	4^2
11th		5^4	5^4	4^3	4^3	4	4	4	4	4^3	4^3	5^2	5^2
13th		6^4	6^4	5^3	5^3	5	5	5	5	5^3	5^3	5^2	5^2

[1] : Scorching at the top edges of the leaf segment.

[2] : Scorching at the top of the leaf segment.

[3] : Slight scorching at the top of the leaf segment.

[4] : Top half of the leaf segment curled inwards from the sides.

[5] : Based on a 1-6 scale of decreasing greeness (see TABLE 27) in 2 replicates (R).

Benzimidazole at 250 ppm in liquid or soft Ionagar media not only did not delay sensecence but also caused scorching of the top and top edges of the leaf segments. At 200 ppm scorching appeared at the top edges of the segments in liquid media (treatments (5) & (6)), and at 150 ppm and above in soft Ionagar media, scorching occurred at the top of the segments (treatments (10), (11) & (12) from the 5th day after detachment. It was established therefore that benzimidazole by itself, at 150 ppm in liquid media and at 100 ppm in soft Ionagar media delayed senescence in leaf segments without scorching and dehydration.

Experiment 3 (see Section 5.1.2.1)
Results in TABLE 35 show that in liquid medium benzimidazole by itself maintained leaf greeness up to the 11th day at 100-200 ppm (treatment (3)-(5)) which was much longer than did all other treatments. However, when mixed with 0.25 concentration Long Ashton nutrient solution (LANS) only the 150 ppm benzimidazole treatment could sustain leaf greeness up to the 9th to 11th day (treatment (10)) (PLATE 13), indicating that benzimidazole functioned well by itself (liquid form) at 100-200 ppm but when combined with 0.25 concentration of LANS it functioned well only at 150 ppm.

On soft Ionagar benzimidazole by itself maintained leaf greeness up to the 7th day at 100-150 ppm without side effects (treatments (15) & (16)). However, when combined with 0.25 concentration of normal LANS, benzimidazole (soft Ionagar form) functioned best at 150 ppm without side effects. Hence soft Ionagar medium containing 150 ppm benzimidazole and 0.25 concentration of LANS appeared as the best medium for detached leaf culture. It is superior to its liquid form in terms of ease in handling and non-contamination and non-interference of the various treatments among leaf segments.

TABLE 35. COLOUR REACTIONS[3] OF THE BASAL SEGMENTS OF 2nd FULLY OPENED TOP LEAVES OF RICE CULTIVAR SHII TO VARIOUS CONCENTRATIONS OF BENZIMIDAZOLE (B_1) IN COMBINATION WITH VARIOUS CONCENTRATIONS OF LONG ASHTON NUTRIENT SOLUTION (LANS) IN LIQUID AND SOFT IONAGAR (SIA) MEDIA.

Day after detachment	Medium	(1) 0 ppm B_1		(2) 50 ppm B_1		(3) 100 ppm B_1		(4) 150 ppm B_1		(5) 200 ppm B_1		(6) 250 ppm B_1	
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
1st	Benzimidazole (B_1)	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		3	3	2	2	1	1	1	1	1	1	1	1
7th		4	4	3	3	1	1	1	1	1	1	2	2
9th		5	5	4	4	1	1	1	1	1	1	3	3
11th		5	5	5	5	2	2	2	2	2	2	4	4
13th		6	6	6	6	4	4	3	3	4	4	5	5
		(7) (1)+0.25 Conc LANS		(8) (2)+0.25 Conc LANS		(9) (3)+0.25 Conc LANS		(10) (4)+0.25 Conc LANS		(11) (5)+0.25 Conc LANS		(12) (6)+0.25 Conc LANS	
1st	B_1 + Long Ashton Nutrient Solution (LANS)	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		3	3	2	2	1	1	1	1	1	1	1	1
7th		4	4	3	3	1	1	1	1	2	2	2	2
9th		5	5	4	4	3	3	1	1	3	3	3	3
11th		5	5	5	5	4	4	3	3	4	4	4	4
13th		6	6	6	6	5	5	4	4	5	5	5	5
		(13) (1) in SIA		(14) (2) in SIA		(15) (3) in SIA		(16) (4) in SIA		(17) (5) in SIA		(18) (6) in SIA	
1st	B_1 in soft Ionagar (SIA)	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		3	3	2	2	1	1	1	1	1	1	1	1
7th		4^2	4^2	3	3	2	2	2	2	2^1	2^1	2^1	2^1
9th		5^2	5^2	5	5	4	4	4	4	4^1	4^1	4^1	4^1
11th		5^2	5^2	5	5	4	4	4	4	4^1	4^1	4^1	4^1
13th		6^2	6^2	6	6	5	5	5	5	5^1	5^1	5^1	5^1
		(19) (7) in SIA		(20) (8) in SIA		(21) (9) in SIA		(22) (10) in SIA		(23) (11) in SIA		(24) (12) in SIA	
1st	B_1 + LANS in SIA	1	1	1	1	1	1	1	1	1	1	1	1
3rd		1	1	1	1	1	1	1	1	1	1	1	1
5th		3	3	2	2	1	1	1	1	1	1	1	1
7th		4	4	2	2	1	1	1	1	1^1	1^1	2^1	2^1
9th		5^2	5^2	4	4	3	3	2	2	3^1	3^1	4^1	4^1
11th		5^2	5^2	5	5	4	4	3	3	4^1	4^1	4^1	4^1
13th		6^2	6^2	6	6	5	5	4	4	5^1	5^1	5^1	5^1

[1] : Scorching at the top edges of the leaf segment.

[2] : Top half of the leaf segment curled inwards from the sides.

[3] : Based on a 1-6 scale of decreasing greeness (see TABLE 27) in 2 replicates (R).

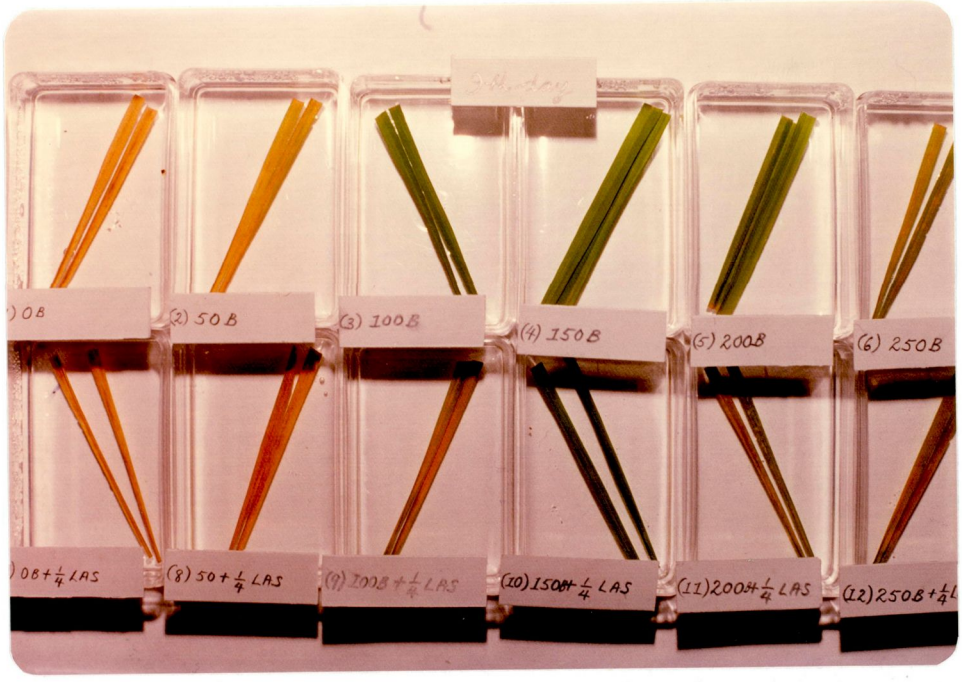


PLATE 13. Colour reactions of the basal segments of 2nd fully opened top leaves (in 2 replicates) of rice cultivar SMII, 9 days after detachment in liquid media of various concentrations of benzimidazole (B) and Long Ashton nutrient solution (LAS) and their combinations.

5.2. Method of Inoculation of Detached Leaf Culture

5.2.1. Materials and Methods

The main aim of this experiment was to determine an inoculation method which would induce good symptoms on detached leaf segments and also to test whether the method of inoculation could differentiate cultivar resistance for Indica and Japonica cultivars.

Cultivar

Three cultivars were used. The Indica types IR22 and T(N)1 were from the Philippines and Taiwan respectively, and a Japonica type, Sasanishihi from Japan. IR22 is generally moderately susceptible and T(N)1 and Sasanishihi are generally susceptible. They were maintained under flooded conditions for 28 days in the glasshouse before being used for the experiment.

Medium

The best medium found was used for this experiment. The soft Ionagar medium consisted of 150 ppm benzimidazole with 0.25 concentration Long Ashton nutrient solution (LANS) in soft Ionagar at 0.5%. Details are given in Section 5.1.2.1 Experiment 1. However, instead of small transparent polystyrene boxes, transparent plastic trays referred to in Section 5.1.1.1 were used. And the LANS used in previous experiments was replaced by the 'low' nitrogen LANS (TABLE 36) and was used for this and the following experiments.

Methods of Inoculation

The methods of inoculation were variations of (i) the scissor and (ii) the pin prick inoculation methods as follows:-

(i) Scissors - basal dip method This is similar to the scissors inoculation method except that a pair of sterile scissors is used and the segment is inoculated by dipping the basal end into standard bacterial suspension (prepared as in Section 1.1) for about ten seconds and immediately stuck into the soft Ionagar by the basal end for a short length at a gradient of about 10^{o}.

TABLE 36. MACRO AND MICRO NUTRIENT COMPOSITION[1] OF HEWITT'S (1966) LONG ASHTON 'LOW' NITROGEN NUTRIENT SOLUTION.

Macro and micro nutrients	Molecular weight	g/2 litres (stock solution)
1. $Ca(NO_3)_2 . 4H_2O$	236	157.4
2. KNO_3	101	67.2
3. $MgSO_4 . 7H_2O$	247	74.0
4. $NaH_2PO_4 . 2H_2O$	156	41.6
5. Ferric Citrate	335	18.2
6. $MnCl_2 . 4H_2O$	198	0.396
7. $CuCl_2 . 4H_2O$	170	0.034
8. $ZnCl_2$	136	0.028
9. H_3BO_3	62	0.372
10. $Na2MoO_4 . 2H_2O$	242	0.022

1: Full concentration standard 'low' nitrogen Long Ashton nutrient solution as mentioned in the text is prepared by mixing 10 ml of the above stock solutions into 900 ml of distilled water.

TABLE 37. SCALE FOR ASSESSING CULTIVAR REACTIONS IN STRAIN TYPING IN DETACHED LEAF CULTURE.

Scale	Description of lesion	Reaction
0	No lesion	Highly resistant (HR)
1	Lesion occupies 1/3 or less of leaf segment	Resistant (R)
2	Lesion occupies 1/3-2/3 of leaf segment	Moderately resistant (M)
3	Lesion occupies2/3 or more of leaf segment	Susceptible (S)

(ii) Scissors - basal normal method This is also similar to the scissors inoculation method except that a pair of scissors which has been dipped into the standard bacterial suspension is used to cut and also inoculate the basal end of the leaf segment.

(iii) Scissors - top dip method This is similar to (i) except that the leaf segment is inoculated by dipping the top end after cutting into standard bacterial suspension for ten seconds.

(iv) Scissors - top normal This is the normal scissors methods and is similar to (ii) above except that the top end of the leaf segment was inoculated.

(v) Scissors - Control Distilled water was introduced into the top end of the leaf segment as in (iv) above.

(vi) Pin prick - modified The pin prick apparatus referred to in Section 3.1.1 (PLATE 5) was used to inoculate the leaf section in the middle region but modified in that pricks were made and the standard bacterial suspension then introduced on to the two pricks from a small strip of filter paper soaked in the bacterial suspension.

(vii) Pin prick - normal The pin prick apparatus (PLATE 5) was used in the normal way as in Section 4.1.1.

(viii) Pin prick - control Similar to (vii) but distilled water was used instead of bacterial suspension.

Procedure

28-day old seedlings of the 3 cultivars were taken from the glasshouse to the laboratory in sufficient quantities. Only the basal 5 cm segment of the second fully opened top leaves were used for the experiment. The rest of the procedure was similar to that in experiment 1 in Section 5.1.2.1 and Section 5.1.1.1. The experiment was done in 3 replicates.

Assessment

The method of assessing cultivar reactions in strain typing using the detached leaf culture method was based on the size of lesion induced on the 5 cm leaf segments as shown in TABLE 37. Assessment was done 11 days after inoculation.

5.2.2. Results

Results in TABLE 38 show that while all methods of inoculation caused some disease symptoms to develop on the Indica type of rice cultivars IR22 and T(N)1, none caused marked symptom on the supposedly susceptible Japonica rice cultivar Sasanishihi.

Good disease symptom developed on cultivars IR22 and T(N)1 inoculated by both the scissors and the pin prick methods and their modifications. However, the pin prick method, on the whole was less good than the scissors method because it caused only moderate disease symptoms (score 2) on susceptible cultivar T(N)1 (TABLES 38 & 37). This method also caused a similar reaction on a moderately resistant cultivar IR22. This indicates that the pin prick method did not differentiate cultivar resistance sufficiently well and hence would not be suitable for use in detached leaf culture.

The scissors method was very successful in causing disease symptoms on detached leaf segments. However, of the various forms of the scissors method, only basal dipping of the leaf segments into the inoculum following cutting produced good results on moderately resistant (IR22) and susceptible T(N)1 cultivars (TABLE 38). The other forms of the scissors method did not differentiate cultivar resistance effectively.

It was of interest that all the leaf segments in the control treatments kept green up to 17 days after detachment as a result of the change to HEWITT'S (1966) 'low' nitrogen Long Ashton nutrient solution.

5.3. Strain Typing using Detached Leaf Culture Technique

5.3.1. Materials and Methods

The aim of this experiment is to attempt to identify and classify *Xanthomonas oryzae* strains by means of the detached leaf culture technique developed through the above experiments in Sections 5.1.1, 5.1.2 and 5.2.

TABLE 38. DISEASE SCORES[1] OF 3 RICE CULTIVARS IN DETACHED LEAF CULTURE INOCULATED WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* BY 6 METHODS OF INOCULATION 11 DAYS AFTER INOCULATION.

Method of inoculation	Cultivar								
	IR22			T(N)1			Sasanishihi		
	Replicate			Replicate			Replicate		
	1	2	3	1	2	3	1	2	3
1. Scissors - basal dip	1	2	2	3	3	3	1	1	1
2. Scissors - basal normal	3	3	3	3	3	3	1	1	1
3. Scissors - top dip	1	2	2	2	2	2	1	1	1
4. Scissors - top normal	2	2	2	1	2	2	1	1	1
5. Scissors - control	1	1	1	1	1	1	1	1	1
6. Pin prick - modified	2	2	2	2	2	2	1	1	1
7. Pin prick - normal	2	2	2	2	3	2	1	1	1
8. Pin prick - control	1	1	1	1	1	1	1	1	1

1 : Based on a 0-3 scale of increasing disease. See TABLE 37 for corresponding disease reactions (1 = Resistant. 2 = Moderately resistant. 3 = Susceptible).

Cultivar

The 6 differential cultivars identified in Section 3 (TABLE 17), together with another cultivar SMII, a Philippines cultivar IR8 and a Japanese cultivar Sasanishihi (Japonica type) were used. The methods of growing and maintenance of seedlings under glasshouse conditions were similar to those in Section 3.1.1.

Medium

The medium used consisted of soft Ionagar (0.5%) with 150 ppm benzimidazole (refer Section 5.1.2.1, Experiment 1) and 0.25 concentration of HEWITT's (1966) 'low' nitrogen Long Ashton nutrient solution detailed in Section 5.2.1 and TABLE 36.

Procedure

All procedures are similar to those in Section 5.1.1.1, except that only 5 cm segments from the lower half of the 2nd fully opened top leaves were used. The experiment was done in 3 replicates.

Assessment

The criterion and method of assessment is given in TABLE 37 in Section 5.2.

5.3.2. Results

Again, the supposedly susceptible Japonica cultivar Sasanishihi did not develop symptoms after inoculation with all strains of *Xanthomonas oryzae*. Its negative reactions to the isolates were similar to the controls (TABLE 39, PLATE 14). Only 6 of the 8 Indica rice cultivars gave normal disease reactions. These were: IR1545-339-2-2, MR1, MR35, IR20, Jaya and T(N)1. The other 2 generally susceptible cultivars IR8 and SMII, were not suitable for use as differential cultivars as they could not differentiate accurately expected differences in virulence among the isolates (TABLE 39). Based on the disease reaction patterns formed on the 6 differential cultivars (TABLE 40) the isolates could be once again classified into 4 strains according to their ranges of virulence: (i) limited

TABLE 39. DISEASE SCORES[1] OF 9 RICE CULTIVARS TO 10 *XANTHOMONAS ORYZAE* ISOLATES IN DETACHED LEAF CULTURE 11 DAYS AFTER INOCULATION

Isolate \ Cultivar	IR1545-339 -2-2	MR1	IR20	MR35	Jaya	T(N)1	IR8	SMII	Sasanishihi
A	1	1	1	1	1	2	3	1	0
K	1	1	1	2	2	2	3	1	0
I	1	1	1	2	2	3	3	2	0
F	2	2	2	2	2	3	2	1	1
L	2	2	2	3	3	3	2	2	0
S	1	1	2	2	2	3	2	1	0
B	2	2	2	3	3	3	2	2	0
C	2	2	3	3	3	3	2	2	0
H	1.6	2	3	3	3	3	2	2	0
M	2	2	3	3	3	3	1	1	0
Control	0	0	0	0	0	1	0	0	0

1 : Mean of 3 replicates. See TABLE 37 for the corresponding disease reactions (1 = Resistant. 2 = Moderately resistant. 3 = Susceptible).

TABLE 40. CLASSIFICATION OF *XANTHOMONAS ORYZAE* ISOLATES BASED ON RANGE OF VIRULENCE[1] ON 6 RICE DIFFERENTIAL CULTIVARS IN DETACHED LEAF CULTURE

Differential cultivar / Range of virulence	IR1545-339-2-2	MR1	MR35	IR20	Jaya	T(N)1	Classification of isolates
I. Very limited	R	R	R	R	R	R	
II. Limited	R	R	R	R	R	M	A
III. Moderately limited	R	R	R-M	R-M	M	M-S	K, I
IV. Moderately wide	R-M	R-M	M-S	M-S	M-S	S	F, L, S, B, H
V. Wide	M	M	S	S	S	S	C, M
VI. Very wide	S	S	S	S	S	S	

1 : Based on diseas reactions (see TABLE 37). R = Resistant. M = Moderately resistant. S = Susceptible.

TABLE 41. COMPARISONS OF *IN VIVO* AND *IN VITRO* STRAIN CLASSIFICATIONS OF *XANTHOMONAS ORYZAE* BASED ON RANGE OF VIRULENCE[1] ON 6 DIFFERENTIAL CULTIVARS

Virulence group	Isolate	IR1545-339-2-2	MR1	MR35	IR20	Jaya	T(N)1	Type of strain classification
II. Limited	A	R	R	R	R	R	M	*In vivo*[2]
		R	R	R	R	R	M	*In vitro*[3]
III. Moderately limited	K, I	R	R	R	R-M	M-S	S	*In vivo*
		R	R	R-M	R-M	M	M-S	*In vitro*
IV. Moderately wide	F, L, S	R	R-M	M-S	S	S	S	*In vivo*
	B, H	R-M	R-M	M-S	M-S	M-S	S	*In vitro*
V. Wide	C, M	M	S	S	S	S	S	*In vivo*
		M	M	S	S	S	S	*In vitro*

1 : For *in vivo* reactions see TABLE 5. For *in vitro* reactions see TABLE 37.

2 : From established strain classification on seedlings (see TABLE 17 in Section 3).

3 : From established laboratory strain classification on detached leaf culture (see TABLE 40) in Section 5).



PLATE 14. Varying reactions if isolates of *Xanthomonas oryzae* on rice cultivars in detached leaf culture showing negative reactions on susceptible Japonica cultivar Sasanishihi (bottom) similar to those of the control (far right.)

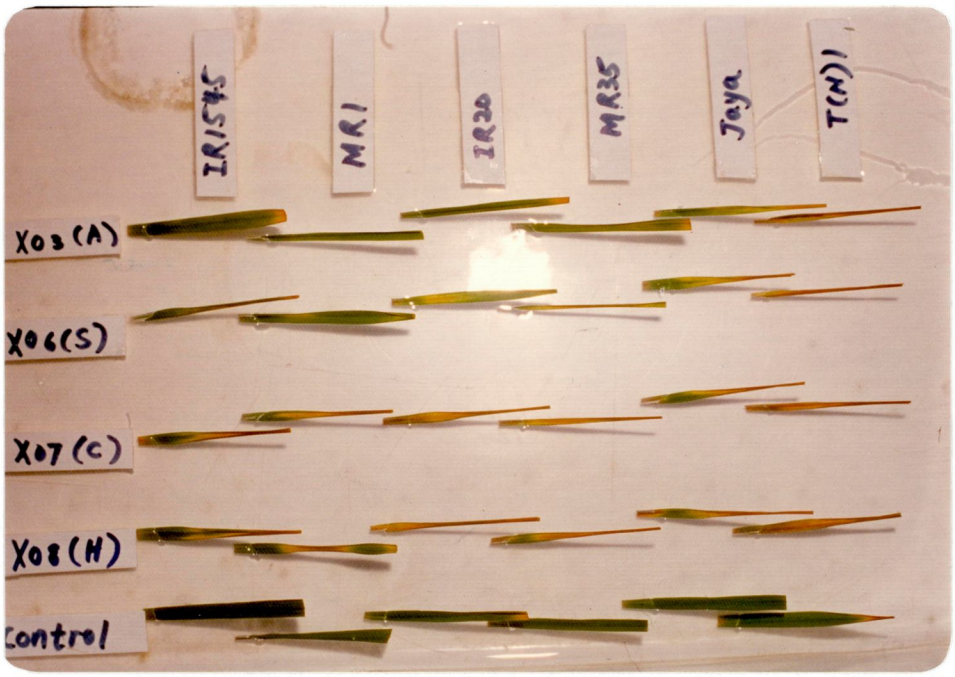


PLATE 15. Typical disease reaction patterns of 4 *Xanthomonas oryzae* strains on 6 differential cultivars in detached leaf culture.

(isolate A) (ii) moderately limited (isolates K, I) (iii) moderately wide (isolates F, L, S, B, H) and (iv) wide (isolates C, M) (PLATE 15). The results were almost similar to those obtained on seedlings of similar set of differential cultivars in Section 3 (TABLE 41). However, classification of strains by means of detached leaf culture (TABLE 41) gave slightly more susceptible reactions for strains of moderately limited and moderately wide range of virulence on more resistant cultivars, whereas the reverse was true for strains of wide range of virulence.

5.4. Discussion and Conclusion

5.4.1. Determination of Best Medium, Type and Segment of Detached Leaf for Detached Leaf Culture.

5.4.1.1. Detached Whole Leaf

The 1st fully opened top leaves tended to curl up much more than did the 2nd leaves, probably because of their greater susceptibility to dehydration.

The fact that yellowing started downwards from the tip, the more delicate part of the leaf blade, suggested that young tissues were not suitable for detached leaf culture as were the 1st leaves compared to the 2nd leaves. It was sensible therefore to choose only the basal 5 cm segment or the lower half of the 2nd fully opened top leaves for detached leaf culture.

5.4.1.2. Medium and Segment of Detached Leaf

The basal segment of the 2nd fully opened top leaves kept its original greeness much longer than did the middle and end segments, probably for the reasons expressed above. Hence the basal segment or the lower half of the leaf was the choice for use in detached leaf culture.

Long Ashton nutrient solution by itself, irrespective of concentration did not delay senescence in detached leaf segments and did not cause scorching. It was effective only in combination with benzimidazole. Benzimidazole delayed senescence

with concentration of benzimidazole important. This suggests that benzimidazole prevented rapid deterioration which normally takes place in detached leaves and was due to the fact that benzimidazole delayed protein degradation in detached leaves (SAMBORSKI *et al.*, 1958). However, at 200 ppm and above benzimidazole caused scorching in leaf segments. The combination of 150 ppm benzimidazole and 0.25 concentration of HEWITT's (1966) Long Ashton nutrient solution in 0.5% Ionagar was shown to be the best medium for detached leaf culture.

5.4.2. Method of Inoculation of Detached Leaf Culture

The pin prick methods did not induce much disease, neither did the scissor method of inoculation of the top end of leaf segments. The scissor method of inoculation of the basal-dip method gave good infections as the method enabled the bacteria, introduced at the basal end, to move upwards along with the flow of water and nutrients from the bottom, and in doing so aided bacterial infection of the leaf segments. The fact that the susceptible Japonica cultivar Sasanishihi was not diseased suggests that such cultivars were not suitable for detached leaf culture purposes.

The change from HEWITT's (1952) formulation (TABLE 30) of Long Ashton nutrient solution to his 1966 formulation (TABLE 36) did significantly delay further the senescence in leaf segments. This was observed in the leaf segments of the control treatments, where greeness remained for 17 days after detachment. This may be due to the absence of iron EDTA in the 1966 formulation which prevented precipitation in the final solution and made more nutrients available to the leaf segments.

5.4.3. Strain Typing using Detached Leaf Culture Technique

The supposedly susceptible Japonica cultivar Sasanishihi again was not diseased by the bacterium irrespective of its virulence, hence confirming the unsuitability of the cultivar in detached leaf culture. The study also revealed that not

all Indica cultivars were suitable. A good example of this was given by two generally susceptible cultivars IR8 and SMII which gave anomalous disease reactions compared with those of cultivar T(N)1 of equal general susceptibility rating.

It needs be mentioned that a detailed strain classification using the detached leaf culture technique would be difficult because the results had shown that it could be used to classify isolates only into broad categories. This was so because the reaction patterns were only based on lesion length on limited leaf segment of 5 cm and hence may not be sufficiently sensitive. However, it was of interest that it gave results in broad categories which were similar to those given by accepted techniques on seedlings and adult plants.

6. MAINTENANCE OF *XANTHOMONAS ORYZAE* CULTURE UNDER LABORATORY CONDITIONS

6.1. Effects of Successive Sub-Culturing and Temporary Storage on the Growth and Virulence of Five Isolates

6.1.1. Materials and Methods

(a) Growth of Cultures

Five *Xanthomonas oryzae* isolates (A, I, F, B, M) ranging from weakly virulent (isolate A) to virulent (isolate M) (refer Section 3, TABLE 16) were cultured on Wakimoto's (1960) potato semisynthetic agar (WPSA) (refer Section 1.1) in test tub slants (15 cm x 14 mm) by streaking one loopful of 48-hour old cultures over their surfaces. Three types of maintenance and short-term storage techniques involving different numbers and intervals of sub-culturing were investigated as follows:

(i) Main treatments 1 (E_1). A total of 4 sub-culturings done within 3 months of different storage periods at -10°c before sub-culturings and between sub-culturings at 3°-6°c (FIG. 5) as follows:

E_1T_1 = 4 sub-culturings, once every month for 3 months.

E_1T_3 = 4 sub-culturings, once every fortnight for 1½ months.

E_1T_3 = 4 sub-culturings, once every week for 3 weeks.

E_1T_4 = 4 sub-culturings, once every 3-4 days for 10 days.

(ii) Main treatments 2 (E_2). Different total number of sub-culturings within 3 months with different storage periods between sub-culturing at 3° - 6°c (FIG.6) as follows:

E_2T_1 = 4 sub-culturings once every month for 3 months (= E_1T_1)

E_2T_2 = 7 sub-culturings once every fornight for 3 months.

E_2T_3 = 13 sub-culturings once every week for 3 months.

E_2T_4 = 25 sub-culturings once every 3-4 days for 3 months.

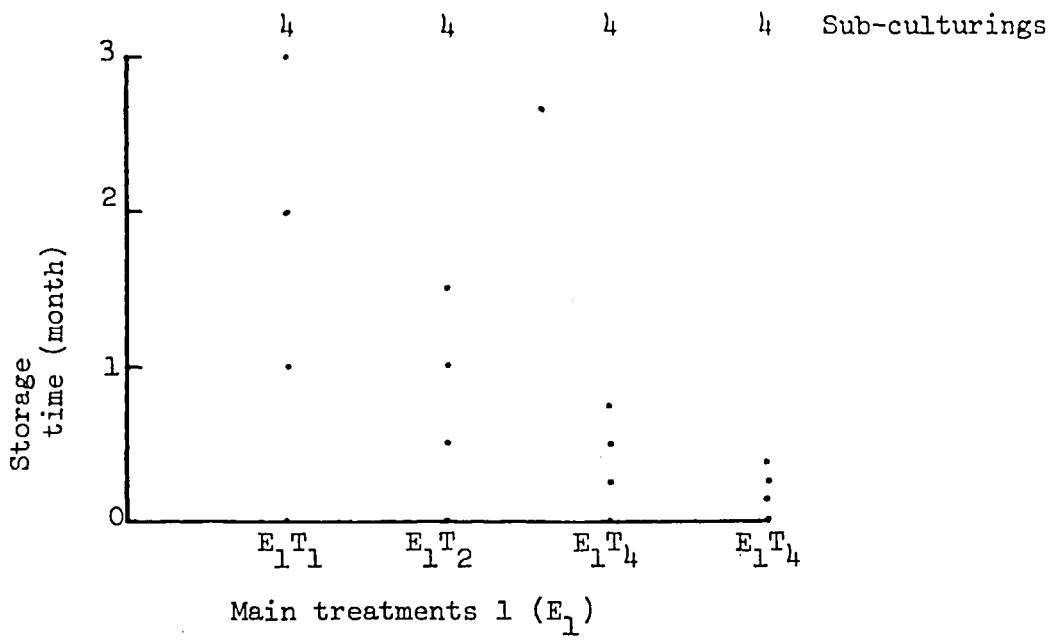


FIG. 5. Main treatments 1 (E_1) with constant number of sub-culturing (.) but different storage periods at 3° to 6°c between sub-culturings and -10°c before sub-culturing for 3-month period.

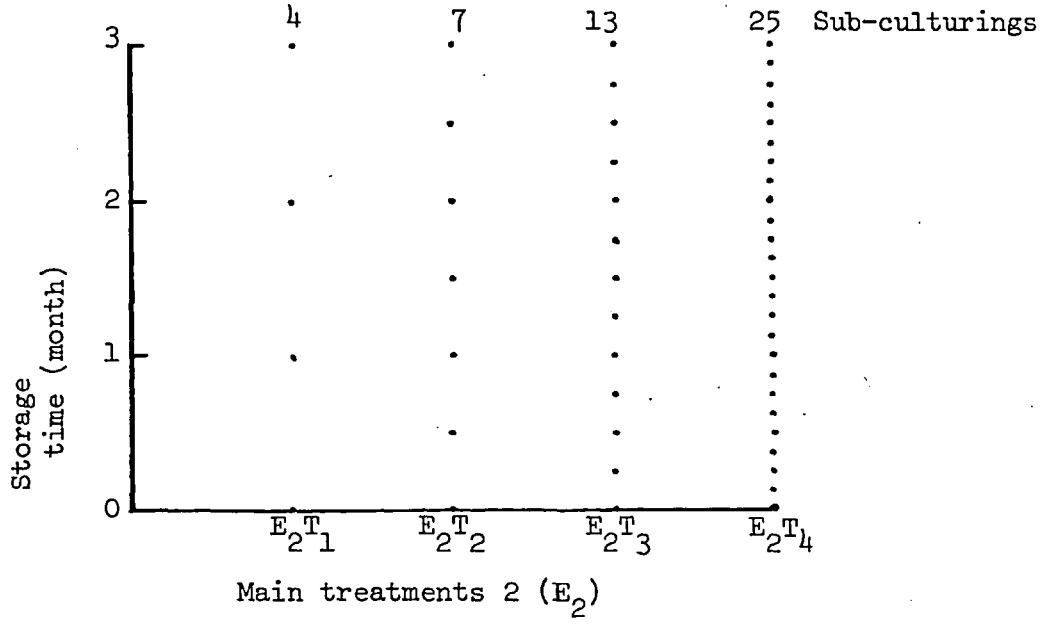


FIG. 6. Main treatments 2 (E_2) with different number of sub-culturing (.) and different storage periods at 3° to 6°c between sub-culturings over a period of 3 months.

(iii) Main treatments 3 (E_3). Two sets of original cultures of the 5 isolates were examined as follows:

E_3O_1 = Original cultures stored at 3^o - 6^oc continuously for 3 months.

E_3O_2 = Original cultures stored at -10^oc continuously for 9 months.

All sub-culturings were done in test tube slants as stated and were kept in a refrigerator at 3^o - 6^oc prior to the first sub-culturings, unless otherwise stated. All sub-cultures were incubated at $26^o \pm 2^o$c for 3 days before being assessed for growth as follows:

1. Visual growth assessment (as in Section 1.1 with slight modification).

Scale	Visual growth description
1	Good
2	Fair
3	Poor
4	Very poor or negligible

2. Optical density measurement (as in Section 1.1).

Before the optical density measurements were carried out, all sub-cultures in suspensions were examined for re-growth by the plating and streaking methods. One loopful of each of the suspensions were streaked on WPSA (refer Section 1.1) plates and 0.1 ml of each of the suspensions was plated out on modified Wakimoto's potato semisynthetic agar (refer Section 1.1) in 4 replicates and observed on the third and fifth day of incubation at $26^o \pm 2^o$c. The optical densities (absorbance at 620 nm) of the various suspensions were measured using a Beckman spectrophotometer and their populations estimated using the calibration graph in FIG. 1 (refer Section 1).

(b) Virulence

Sufficient standard bacterial suspensions of all the isolates were prepared (as in Section 1.1) for all treatments to inoculate seedlings of susceptible cultivar T(N)1 by the mass pin prick method (refer Section 3.1.1, PLATE 4).

The experiment was done in a split-plot design with 8 replicates. Seedlings were grown by direct seeding at the distance of 3 x 3 cm. They were inoculated 26 days after sowing and assessed for disease reactions 14 days after inoculation using the scale in TABLE 5. The materials and methods used for planting and assessment were similar to those in Section 3.1.1.

The experiments were repeated with a weakly virulent strain (isolate I) and a virulent strain (isolate M) (refer TABLE 16). The final sub-cultures were allowed to grow for a further 2 days at $26^{o} \pm 2^{o}$c before being sub-cultured again and incubated for 3 days. Growth and virulence measurements were similar to (a) and (b) above. The experiments were conducted in a randomised complete block design with 3 replicates. Materials and methods used were similar to preceeding experiments in (a) and (b).

6.1.2. Results

Results on the cultural growths of the 5 isolates assessed visually (TABLE 42) and by means of their optical densities (TABLE 43), indicate that with minimal frequency of sub-culturing (4 times) and different storage periods before sub-culturing at -10^{o}c and between sub-culturing at $3^{o} - 6^{o}$c for 3 months, their growths were not affected irrespective of their virulence ($E_1T_1 - E_1T_4$). However, if the frequency of sub-culturing was increased with similar storage periods between sub-culturings at $3^{o} - 6^{o}$c ($E_2T_1 - E_2T_4$), only very frequent sub-culturing i.e. 25 sub-culturings done once every 3-4 days (E_2T_4) had some adverse effect on the growth of most strains irrespective of virulence (TABLES 42 & 43). A reduction in the frequency of sub-culturing to 13 at weekly intervals (E_2T_3) affected only the weakly virulent strain (isolate A in TABLES 42 & 43). Further reduction in the frequency of sub-culturing to 7 and 4 by sub-culturing at fortnightly and monthly intervals respectively (E_2T_2 & E_2T_1 respectively in TABLES 42 & 43) did not affect the growth of all strains.

TABLE 42. VISUAL GROWTH ASSESSMENTS[1] OF 5 *XANTHOMONAS ORYZAE* ISOLATES UNDER DIFFERENT STORAGE AND MAINTENANCE CONDITIONS

Isolate \ Treatment[2]	E_1T_1	E_1T_2	E_1T_3	E_1E_4	E_2T_1	E_2T_2	E_2T_3	E_2T_4	E_3O_1	E_3O_2
A	2	2	2	2	2	2	2.7	2.7	4	2
I	1	1	1	1	1	1	1	2	4	2
F	1	1	1	1	1	1	1	2	3	1
B	1	1	1	1	1	1	1	1	2	1
M	1	1	1	1	1	1	1	2	1	1

1 : Mean of 3 replicates, based on a 1-4 scale of decreasing growth
(1 = Good growth. 4 = Very poor or negligible growth)

2 : E_1T_1 = 4 sub-culturings, once every month for 3 months
E_1T_2 = 4 sub-culturings, once every fortnight for 1½ months
E_1T_3 = 4 sub-culturings, once every week for 3 weeks
E_1T_4 = 4 sub-culturings, once every 3 to 4 days for 10 days

E_2T_1 = 4 sub-culturings, once every month for 3 months (= E_1T_1)
E_2T_2 = 7 sub-culturings, once every fortnight for 3 months
E_2T_3 = 13 sub-culturings, once every week for 3 months
E_2T_4 = 25 sub-culturings, once every 3 to 4 days for 3 months

E_3O_1 = Original cultures stored at 3° to 6°c for 3 months
E_3O_2 = Original cultures stored at -10°c for 9 months

TABLE 43. OPTICAL DENSITY MEASUREMENTS[1] OF 5 *XANTHOMONAS ORYZAE* ISOLATES UNDER DIFFERENT STORAGE AND MAINTENANCE CONDITIONS

Isolate \ Treatment[2]	E_1T_1	E_1T_2	E_1T_3	E_1T_3	E_2T_1	E_2T_2	E_2T_3	E_2T_4	E_3O_1	E_3O_2
A	1.8	1.8	1.7	1.7	1.8	1.7	1.5	1.7	0.2	1.9
I	2.0	2.0	2.0	2.0	1.8	1.7	1.8	1.5	0.2	1.6
F	2.0	1.9	1.9	2.0	1.7	1.8	1.8	1.1	0.8	1.9
B	1.9	1.9	1.9	1.9	1.7	1.7	1.9	1.9	1.8	1.9
M	2.0	2.0	2.0	1.9	2.0	2.0	1.8	1.6	1.8	2.0

1 : Mean of 3 replicates, measured by a Beckman spectrophotometer (at 620 nm) with an absorbance scale of 0 - 2.0 (maximum turbidity).

2 : See TABLE 42.

TABLE 44. DISEASE SCORES[1] OF 5 *XANTHOMONAS ORYZAE* ISOLATES UNDER DIFFERENT STORAGE AND MAINTENANCE CONDITIONS ON SUSCEPTIBLE RICE CULTIVAR T(N)1

Isolate \ Treatment[2]	E_1T_1	E_1T_2	E_1T_3	E_1T_4	E_2T_1	E_2T_2	E_2T_3	E_2T_4	E_3O_1	E_3O_2
A	3.0	3.0	3.0	2.5	2.9	3.0	3.0	2.5	0	2.6
I	3.3	3.4	3.1	3.1	3.0	2.9	2.8	3.0	0.4	2.5
F	3.5	3.5	3.5	3.0	3.0	3.0	3.4	4.0	3.1	3.1
B	4.8	4.4	3.8	3.8	3.6	3.3	4.0	4.9	4.4	4.0
M	4.8	4.5	4.4	4.5	4.6	4.0	4.3	4.3	4.3	4.3

1 : Mean of 8 replicates, based on a 0-5 scale of increasing disease (see TABLE 5).

2 : See TABLE 42.

However, in terms of re-growth from suspensions, colony formation by plating appeared to be similar for all treatments (E_1 & E_2) as typified by isolate M, on the 3rd day and the 5th day of incubation. Growth from streaking appeared to confirm the above results as typified by a weakly virulent strain (isolate I) and a virulent strain (isolate M).

On the other hand, continuous refrigeration for 3 months at 3^{o} - 6^{o}c (E_3O_1) was shown to be detrimental to cultural growth of weakly virulent (isolates A, I) and moderately virulent (isolate F) strains (TABLES 42 & 43), whereas continuous storage for 9 months at -10^{o}c (E_3O_2) had little or no effect on the cultural growths of all strains.

However, despite differences in visual cultural growths among the various treatments (TABLE 42), supported by their optical densities in TABLE 43, the calibration graph in FIG. 1 shows that the number of viable bacterial cells for all isolates in all treatments were about 10^9 cells/ml or more except the weakly virulent and moderately virulent strains stored continuously at 3^{o} - 6^{o}c for 3 months (E_3O_1) which gave much lower viable cell counts. The results tally with those given by assessments based on visual cultural growth and optical density mentioned earlier. Results on the amounts of disease produced also indicate that all variations in the frequency of sub-culturing and the intervals between sub-culturings despite some physical differences stated above, did not matter much, except for treatment involving continuous refrigeration at 3^{o} - 6^{o}c (TABLE 44). This treatment, which had been shown to be detrimental to weakly virulent strains (isolates A & I of main treatment E_3O_1 in TABLES 42 & 43) was found to adversely affect their virulence (TABLE 44, FIG. 7). The normal disease scores of isolates A and I were reduced from about 3 (as evidenced from all other treatments) to 0 and 0.4 respectively. Those of moderately virulent (isolates F & B) and virulent (isolates M) strains remained almost similar (PLATE 16).

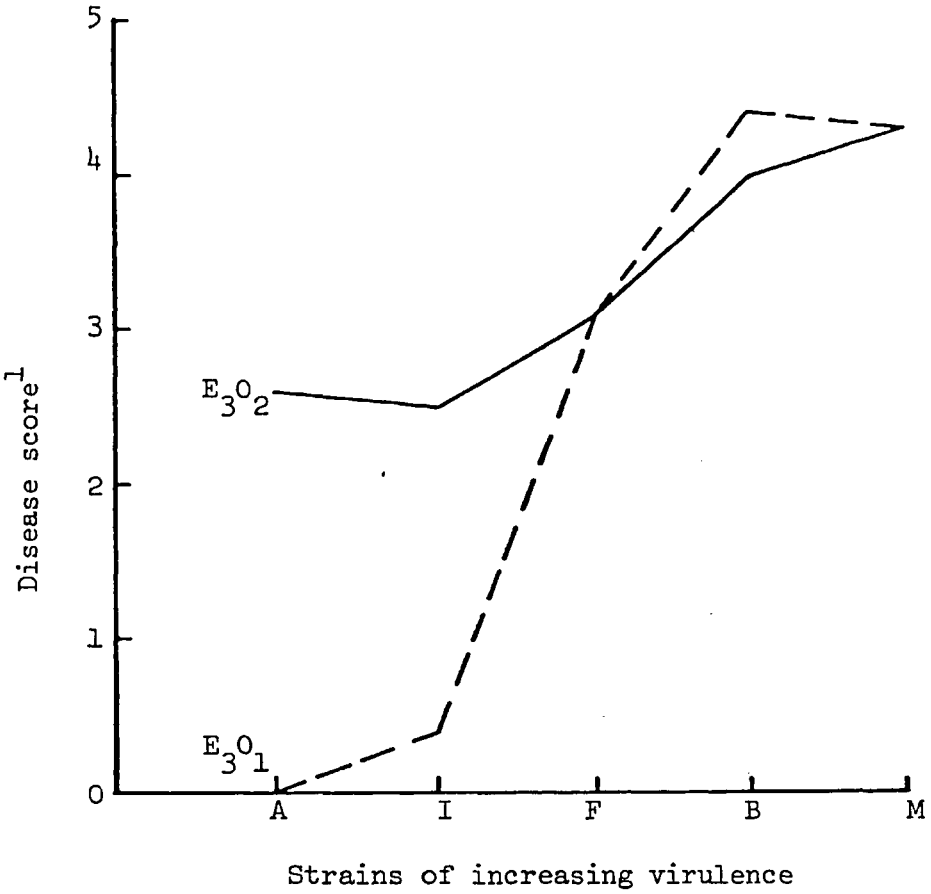


FIG. 7. Effects of continuous refrigeration at 3° - 6°c (E_3O_1) and deep freezing at -10°c (E_3O_2) for 3 and 9 months respectively on the virulence of weakly virulent to virulent strains of *Xanthomonas oryzae*.

1 : 0-5 scale of increasing disease as detailed in TABLE 5.

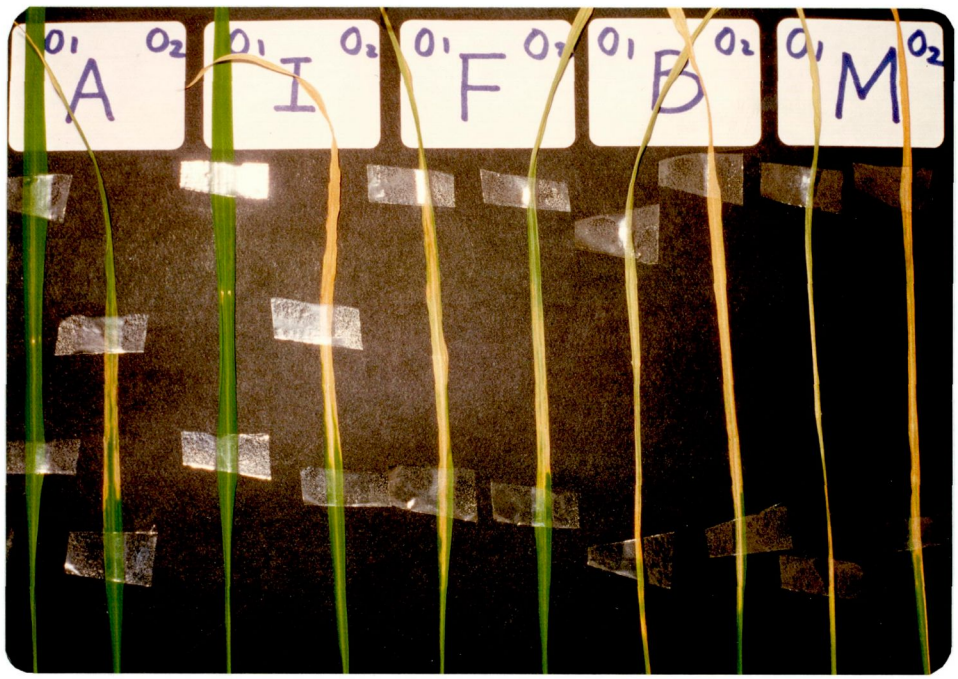


PLATE 16. Disease reactions of 5 isolates of *Xanthomonas oryzae* on a susceptible cultivar T(N)1 showing detrimental effect of continuous refrigeration (3^{o} - 6^{o}c) for 3 months (O_1) on the virulence of only weakly virulent strains (isolates A, I), while continuous storage at -10^{o}c (O_2) for 9 months had no such adverse effect on all strains irrespective of virulence.

Results from another experiment using only a weakly virulent (isolate I) and a virulent (isolate M) strains show that with 2 successive sub-culturings at 2 days' interval done at the end of the final sub-culturings from preceeding experiments and kept at $26^{o} \pm 2^{o}c$, the growth (TABLES 45 & 46) and virulence (TABLE 47) of the virulent strain (isolate M) were not affected by all the treatments, while those of the weakly virulent strain (isolate I) were similarly adversely affected only by continuous refrigeration at $3^{o} - 6^{o}c$ (PLATE 17). The overall results suggest that:

1. *Xanthomonas oryzae* cultures grown on solid agar media (Wakimoto's potato semisynthetic agar) (TABLE 3) needs be regularly sub-cultured either at monthly, fortnightly, weekly or every 3-4 days, irrespective of the number of sub-culturings done, and stored at $3^{o} - 6^{o}c$ 2 days after each sub-culturing, in order to maintain their growth and virulence.

2. Storage at $-10^{o}c$ could maintain their growth characteristics and virulence irrespective of strain virulence, for longer periods.

6.2. Cultural Differentiation and its Effects on Virulence

6.2.1. Materials and Methods

Experiment 1

The study used the strain with the widest spectrum of virulence (isolate M) in a 7-fold dilution series. 0.1 ml of the 7th dilution of standard bacterial suspension (refer Section 1.1) was plated out on to the MWPSA plates (refer TABLE 3 in Section 1.1) for good single colony growths and incubated for 3 days at $26^{o} \pm 2^{o}c$. The 2 main forms of colonies which grew were described and sub-cultured by the zig-zag streaking method on to dry WPSA slants for copious surface growths (refer TABLE 3 in Section 1.1) and incubated for 3 days. Standard bacterial suspension prepared from each type of cultures was inoculated on 8 first fully opened top leaves of 27-day old seedlings of a susceptible cultivar T(N)1 by means of the pin prick method (refer Section 4.1.1).

TABLE 45. VISUAL GROWTH ASSESSMENTS[1] OF A WEAKLY VIRULENT (ISOLATE I) AND A VIRULENT (ISOLATE M) STRAIN OF *XANTHOMONAS ORYZAE* UNDER DIFFERENT STORAGE AND MAINTENANCE CONDITIONS

Isolate \ Treatment[2]	$E_1T_1^1$	$E_1T_1^2$	$E_1T_1^3$	$E_1T_1^4$	$E_2T_1^1$	$E_2T_1^2$	$E_2T_1^3$	$E_2T_1^4$	$E_3O_1^1$	$E_3O_1^1$
I	1	1	1	1	1	1	1	1	4	1
M	1	1	1	1	1	1	1	1	1	1

1 : Mean of 3 replicates based on a 1-4 scale of decreasing growth (1 = Good growth, 4 = very poor or negligible growth).

2 : See TABLE 42.

TABLE 46. OPTICAL DENSITY MEASUREMENTS[1] OF A WEAKLY VIRULENT (ISOLATE I) AND A VIRULENT (ISOLATE M) STRAIN OF *XANTHOMONAS ORYZAE* UNDER DIFFERENT STORAGE AND MAINTENANCE CONDITIONS

Isolate \ Treatment[2]	$E_1T_1^1$	$E_1T_1^2$	$E_1T_1^3$	$E_1T_1^4$	$E_2T_1^1$	$E_2T_1^2$	$E_2T_1^3$	$E_2T_1^4$	$E_3O_1^1$	$E_3O_1^2$
I	1.9	1.9	1.9	2.0	1.8	1.8	1.9	2.0	0.1	1.9
M	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	1.9	2.0

1 : Mean of 3 replicates, measured by a Beckman spectrophotometer (at 620 nm) with an absorbance scale of 0 - 2.0 (maximum turbidity).

2 : See TABLE 42.

TABLE 47. DISEASE SCORES[1] OF A WEAKLY VIRULENT (ISOLATE I) AND A VIRULENT (ISOLATE M) STRAINS OF *XANTHOMONAS ORYZAE* UNDER DIFFERENT STORAGE AND MAINTENANCE CONDITIONS ON SUSCEPTIBLE RICE CULTIVAR T(N)1

Isolate \ Treatment[2]	$E_1T_1^1$	$E_1T_1^2$	$E_1T_1^3$	$E_1T_1^4$	$E_2T_1^1$	$E_2T_1^2$	$E_2T_1^3$	$E_2T_1^4$	$E_3O_1^1$	$E_3O_1^2$
I	3.4	3.3	3.1	3.1	3.0	3.0	3.0	3.0	0	2.6
M	4.8	4.5	4.3	4.1	4.1	4.1	4.1	4.0	4.3	4.4

1 : Mean of 8 replicates, based on a 0-5 scale of increasing disease (see TABLE 5).

2 : See TABLE 42.

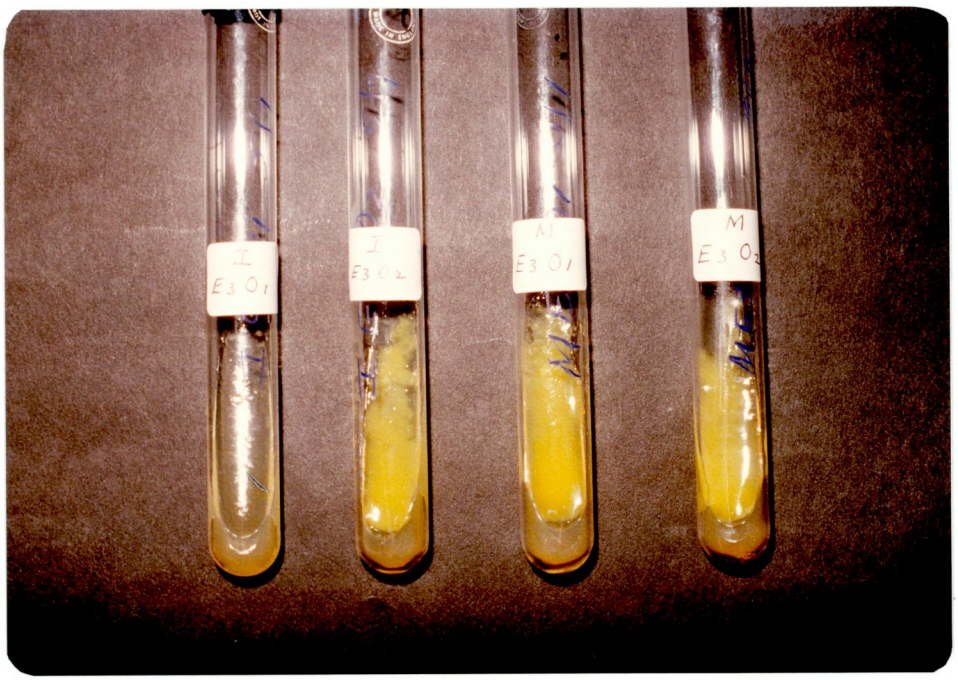


PLATE 17. Cultural growth of a weakly virulent strain (isolate I) and a virulent strain (isolate M) of *Xanthomonas oryzae* which had been subjected to continuous refrigeration (E_3O_1) for 3 months and continuous storage at - 10°c (E_3O_2) for 9 months, showing the detrimental effect of the former only on weakly virulent strains, with the latter able to maintain growths of both strains.

Experiment 2

The plating from original culture was repeated for more detailed study in which colonies of 3 different sizes were cultured to test their virulence after 4 days incubation at 26 ± 2°c. Twelve colonies of each type were randomly picked, cultured and incubated for 48 hours before being used to inoculate the flag leaves of a susceptible cultivar T(N)1. Five flag leaves were inoculated with standard suspension of each colony by means of the pin prick method (refer Section 4.1.1). The experiment was repeated using standard suspensions of cultures sub-cultured from those grown from single colonies.

Pure cultures from single colonies of the different types were plated out separately after 5 weeks storage at 3-6°c and observed for colony variations within each colony type after 4 days incubation. Colonies which survived were counted and their diameters measured with the help of a colony counter. Standard suspensions prepared from cultures of 3 colonies of each type which survived were again tested for virulence where the pin prick method of inoculation was used to inoculate 8 first fully opened top leaves of 27-day old seedlings of susceptible cultivar T(N)1.

Disease assessments for the flag leaves and seedlings were made 20 and 14 days after inoculation respectively based on a 0-5 disease scale given in TABLE 5.

6.2.2. Results

Bacterial colonies which grew after 3 days incubation in Experiment 1, were composed of 2 distinct main forms (TABLE 48), the smaller yellow colonies the avirulent form while the larger yellow colonies (with or without paler halo around the periphery) being the virulent form (PLATE 18). The only other colony difference between the avirulent and virulent forms (whose mean diameters were < 0.5 mm and > 0.5 mm respectively after 3 days incubation) was that after 5 days

TABLE 48. COLONY CHARACTERISTICS OF THE VIRULENT AND AVIRULENT FORMS OF A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* AFTER 3-5 DAYS OF INCUBATION ON MWPSA[1] PLATE

Character	Avirulent form	Virulent form
1. Shape	Round	Round
2. Diameter	$\underline{c}$ 0.5 mm^2 $\underline{c}$ 1-2 mm^3	$\underline{c}$ 0.5 mm or more^2 $\underline{c}$ 3-4 mm^3
3. Margin	Regular	Regular
4. Surface	Convex, glossy, shiny	Convex, glossy, shiny
5. Colour	Yellow	Pale yellow with or without paler halo around periphery

1 : MWPSA = Modified Wakimoto's (1960) potato semisynthetic agar (see TABLE 3).

2 : After 3 days of incubation at 26° ± 2°c.

3 : After 5 days of incubation at 26° ± 2°c.

TABLE 49. CULTURAL CHARACTERISTICS OF THE VIRULENT AND AVIRULENT FORMS OF A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* AFTER 2 DAYS AFTER INCUBATION ON WPSA[1] SLANTS AND THEIR VIRULENCE ON A SUSCEPTIBLE RICE CULTIVAR T(N)1

Character	Avirulent form	Virulent form
1. Growth	Slow, definite	Profused, spreading
2. Colour	Yellow	Pale yellow
3. Optical density[2]	2.0 ± 0 (max)	2.0 ± 0 (max)
4. Disease score[3]	2.0 ± 0.4	4.2 ± 0.1
5. Disease Reaction[4]	Resistant	Susceptible

1 : WPSA = Wakimoto's (1960) potato semisynthetic agar (see TABLE 3).

2 : Mean of 3 replicates given by a Beckman spectrophotometer (absorbance at 620 nm).

3 : Mean of 8 based on a 0-5 scale of increasing disease (see TABLE 5).

4 : Based on the mean disease scores (see TABLE 5).



PLATE 18. Two distinct forms of colony of a virulent strain of *Xanthomonas oryzae*, the smaller yellow ones being the avirulent form while the larger pale yellow ones being the virulent form. The virulent colonies had coalesced after 5 days of incubation.

incubation, colonies of both forms had grown but the avirulent form grew more slowly to a mean diameter of c 1-2 mm, whereas the virulent form grew faster to a mean diameter of c 3-4 mm.

When the 2 forms of colony were cultured on WPSA slants and after 3 days incubation, the colour difference was apparent again with both forms having similar maximum optical densities (absorbance) of 2.0 (TABLE 49). Their virulence tests (TABLE 49) confirmed that the yellow, smaller colonies were the avirulent form as it produced a resistant disease reaction (mean disease score of 2.0), while the pale yellow, larger colonies were the virulent form producing a susceptible disease reaction (mean disease score of 4.2) on a susceptible cultivar.

Results from Experiment 2 revealed that 3 different colony types could in fact be isolated, based largely on differences in size. On the 4th day of incubation most colonies fell within the diameter ranges of 0.5 - 1 mm (small colonies, T_1) 1 - 2 mm (medium-sized colonies, T_2) and 2.5 - 4 mm (large colonies, T_3). The small colonies appeared slightly more yellowish than the medium-sized and large colonies. All 12 colonies of each colony type gave fairly constant disease scores with minimal standard deviations (TABLE 50). The small colonies appeared to be the avirulent type giving a mean disease score of 2.4 and a resistant disease reaction whereas the medium-sized and the large colonies appeared to be the virulent type as they produced constantly similar high disease scores averaging at 4.6 and 4.7 respectively giving a susceptible disease reaction (TABLE 50).

However re-plating of the 3 colony types after being stored for 5 weeks at 3-6^{o}c showed that the bacterium of the avirulent small colonies was not viable whereas that of the virulent

TABLE 50. DISEASE SCORES[1] OF 12 COLONIES OF EACH OF THE 3 TYPES OF COLONY OF *XANTHOMONAS ORYZAE* ON FLAG LEAVES OF A SUSCEPTIBLE RICE CULTIVAR T(N)1

Colony no.	Colony type[2]		
	T_1	T_2	T_3
1	2.6 ± 0.5	3.6 ± 0.5	4.4 ± 0.5
2	1.4 ± 0.5	4.4 ± 0.5	4.4 ± 0.5
3	2.6 ± 0.5	5.0 ± 0	5.0 ± 0
4	2.4 ± 0.5	5.0 ± 0	4.8 ± 0.4
5	2.2 ± 0.4	4.6 ± 0.5	4.4 ± 0.5
6	2.4 ± 0.5	4.4 ± 0.5	5.0 ± 0
7	2.4 ± 0.5	4.6 ± 0.5	4.8 ± 0.4
8	2.6 ± 0.5	4.4 ± 0.5	4.8 ± 0.4
9	2.6 ± 0.5	4.8 ± 0.5	5.0 ± 0
10	2.4 ± 0.5	4.8 ± 0.4	4.8 ± 0.4
11	2.4 ± 0.5	4.8 ± 0.4	4.8 ± 0.4
12	2.2 ± 0.4	4.4 ± 0.5	4.6 ± 0.5
Mean	2.4 ± 0.3	4.6 ± 0.4	4.7 ± 0.2
Disease reaction[3]	R	S	S

1 : Mean of 5 based on a 0-5 scale of increasing disease (see TABLE 5), 20 days after inoculation.

2 : T_1 = Small colonies mainly within the diameter range of 0.5-1 mm.
T_2 = Medium-sized colonies mainly within the diameter range of 1-2 mm.
T_3 = Large colonies mainly within the diameter range of 2.5-4 mm.
Colony sizes were assessed after 4 days incubation.

3 : R = Resistant, S = Susceptible (see TABLE 5).

medium-sized and large colonies was (TABLE 51). Colony counts and diameter measurements of the virulent medium-sized and large colonies show that the virulent large colonies produced more than twice the number of colonies than that of the virulent medium-sized colonies which was significant at the 1% level and their mean colony sizes nearly twice as large as that of the virulent medium-sized colonies (TABLE 51). Their virulence however was the same as before, producing a susceptible disease reaction (TABLES 51 & 52). Colonies were uniform in size with about 85% of the medium-sized colonies falling within the 1-2.4 mm diameter range and also about 85% of the large colonies falling within the 2.4-4 mm diameter range (TABLE 52). Most of the 15% of the colonies of both types were of unusually smaller sizes.

6.3. Discussion

Although no obvious effects were observed on the growth and virulence of virulent strains after continuous storage at 3^{o} - 6^{o}c for 3 months, it was possible, even when regularly sub-cultured to maintain their growth and virulence that their virulence, as shown in Section 4 (refer Section 4.7, last paragraph), could be adversely affected. The fact that this did not happen in this study probably shows that the period involved (3 months) was not sufficiently long to cause detrimental effects on virulent strains. However as pointed out by FUJII (1976), virulent strains when sub-cultured regularly tend to give rise to its avirulent mutants which increase in population gradually during successive sub-culturings on artificial media. And indeed in more detailed studies 3 types of colony of definite sizes were identified. The medium-sized and large colonies with diameters varying from 1 - 4 mm on the 4th day of incubation, were shown to be the virulent forms and the small colonies with diameters of 1 mm or less, the avirulent forms confirming results of preliminary investigations in Experiment 1. Re-plating from their pure cultures showed that while about 85% of the colonies of only the medium-sized and large colonies were

TABLE 51. NUMBERS AND SIZES[1] OF COLONIES RE-PLATED FROM PURE CULTURES OF THE SMALL (T_1), MEDIUM-SIZED (T_2) AND LARGE (T_3) COLONIES[2] OF *XANTHOMONAS ORYZAE* AFTER BEING STORED AT 3° - 6°c FOR 5 WEEKS AND RESULTS OF CHI-SQUARE TESTS.

Plate No.	T_1		T_2		T_3		X^2 test for difference in colony no. between T_2 & T_3
	No.	Diameter	No.	Diameter	No.	Diameter	
1	0	-	$7x10^8$	1.2±0.3	$29x10^8$	2.9±0.5	13.44 **
2	0	-	$10x10^8$	2.1±0.4	$36x10^8$	3.2±0.5	14.70 **
3	0	-	$16x10^8$	1.8±0.6	$34x10^8$	2.6±0.8	6.48 *
4	0	-	$20x10^8$	1.6±0.4	$31x10^8$	3.2±0.7	2.38 ns
5	0	-	$17x10^8$	1.5±0.4	$40x10^8$	3.6±0.6	9.28 **
6	0	-	$16x10^8$	1.9±0.6	$31x10^8$	3.5±0.8	4.78 *
7	0	-	$18x10^8$	1.9±0.5	$27x10^8$	3.6±0.6	1.80 ns
8	0	-	$15x10^8$	2.3±0.5	$46x10^8$	3.0±0.9	15.76 **
Mean	0	-	$15±4x10^8$	1.8±0.3	$34±6x10^8$	3.2±0.3	
Overall X^2 test							68.62 **

1 : Diameters of all colonies were measured in mm with the help of a colony counter, 4 days after incubation.

2 : See TABLE 50.

TABLE 52. PERCENTAGE OF MEDIUM-SIZED AND LARGE COLONIES RE-PLATED FROM PURE CULTURES OF THE MEDIUM-SIZED (T_2) AND LARGE (T_3) COLONIES[1] OF *XANTHOMONAS ORYZAE* AFTER BEING STORED AT 3° - 6°c FOR 5 WEEKS AND THEIR VIRULENCE ON SEEDLINGS OF A SUSCEPTIBLE RICE CULTIVAR T(N)1.

Plate/Leaf no.	T_2				T_3			
	% colony no. (1 - 2.4 mm diameter)	Disease score[2]			% colony no. (2.5 - 4 mm diameter)	Disease score[2]		
		Colony no.				Colony no.		
		1	2	3		1	2	3
1	86	4	4	5	86	4	4	5
2	80	4	4	5	92	4	4	5
3	88	4	4	5	65	5	4	5
4	90	4	5	5	81	4	5	5
5	88	5	5	5	85	4	5	5
6	81	5	5	5	84	4	5	5
7	89	4	5	5	96	5	4	5
8	67	4	5	5	80	4	5	4
Mean	84 ± 7	4.3±0.4	4.6±0.5	5.0±0	84 ± 9	4.3±0.4	4.5±0.5	4.8±0.4
Disease reaction[3]		S	S	S		S	S	S

[1] : See TABLE 51.

[2] : Based on a 0-5 scale of increasing disease (see TABLE 5), 14 days after inoculation.

[3] : S = Susceptible (see TABLE 5).

recovered, most of the 15% of the remaining colonies of each type were small colonies. However, as the avirulent small colonies also tended to lose their viability completely and the fact that pure cultures from colonies of virulent medium-sized and large colonies gave rise to about 15% small colonies after a period of storage, confirmed that pure cultures tended to differentiate into avirulent mutants which could undermine its virulence.

As the avirulent mutants gave very little disease and its proportions in the original virulent cultures undermines its original virulence (FUJII, 1976), it is logical to expect that virulence of a virulent strain, which has been sub-cultured successively over a period of time, would decrease proportionately. It seems therefore that storage of original cultures for short or long periods at $-10^{o}c$ would be best. The next best method would be to sub-culture at monthly, fortnightly or weekly intervals, with storage after growth at $3^{o} - 6^{o}c$.

The presence of virulent and avirulent mutants and their proportions within an inoculum, therefore has direct bearing to the ultimate amount of disease produced in the plant, which could be explained by two schools of thought. One is the phenomenon of induced - resistance or cross protection (WOOD, 1978), where the presence of equally large population of avirulent mutants causes the host cells to become resistant to the virulent form. The second, is an equally convincing explanation (FUJII, 1976) that the presence of an equal amount of avirulent population not only inhibits the multiplication of the virulent form but multiplies even faster than without the virulent form. This has been shown experimentally in pure liquid cultures as well as in rice plants where the amount of disease that could be produced by the virulent form was shown to be reduced drastically in proportion to the ratio of the dilution.

7. SOME ASPECTS OF CULTURAL CONTROL OF *XANTHOMONAS ORYZAE*

7.1. Seed Transmission

7.1.1. Materials and Methods

The experiment was conducted in circular pots (33 x 25 cm) in randomised complete block design where the treatments were replicated 3 times. Each treatment of each of 3 replicates consisted of 10 pots. The 3 treatments were as follows:

T_1 = Seeds from plants inoculated at early milk ripe stage by dipping the panicles in standard bacterial suspension for 1 minute (refer Section 1.1).

T_2 = Seeds from plants inoculated at the booting stage by the scissors (shearing = clip-inoculation) method (refer Section 3.1.1).

T_3 = Seeds from un-inoculated (disease-free) plants (control).

Seeds from susceptible cultivar T(N)1, collected from the 3 treatments were sowed in similar pots containing typical rice soil and the seedlings were maintained for 25 days and fertilized with 0.5 kg 'amophos' fertilizer (11N : 48p) per 23 sq.m. as basal dressing and at second week of growth. The seedlings were transplanted into similar pots after 25 days at 2 seedlings per pot. The plants were then fertilized with 150 Kg N/ha (urea), 50 Kg P_2O_5/ha (double superphosphate) and 37 KgK_2O/ha (muriate of potash). Urea was applied in 3 equally split applications basal dressing, first top dressing at active tillering stage and second top dressing at the young ear formation stage, while the double superphosphate and the muriate of potash were applied only as basal dressings. The experiment was conducted in an insect-proof green-house.

The effects of the 3 treatments on plant growth were studied by taking the percentage germination at 15 days after sowing, seedling height at 15, 22 and 29 days after sowing, tiller count at 47 and 59 days after sowing and panicle count at milk ripe stage and plant height at 47, 59 and 81 days after sowing.

The plants were also observed for Kresek symptoms at 22 and 29 days after sowing. Effects on yields were studied by measuring panicle number, panicle weight, weight of grains/hill, 1000 grain weight, % filled grain weight and total yield based on five-hill samples.

Throughout the experiment the plants were recorded for bacterial blight symptoms. Seeds from these treatments were also tested in the laboratory for the pathogen using a modification of the improved method for detecting *Xanthomonas oryzae* in rice seeds as proposed by HSIEH *et al.*, (1974). The method basically involved touching the sterilised seed abscission surfaces on WPSA plates (refer Section 1) for 3-5 seconds and then observing for bacterial growths after 4 days incubation.

7.1.2. Results

(i) Laboratory tests

The tests for the presence of *Xanthomonas oryzae* pathogen in infected seeds and seeds from infected plants gave negative results. The many yellow bacterials colonies isolated from all treatments including the control were found to be saprophytes.

(ii) Germination percentage 15 days after sowing (TABLE 53)

The percentage germination at 15 days after sowing (DAS) was adversely affected by bacterial infection of developing seeds through direct contact (T_1) and the plants (T_2). Percentages of germination of T_1 (63%) and T_2 (70%) were not significantly different (at the 5% level) but both were significantly lower (at the 1% level) than that of the control (93%). The results indicate that rice seeds could be infected with *Xanthomonas oryzae* either through direct contact as in T_1 or through severely infected plants as in T_2.

TABLE 53. MEAN PERCENTAGE SEED GERMINATION AT 15 DAS[1], SEEDLING HEIGHT AT 15, 22 AND 29 DAS AND PLANT HEIGHT AT 47, 59 AND 81 DAS WITH RESULTS OF F-TEST AND LEAST SIGNIFICANT DIFFERENCE (LSD) TEST

Treatment[2]	% germination	Seedling height (cm)			Plant height (cm)		
		15 DAS	22 DAS	29 DAS	47 DAS	59 DAS	81 DAS
T_1	62.7	11.9	22.6	28.6	54.8	72.5	81.9
T_2	70.0	21.5	34.0	38.6	58.8	76.9	85.2
T_3 (control)	92.7	25.2	36.6	42.5	62.6	80.4	91.3
Grand mean	75.1	19.5	31.1	36.5	58.7	76.6	86.2
SD/obs[3]	3.9	1.1	1.8	3.4	1.7	1.2	3.1
CV[4](%)	5.2	5.6	5.7	9.2	2.9	1.6	3.6
F-test[5]							
Replicate	2.7 NS	4.1 NS	2.9 NS	0.3 NS	1.2 NS	3.7 NS	0.5 NS
Treatment	48.6 **	117.4 **	53.8 **	13.9 **	15.7 **	30.6 **	7.1 **
LSD 5%	8.8	2.5	4.0	7.6	3.9	2.8	7.0
LSD 1%	14.6	4.1	6.8	12.8	6.4	4.5	11.6
LSD test[6]	T_1a T_2a T_3 b	T_1a T_2 b T_3 c	T_1a T_2 b T_3 b	T_1a T_2 b T_3 b	T_1a T_2 b T_3 b	T_1a T_2 b T_3 c	T_1a T_2ab T_3 b

1 : DAS = Days after sowing

2 : T_1 = Seeds from plants inoculated at milk ripe stage by direct soaking of panicles in bacterial suspension. T_2 = Seeds from plants inoculated at booting stage by the scissors method. T_3 = Seeds from disease-free plants (control).

3 : SD/obs = Standard deviation per observation. 4 : CV = Coefficient of variation. 5 : NS = Not significant at the 5% level. ** = Significant at the 1% level. 6 : Treatments with common letters are not significant at the 5% level. Those with different letters are significant either at the 5% or 1% levels.

(iii) Percentage Kresek at 22 and 29 days after sowing

Kresek (seedling death) was observed in T_1 at 22 days after sowing and was of the order of 3.3%. At 29 days after sowing, however, Kresek rose to 4.4%. Treatments T_2 and T_3 had no Kresek. The results show that through direct contact of bacteria with developing seeds, the pathogen could be transmitted to cause Kresek. However, even in this case, the percentage Kresek was very low indeed, suggesting the relatively unimportant role of seed contamination as a source of transmission of the pathogen.

(iv) Seedling height measured at 15, 22 and 29 days after sowing (TABLE 53)

The height of seedlings increased progressively from 15, 22 up to 29 DAS for all the 3 treatments. At each of these stages of growth the general trend was that the T_1 seedlings were significantly shorter than those of T_2 and T_3. Although the T_2 seedlings were significantly shorter than those of T_3 initially (15 DAS), their differences were not significant at later stages of growth (22 & 29 DAS). The results therefore indicate that direct soaking of developing grains in bacterial suspension could decrease significantly seedling height.

(v) Plant height measured at 47, 59 and 81 DAS (TABLE 53)

Plant height measured from the soil surface up to the tallest leaf tips was affected significantly by the 2 treatments. Although plant height increased progressively from 47, 59 and 81 DAS the significant differences among the 3 treatments were maintained throughout. Plants of T_1 were the shortest (82 cm) of the 3 treatments but was not significantly different from that of T_2 (85 cm) which was significantly shorter than that of the control, T_3 (91 cm). Here again direct soaking of developing grains in bacterial suspension affected the grain such that plants grown from such seeds were significantly stunted.

(vi) Tiller count at 47 and 59 days after sowing (TABLE 54)
The results in TABLE 54 show that differences between the treatments were not significant (at the 5% level).

(vii) Panicle count at milk ripe stage (TABLE 54)
Differences in the number of panicles per hill between the treatments were not significant (at the 5% level).

(viii) Effects on yield components and total yield of rice (TABLE 55)
Among the yield components and total yield studied, only the % filled grain and total yield were affected significantly (at nearly the 5% & the 5% levels respectively) by seed and plant infections (T_1 & T_2 respectively) (TABLE 55), with T_1 having a greater adverse effects. The % filled grain (68%) and the total yield (66g) of T_1 were significantly lower (at the 5% level) than those of the controls, T_3 being 75% and 109g respecitvely. Those of T_1 and T_2 were not significantly different (at the 5% level). This indicates again that direct contact between developing grains and the bacterium could impair plant growth such that even the yield of rice was significantly reduced.

(ix) Disease assessment
Despite significant reductions in % germination, seedling height, plant height, % filled grain and the total yield, brought about by direct seed infection, no disease symptom was observed in plants of any of the treatments. Hence disease assessment was not possible.

7.2. Seed Sources and Transplanting Practises as Means of Disease Transmission and Dissemination in the Field

7.2.1. Materials and Methods

The experiments were conducted in rice fields in split-plot design with 3 replicates. 25-day seedlings were transplanted at a planting distance of 25 x 25 cm into plots of 5 x 2 hills (10 hills per treatment). Fertilizer rates, methods of application and transplanting were similar to those in previous experiment (Section 7.1.1). The 7 sub-plot treatments were as follows:-

TABLE 54. MEAN TILLER NUMBER AT 47 DAS[1] AND 59 DAS AND PANICLE NUMBER AT MILK RIPE STAGE WITH RESULTS OF F-TEST

Treatment[2]	Tiller number (per hill)		Panicle number (per hill)
	47 DAS	59 DAS	
T_1	10.9	17.6	18.2
T_2	11.3	17.6	19.0
T_3(control)	12.9	18.2	20.1
Grand mean	11.7	17.8	19.1
SD/obs[3]	1.7	1.3	1.7
CV[4](%)	14.4	7.1	9.0
F-test[5]			
Replicate	0.0 NS	0.2 NS	2.6 NS
Treatment	1.1 NS	0.3 NS	0.9 NS

1 : Days after sowing

2 : T_1 = Seeds from plants inoculated at milk ripe stage by direct soaking of panicles in bacterial suspension. T_2 = Seeds from plants inoculated at booting stage by the scissors method. T_3 = Seeds from disease-free plants (control).

3 : SD/obs = Standard deviation per observation.

4 : CV = Coefficient of variation.

5 : NS = Not significant at the 5% level.

TABLE 55. MEAN VALUES OF YIELD COMPONENTS AND TOTAL YIELD OF RICE WITH RESULTS OF F-TEST AND LEAST SIGNIFICANT DIFFERENCE (LSD) TEST

Treatment[2]	Yield components and total yield							
	(i)	(ii)	(iii)	(iv)	(v)	(vi)[3]	(vii)	(viii)
T_1	17.6	21.0	1.1	19.3	16.6	67.5	22.3	65.9
T_2	15.1	17.9	1.2	16.4	14.8	71.6	20.6	82.6
T_3	18.1	27.2	1.5	25.3	23.6	75.2	26.1	108.7
Grand mean	17.1	22.0	1.3	20.4	18.4	71.4	23.0	85.7
SD/obs[4]	2.9	4.5	0.2	4.2	5.2	2.6	2.5	13.6
CV[5](%)	17.0	20.4	18.0	20.8	28.0	3.6	10.9	15.9
F-test[6]								
Replicate	0.7NS	0.8NS	0.0NS	0.8NS	0.6NS	1.2NS	0.1NS	0.9NS
Treatment	1.2NS	3.3NS	1.8NS	3.4NS	2.4NS	6.7†	3.8NS	7.6*
LSD 5%	-	-	-	-	-	5.9	-	30.8
LSD 1%	-	-	-	-	-	9.8	-	51.1
LSD test[7]						T_1 a T_2 ab T_3 b		T_1 a T_2 ab T_3 b

[1] : (iv) = Panicle no./hill.
(ii) = Total panicle wt./hill (g).
(iii) = Mean wt./panicle (g).
(iv) = Wt. of grains/hill (g).
(v) = Wt. of filled grains/hill (g).
(vi) = % filled grain.
(vii) = Mean grain wt. (mg).
(viii) = Total rice yield of 5 hills (g).

[2] : T_1 = Seeds from plants inoculated at milk ripe stage by direct soaking of panicles in bacterial suspension.
T_2 = Seeds from plants inoculated at booting stage by the scissors method.
T_3 = Seeds from disease-free plants (control).

[3] : Arcsin transformed data ($\sin^{-1}\sqrt{\%}$)

[4] : SD/obs = Standard deviation per observation.

[5] : CV = Coefficient of variation.

[6] : NS = Not significant at the 5% level. * Significant at the 5% level.
† = Significant at nearly 5% level.

[7] : Treatments with common letters are significant at the 5% level. Those with different letters are significant either at the 5% or 1% level.

T_1 = Seedlings of susceptible cultivar T(N)1, from area (Permatang Rambai), where disease is endemic.

T_2 = Seedlings of susceptible cultivar T(N)1, from disease-free area (Bumbong Lima).

T_3 = Seedlings of susceptible cultivar T(N)1, from seeds soaked in standard bacterial suspension (refer Section 1.1) for one minute at milk ripe stage (seeds sown in pots).

T_4 = Seedlings of susceptible cultivar T(N)1, from seeds collected from plants artificially inoculated with standard bacterial suspension (refer Section 1.1) at booting stage by the scissors method (refer Section 3.1.1) (seeds sown in pots).

T_5 = Seedlings of susceptible cultivar T(N)1, from seeds collected from disease-free plants.

T_6 = Seedlings from seeds collected from severely (naturally) infected advanced genetic line (Mas x IR8) x (BPI-76 x DGWG) - RU210-36-3-7-3).

T_7 = Seedlings from seeds collected from another severely (naturally) infected advanced genetic line (Mayang Rambai x Ria - RU111-57-1-3-5).

The 2 main treatments applied on these 7 sub-plot treatments were as follows:-

MT_1 = Seedlings transplanted with leaf tips uncut.

MT_2 = Seedlings transplanted with leaf tips cut.

The experiments were carried out in 2 locations as follows:-

(i) Permatang Rambai, where disease is endemic.

(ii) Bumbong Lima, a disease-free area.

The disease was scored at milk ripe stage by assessing the disease incidence and the disease intensity index. The disease incidence was obtained by assessing the percentage infected plants out of the total number of plants per sub-treatment. The disease intensity indices were obtained using the formula as follows:-

$$\text{Disease intensity index (DII)} = \frac{\text{Mean disease score x\% Disease Severity}}{100}$$

(HO, 1976, 1978).

Disease incidence was assessed on all the 10 hills per sub-treatment. A total of 20 tillers were randomly picked (2 tillers/hill) for assessing % disease severity using FIG. 8. The same tillers were assessed for the mean disease score by averaging the scores of all the five leaves per tiller obtained using the 0-9 disease scale (HO & LIM, 1977a) in TABLE 56.

7.2.2. Results

The percentage disease incidence (TABLE 57) and the disease intensity indices (TABLE 58) in the disease endemic area (Permatang Rambai) both indicate that cutting of seedling leaf tips before transplanting gave a significant effect (at the 10% level) it shows (TABLES 57 & 58) that the various possible seed sources of disease transmission investigated (sub-treatments) reacted differently under each of the leaf tip cutting practices.

Within the "leaf tip uncut" main treatment (MT_1), although the disease did appear on plants grown from seeds which had been artificially inoculated by contact (T_3) or through severe artificially infected plants (T_4) or from plants which were severely and naturally infected (T_6 & T_7), disease for the latter (T_6 & T_7) was significantly more severe (at the 5% level) than that for the former (T_3 & T_4). This indicates that seeds from severely and naturally infected plants could be a potential source of inoculum in the field. This was possibly due to the naturally occurring wild types of the pathogen being more virulent than the one from artificially inoculated seeds and plants. Within the "leaf tips cut" main treatment (MT_2) plants of all treatments were infected to varying degrees (TABLES 57 & 58), indicating that in a disease endemic area, cultural practices such as cutting of seedling leaf tips before transplanting is of primary importance in transmitting the pathogen. It is interesting to note that seedlings from disease endemic area (T_1) and seedlings from severely and naturally infected plants (T_6 & T_7) were among the more severely infected. The results on disease intensity indices (TABLE 58) confirm similar trends.



No. of leaves infected/tiller	0	1	2	3	4	5
Average percentage of leaf or leaf area infected	0	20	35	55	75	90
Percentage range of no. of leaves or leaf area infected	0	20	30-40	50-60	70-8)	81-100
Severity	No infection	Very light	Light	Moderately severe	Severe	Very severe

Procedure: Select random sample of tillers Growth stage: Mile ripe-dough ripe stage Assessment: Assess whole tiller

FIG.8. Disease severity assessment key for bacterial blight of rice (CHIN & HO, 1973)

TABLE 56. DISEASE SCALE FOR ASSESSING DISEASE REACTIONS OF MATURE RICE CULTIVARS TO BACTERIAL BLIGHT DISEASE (HO & LIM, 1977a)

Scale (disease score)	Description of attack
0	No lesion observed
1	Trace lesion on leaf blade
2	Less than quarter of leaf blade destroyed
3	Between quarter and half of leaf blade destroyed
4	Half of leaf blade destroyed
5	Between half and three-quarter of leaf blade destroyed
6	Three-quarter of leaf blade destroyed
7	From three-quarter of leaf to entire leaf blade destroyed
8	Entire leaf blade plus up to half of leaf sheath destroyed
9	Entire leaf blade plus more than half leaf sheath destroyed

TABLE 57. MEAN PERCENTAGE DISEASE INCIDENCES[1] FOR THE EFFECTS OF CULTURAL PRACTICES ON THE TRANSMISSION OF BACTERIAL BLIGHT DISEASE IN THE FIELD IN DISEASE ENDEMIC AREA (PERMATANG RAMBAI) AND RESULTS OF STATISTICAL ANALYSES.

Main treatment[3]	Sub-treatment[2]							Main treatment mean
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	
MT_1 (Leaf tips uncut)	0	0	8.67	6.00	0	42.67	38.33	13.6
MT_2 (Leaf tips cut)	38.33	32.67	18.00	6.00	23.33	32.67	35.00	26.57
Sub-treatment mean	19.17	16.33	13.33	6.00	11.67	37.67	36.67	
LSD test[4]								
MT_1	T_1 0	T_2 0	T_5 0	T_4 6.00	T_3 8.67	T_7 38.33	T_6 42.67	
MT_2	T_4 6.00	T_3 18.00	T_5 23.33	T_2 32.67	T_6 32.67	T_7 35.00	T_1 38.33	

Standard deviation per observation (main treatment) = 7.79
Standard deviation per observation (sub-treatment) = 8.11

LSD 5% for main treatment mean difference = 15.53
LSD 1% for main treatment mean difference = 24.68
LSD 5% for (sub-treatment) mean difference = 13.64
LSD 1% for (sub-treatment) mean difference = 18.54

[1] : Mean of 30 plants from 3 replicates.

[2] : T_1 = From disease endemic area. T_2 = From disease-free area. T_3 = From seeds soaked in inoculum. T_4 = From seeds of artificially infected plants. T_5 = From seeds of disease-free plants. T_6 & T_7 = From seeds of 2 severely and naturally infected advanced genetic lines.

[3] : MT_1 differs from MT_2 at the 10% level. Interaction between the main and sub-treatments is significant at the 1% level.

[4] : LSD = Least significant difference test. Means underscored are not significantly different at the 5% level. Results were analysed by the parametric F-test based on arcsin transformed data, and the LSD test.

TABLE 58. MEAN DISEASE INTENSITY INDICES[1] FOR THE EFFECTS OF CULTURAL PRACTICES ON THE TRANSMISSION OF BACTERIAL BLIGHT DISEASE IN THE FIELD IN DISEASE ENDEMIC AREA (PERMATANG RAMBAI) AND RESULTS OF STATISTICAL ANALYSES

Main treatment[3]	Sub-treatment[2]							Main treatment mean
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	
MT_1 (Leaf tips uncut)	0	0	1.47	0.20	0	3.53	4.03	1.32
MT_2 (Leaf tips cut)	4.27	2.10	0.80	0.27	0.97	2.47	2.60	1.92
Sub-treatment mean	2.13	1.05	1.13	0.23	0.48	3.00	3.32	
LSD test[4]								
MT_1	T_1 0	T_2 0	T_5 0	T_4 0.20	T_3 1.47	T_7 3.53	T_1 4.03	
MT_2	T_4 0.27	T_3 0.80	T_5 0.97	T_2 2.10	T_6 2.47	T_7 2.60	T_1 4.27	

Standard deviation per observation (main treatment) = 2.03
Standard deviation per observation (sub-treatment) = 1.02

LSD 5% for main treatment mean difference = 2.94
LSD 1% for main treatment mean difference = 5.60
LSD 5% for sub-treatment mean difference = 1.72
LSD 1% for sub-treatment mean difference = 2.34

1 : Mean of 60 tillers from 3 replicates.

2 : T_1 = From disease endemic area. T_2 = From disease-free area. T_3 = From seeds soaked in inoculum. T_4 = From seeds of artificially infected plants. T_5 = From seeds of disease-free plants. T_6 & T_7 = From seeds of 2 naturally infected (very severe) advanced genetic lines.

3 : MT_1 & MT_2 are significantly different at the 10% level. Interaction between the main and sub-treatments is significant at the 1% level.

4 : LSD = Least significant difference test. Means underscored are not significantly different at the 5% level. Results were analysed by the parametric F-test based on arcsin transformed data, and the LST test.

In disease-free area however, cutting of leaf tips prior to transplanting (MT_2) was not important as a means of transmitting the pathogen, as its effects measured by percentage disease incidence (TABLE 59) and the disease intensity indices (TABLE 60), were not significantly different (at the 5% level) from the "leaf tips uncut" treatment (MT_1). Both parameters show that no disease was observed for treatments involving seedlings from the disease endemic area (T_1), non-endemic area (T_2), disease-free plants (T_5) and seedlings from artificially inoculated seeds (T_3) and plants (T_4). But significantly higher disease (at the 5% level) was recorded in the 2 treatments (T_6 & T_7) in which seedlings were derived from seeds of severe naturally infected plants. This again supports the possibility that the naturally occurring wild types of the pathogen were more virulent than the one from artificially inoculated seeds and plants, and hence is the more important factor in disease initiation and dissemination in the field where disease is not endemic.

7. 3. Discussion

The controversy about seed transmission of the pathogen mentioned in the introduction could be reconciled by the results of the present studies which show that seeds infected artificially either directly or through plant infection did not cause disease whereas naturally infected seeds did so to a limited extent. This may suggest that naturally occurring strains of the bacterium were more virulent than the strain artificially introduced on to seeds and plants, the virulence of which may have decreased through repeated culturing and storage in the laboratory. Even then the amount of disease caused by the naturally occurring strain was so low that seed transmission of the pathogen in the tropics is likely to be unimportant. This confirms results of ANON. (1968) and EAMCHIT *et al.* (1969) who attributed this to the fact that the bacterium could survive in seeds for only a few weeks (3-5 weeks) at such high temperatures of the tropics (28° - 36°).

TABLE 59. MEAN PERCENTAGE DISEASE INCIDENCES[1] FOR THE EFFECTS OF CULTURAL PRACTICES ON THE TRANSMISSION OF BACTERIAL BLIGHT DISEASE IN THE FIELD IN DISEASE-FREE AREA (BUMBONG LIMA) AND RESULTS OF STATISTICAL ANALYSES

Main treatment[3]	Sub-treatment[2]							Main treatment mean
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	
MT_1 (Leaf tips uncut)	0	0	0	0	0	8.87	8.87	2.53
MT_2 (Leaf tips cut)	0	0	0	0	0	28.07	13.07	5.88
Sub-treatment mean	0	0	0	0	0	18.47	10.97	
LSD test[4]	T_1 0	T_2 0	T_3 0	T_4 0	T_5 0	T_7 10.97	T_6 18.47	

Standard deviation per observation (main treatment) = 8.09
Standard deviation per observation (sub-treatment) = 10.41

LSD 5% for sub-treatment mean difference = 12.36
LSD 1% for sub-treatment mean difference = 16.80

1 : Mean of 30 plants from 3 replicates.

2 : T_1 = From disease endemic area. T_2 = From disease-free area. T_3 = From seeds soaked in inoculum. T_4 = From seeds of artificially infected plants. T_5 = From seeds of disease-free plants. T_6 & T_7 = From seeds of 2 naturally infected (very severe) advanced genetic lines.

3 : MT_1 & MT_2 are not significant at the 5% level.

4 : LSD = Least significant difference test. Means underscored are not significantly different at the 5% level. Results were analysed by the parametric F-test based on arcsin transformed data, and the LSD test.

TABLE 60. MEAN DISEASE INTENSITY INDICES[1] FOR THE EFFECTS OF CULTURAL PRACTICES ON THE TRANSMISSION OF BACTERIAL BLIGHT DISEASE IN THE FIELD IN DISEASE-FREE AREA (BUMBONG LIMA) AND RESULTS OF STATISTICAL ANALYSES

Main treatment[3]	Sub-treatment[2]							Main treatment mean
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	
MT_1 (Leaf tips uncut)	0	0	0	0	0	0.57	0.63	0.17
MT_2 (Leaf tips cut)	0	0	0	0	0	2.61	1.05	0.52
Sub-treatment mean	0	0	0	0	0	1.59	0.84	
LSD test[4]	T_1 0	T_2 0	T_3 0	T_4 0	T_5 0	T_7 0.84	T_6 1.59	

Standard deviation per observation (main treatment) = 0.75
Standard deviation per observation (sub-treatment) = 0.85

LSD 5% for sub-treatment mean difference = 1.01
LSD 1% for sub-treatment mean difference = 1.37

1 : Mean of 60 tillers from 3 replicates.

2 : T_1 = From disease endemic area. T_2 = From disease-free area. T_3 = From seeds soaked in inoculum. T_4 = From seeds of artificially infected plants. T_5 = From seeds of disease-free plants. T_6 & T_7 = From seeds of 2 naturally infected (very severe) advanced genetic lines.

3 : MT_1 & MT_2 are not significantly different at the 5% level.

4 : LSD = Least significant difference test. Means underscored are not significantly different at the 5% level. Results were analysed by the parametric F-test based on arcsin transformed data, and the LSD test.

Cultural practices such as cutting of seedling leaf tips prior to transplanting could be a factor in transmission of the pathogen in the field as OU (1972) has stated, cutting of the vascular bundles of the leaves permits entry of the bacterium into the plant. However, this ocurred only in the disease endemic area and suggests that the rice field water harbours the pathogen. This being so, it can be expected that seedlings from endemic area, treated similarly, to be most severely infected when planted in disease endemic area, as was the case in T_1 in TABLES 57 and 58. However, it is noted that the amount of disease caused was still very low which could be due to the low inoculum in the irrigation water in canals and rice fields.

While the syndrome was not expressed in the adult plants grown from artificially inoculted seeds, inoculation did decrease seed germination, growth and yield of the rice plants. The bacterium had obviously affected the seeds and hence plant performance. This may be because the inoculum within the seeds was either not sufficiently high or the bacteria within the seeds did not survive through to the following season. However, the fact that the bacterium could not be isolated from the infected seeds suggests the latter to be a more likely factor.

The overall result suggests that when one uses seeds from severe naturally infected plants or seedlings from disease endemic area and transplants with leaf tips cut in an endemic area, then a very conducive environment for disease epidemic is created, the occurrence of which now depends on the level of inoculum in the rice field water.

8. CHEMICAL CONTROL OF *XANTHOMONAS ORYZAE*

8.1. *In Vitro* Assay of Three Bactericides

8.1.1. Materials and Methods

Two commercially available bactericides and one experimental bactericide were tested. They were as follows (APPENDIX 1):

(i) Celdion 50% a.i. WP (Takeda Chemical Industries Ltd., Japan)

(ii) Phenazine 10% a.i. WP (Nihon Nohyaku Co. Ltd., Japan)

(iii) R115799 25% a.i. WP (ICI, Jealott's Hill, England)

They were tested at 0, 1, 5, 25, 50 and 100 ppm a.i. Appropriate amounts for 100 ml agar medium were weighed in sterile McCartney bottles containing 10 ml sterile distilled water and dissolved by shaking thoroughly. Aliquots of 90 ml modified Wakimoto's potato semisynthetic agar (TABLE 3) in conical flasks were autoclaved at 120°c for 15 minutes. When the agar was sufficiently cool, each aliquot was mixed with appropriate bactericide solutions and c 10 ml poured into each of a series of sterile petri dishes. Eight plates were prepared for each concentration and left to dry for three days before use.

Cells from two-day old slant cultures of a virulent strain of *X. oryzae* (Isolate M) on Wakimoto's potato semisynthetic agar (TABLE 3) were dispersed in 10 ml sterile distilled water per tube culture by means of a rotary mixer. Five slant cultures were used for final suspension from which a seven-fold dilution series was prepared, each dilution being followed by thorough dispersion of cells with a rotary mixer. 0.1 ml from the seventh dilution was placed on agar plates containing various concentrations of the bactericides. The suspension was spread thoroughly over the agar with a sterile L-shaped glass rod. There were two replicates, each of four plates for each treatment, arranged in a strip-plot design.

Inoculated plates were incubated at 26° ± 2°c for four days. Bacterial colonies which grew on agar surfaces were counted

by means of a colony counter and their average sizes estimated visually. Further observations were made on the seventh and tenth day of incubation.

Bactericides which had no obvious effect on the bacterium at any concentration were tested at higher concentrations of 500, 1000, 1500, 2000, 2500 and 3000 ppm a.i. following similar procedures.

8.1.2. Results and Discussion

The analysis of variance (TABLE 61) shows a significant difference (at the 5% level) between replicates indicating that replication increased the precision of the experiment by reducing experimental error. The analysis further shows significant differences among bactericides (at the 1% level), concentrations (at the 5% level) and interactions (at the 1% level).

Phenazine showed very high *in vitro* activity against *X. oryzae*. At 1 ppm and higher no colony was observed even after ten days incubation (TABLE 61) compared to controls (0 ppm) which gave a significantly higher (at the 5% level) average population of 2 to 3 x 10^9 cells per ml of suspension.

Celdion had very low activity even at 100 ppm. Bacterial populations for all concentrations were within the normal population of 2 to 3 x 10^9 cells per ml of suspension which were not significantly different (at the 5% level) to each other (TABLE 61). However, at 50-100 ppm, colonies were substantially smaller than in the controls (TABLE 62). Further tests (TABLE 63) showed that at 500 ppm, growth was inhibited for four days. After 7 days, however, numbers of colonies were $\underline{c}$ 25% of controls with diameters reduced from 2-5 mm (control) to 0.5 mm.

TABLE 61. *IN VITRO* EFFECTS OF THREE BACTERICIDES ON POPULATIONS[1] OF *XANTHOMONAS ORYZAE* AFTER INCUBATION FOR FOUR DAYS

Bactericide / Concentration (ppm a.i.)	Phenazine (10% a.i. WP)	Celdion (50% a.i. WP)	R115799 (25% a.i. WP)
0 (control)	$2.1 \pm 0.5 \times 10^9$ a	$2.7 \pm 0.6 \times 10^9$ a	$2.6 \pm 0.5 \times 10^9$ a
1	0 b	$2.4 \pm 0.7 \times 10^9$ a	$1.1 \pm 0.7 \times 10^9$ b
5	0 b	$2.6 \pm 0.8 \times 10^9$ a	$0.7 \pm 0.5 \times 10^9$ b
25	0 b	$2.9 \pm 0.6 \times 10^9$ a	0 c
50	0 b	$2.9 \pm 0.4 \times 10^9$ a	0 c
100	0 b	$2.4 \pm 0.7 \times 10^9$ a	0 c

Source of variation	Results of statistical tests[2]	Coefficient of variation (%)
1. Replicate	*	
2. Bactericide	**	4.7
3. Concentration	*	13.0
4. Interaction	**	10.4

[1] : Each reading was an average of 8 plates from 2 replicates. Figures refer to number of viable bacterial cells/ml. Means with common letters are not significantly different at the 5% level.

[2] : Analysis of variance was based on logarithmic transformation of the original data (Log (x+1)).

* : Significant at the 5% level.

** : Significant at the 1% level.

TABLE 62. *IN VITRO* EFFECTS OF THREE BACTERICIDES ON THE SIZE[1] OF *XANTHOMONAS ORYZAE* COLONIES AFTER INCUBATION FOR FOUR DAYS

Bactericide / Concentration (ppm a.i.)	Phenazine (10% a.i. WP)	Celdion (50% a.i. WP)	R115799 (25% a.i. WP)
0 (control)	0.5 - 2 mm	0.5 - 2 mm	0.5 - 2 mm
1	-	0.5 - 2 mm	0.5 - 1 mm
5	-	0.5 - 2 mm	0.5 mm
25	-	0.5 - 2 mm	-
50	-	0.5 - 1 mm	-
100	-	0.5 mm	-

[1] : Range of diameter (mm)

TABLE 63. *IN VITRO* EFFECTS OF HIGH CONCENTRATIONS OF CELDION 50% a.i. WP ON POPULATIONS[1] AND SIZE[2] OF *XANTHOMONAS ORYZAE* COLONIES AFTER INCUBATION FOR 4, 7 AND 10 DAYS

Concentration (ppm a.i.)	Incubation time			
	4 days		7 days	
	Colony number	Diameter	Colony number	Diameter
0 (control)	$2.6\pm0.6\times10^9$	0.5-2 mm	$2.6\pm0.6\times10^9$	2-5 mm
500	0	-	$0.6\pm0.5\times10^9$	0.5 mm
1000	0	-	0 (0.4×10^8)[3]	- (0.5 mm)[3]
1500	0	-	0 (0.1×10^8)[3]	- (0.5 mm)[3]
2000	0	-	0	-
2500	0	-	0	-
3000	0	-	0	-

[1] : Each reading was an average of 8 plates from 2 replicates. Figures refer to number of viable bacterial cells/ml.

[2] : Range of diameter (mm).

[3] : Apparent only after incubation for 10 days.

At 1000 ppm and 1500 ppm bacterial populations were reduced to 2 to 0.3% of controls respectively with colonies of 0.5 mm diameter evident only ten days after incubation. There was complete inhibition of growth at 2000 ppm and above.

R115799 showed high *in vitro* activity. At 1 ppm it reduced bacterial populations by more than 50% and reduced colony diameter significantly (TABLES 61 & 62) compared with controls. At 5 ppm bacterial populations were reduced significantly (at the 5% level) by 75% (TABLE 61) and colony diameter was reduced to 0.5 mm (TABLE 62). There was complete inhibition of growth at 25 ppm and above.

The highly significant interaction effects (TABLE 61) show that the rates of reduction in bacterial population in response to increasing concentration varied greatly with different bactericides (FIG. 9). The rate of reduction was shown to be very slow for Celdion, fast for R115799 and very fast for Phenazine. From the average bacterial population of 1.4053 (logarithmic transformed scale) obtained from all the controls (TABLE 61), the value for 50% killed could be worked out to be 0.7027 (logarithmic transformed scale), which on the graph (FIG. 9) gives the estimated ED 50 values for Phenazine and R115799 as 0.5 ppm and 5.5 ppm a.i. respectively. The estimated LD_{50} value for Celdion could not be obtained as the bactericide was not effective even at 100 ppm (TABLES 61 & 62).

As phenazine has been shown to be highly bacteriotoxic and phytotoxic and Celdion shown to have little *in vitro* activity while being not effective against the disease (Sections 8.3 & 8.4) only the ED 50 value of R115799 was more accurately determined by the liquid culture dilution plate method in Section 8.2 below.

8.2. Determination of ED 50 Value for Bactericide 25% a.i. WP

8.2.1. Materials and Methods

The method used was the liquid culture dilution plate method modified from the YAKUSHIJI & WAKAE's (1971) method. Forty-

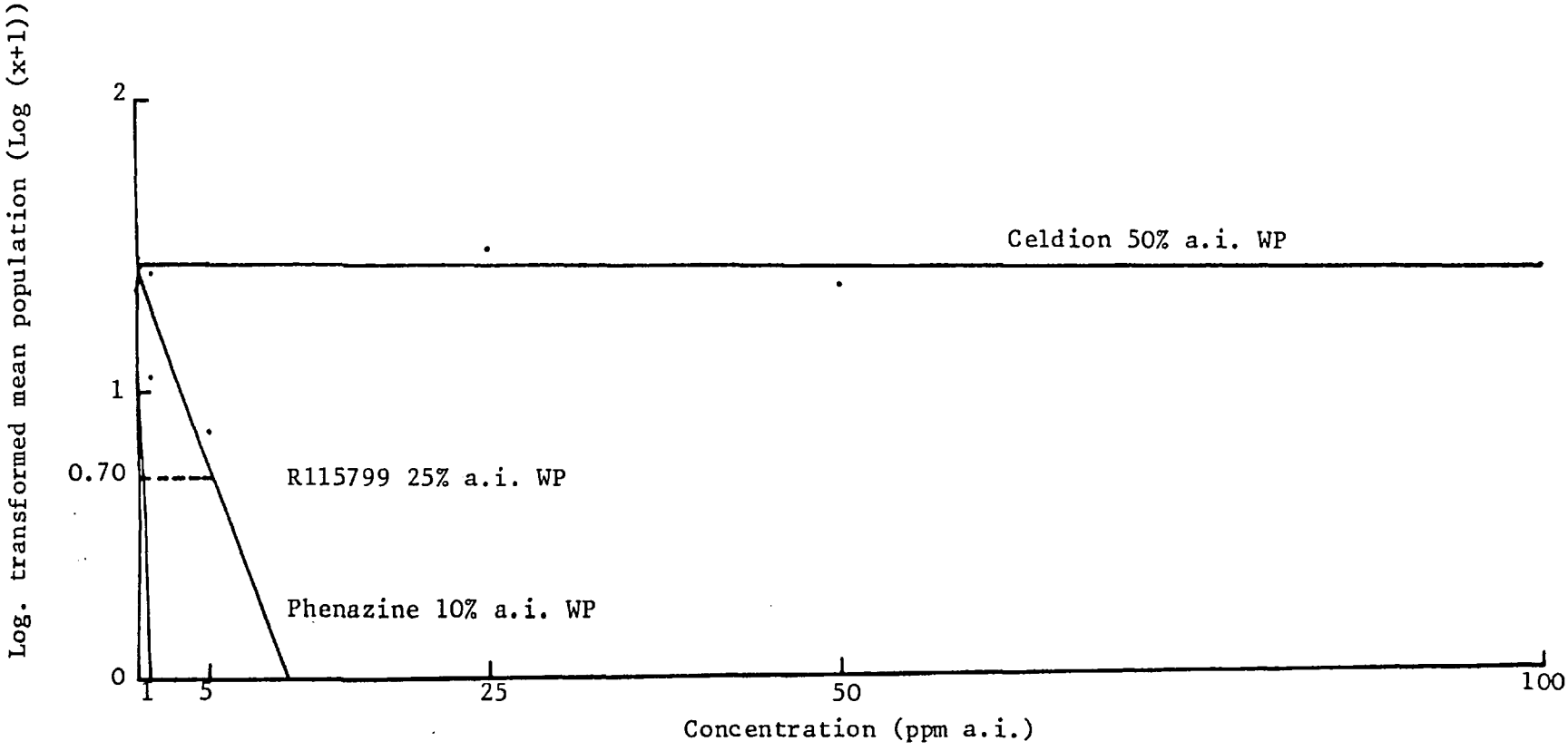


FIG.9. Effects of different concentrations (ppm a.i.) of three bactericides on the mean populations (logarithmic transformed scale) of *Xanthomonas oryzae* after incubation for four days on agar plates.

eight hour old slant culture of a virulent strain of *X. oryzae* was used to prepare suspension by adding 10 ml sterile distilled water per slant and dispersing it with a hirlimixer for 2 minutes after loosening the culture from the agar surface by means of a blunt and rounded glassrod. The suspension was plated out at the 7th dilution of 1:9 mixture of bacterial suspension to sterile distilled water to determine the viability of the suspension which was assessed 4 days after incubation at $26^{\circ} \pm 2^{\circ}$c. The suspension was used to inoculate various concentrations of bactericide at 0, 1, 5, 25 and 50 ppm a.i. as follows.

YAKUSHIJI & WAKAE's (1971) synthetic liquid culture medium (sucrose 0.5%, Na-glutamate 0.1%, L-cysteine 0.001%, $Mg\ Cl_2 \cdot 6\ H_2O$ 0.01%, $Fe\ SO_4 \cdot 7\ H_2O$ 0.001%, $Mn\ SO_4 \cdot 4\ H_2O$ 0.001%, $KH_2\ PO_4$ 0.005%, $K_2\ HPO_4$ 0.005%, pH 6.8) was boiled thoroughly in water bath for about one hour and filtered through a double-layered filter paper. Aliquots of 100 ml were measured into 250 ml conical flasks for all the concentrations tested. Appropriate amounts of bactericide at various concentrations were added into the sterile liquid culture media accordingly. The mixtures were thoroughly mixed in a shaker for 30 minutes after which aliquots of 10 ml of each concentration was pipetted out into each sterile 14 ml McCartney bottle to which one drop of the bacterial suspension prepared as above was added by means of a sterile 1 ml pipette and thoroughly mixed. This was prepared in 3 replicates and subject to continuous shaking for 32 hours at $26^{\circ} \pm 2^{\circ}$c.

The optical densities (absorbance at 620 nm) of all samples including the uninoculated remainder of the mixtures were measured using the Beckman spectrophotometer. Dilution series were also made for all samples as before and the numbers of vaiable bacterial cells were assessed at the various dilutions indicated in TABLE 64, after 4 days incubation at $26^{\circ} \pm 2^{\circ}$c.

8.2.2. Results and Discussion

The test for viability of the suspension used as the inoculum shows that the bacterium was viable consisting of c 2.1 x 10^9 cells/ml. The YAKUSHIJI & WAKAE's (1971) synthetic liquid medium proved to be a suitable medium for the growth of the bacterium and for optical density measurements because of its clearity and hence was suitable for use in the determination of ED 50 values.

The optical densities for the uninoculated controls (TABLE 64) show that the optical density increased with increase in concentration of bactericide. As such the difference in the optical densities between the inoculated and the uninoculated treatments was more relevant for the assessment of percentage growth of the bacterium in liquid cultures which was found to decrease with increase in concentration of bactericide. This was evident as shown by the optical density and the liquid culture dilution plate methods of assessment (TABLE 64). However, the decrease in percentage growth was found to be more when assessed by the liquid culture dilution plate method than by the optical density method (FIG. 10). Such discrepancy was most probably due to the fact that while the optical density measured the total amount of live and dead cells and that assessed by the liquid culture dilution plate method gave only the total number of viable cells in liquid culture (KIRALY *et al.*, 1974). As such the latter method would then be more accurate for the assessment of ED 50 values which for bactericide R115799 25% a.i. WP, was found to be about 0.6 ppm a.i. (FIG. 10), compared to the optical density method and the dilution plate method involving bactericide - incorporated agar plate (refer Section 8.1) which gave an excessively high ED 50 value of 5.5 ppm a.i.

TABLE 64. OPTICAL DENSITIES AND NUMBERS OF VIABLE BACTERIAL CELLS OF THE UNINOCULATED AND INOCULATED 32-HOUR LIQUID CULTURES OF *XANTHOMONAS ORYZAE*[1] AT VARIOUS CONCENTRATIONS OF BACTERICIDE R115799 25% a.i. WP

Concentration (ppm a.i.)	Optical density (absorbance at 620 nm)[2]				Viable bacterial cell count[3]	
	Uninoculated controls	Inoculated	Difference	% growth	No./ml	% growth
1. 0	0.003±0.002	0.101±0.003	0.098	100	$69\pm10\times10^8$	100
2. 1	0.011±0.001	0.055±0.008	0.044	32	$15\pm5\times10^8$	22
3. 5	0.055±0.009	0.077±0.017	0.022	4	$3\pm1\times10^3$	<0.1
4. 25	0.161±0.002	0.181±0.005	0.02	0	0	0
5. 50	0.316±0.002	0.334±0.001	0.018	0	0	0

[1] : Cultured in YAKUSHIJI & WAKAE's (1971) synthetic liquid medium (sucrose 0.5%, Na-glutamate 0.1%, L-cysteine 0.001%, $Mg\ Cl_2 \cdot 6\ H_2O$ 0.01%, $Fe\ SO_4 \cdot 7\ H_2O$ 0.001%, $Mn\ SO_4 \cdot 4\ H_2O$ 0.001%, KH_2PO_4 0.005%, K_2HPO_4 0.005%, pH 6.8).

[2] : Mean of 3 replicates measured by a Beckman spectrophotometer after 32 hours of continuous shaking cultures.

[3] : Mean of 12 plates from 3 replicates assessed 4 days after incubation at $26^\circ \pm 2^\circ$c. Plating was done at the 7th, 7th, 2nd, 0 and 0 dilutions for concentrations of bactericide at 0, 1, 5, 25 and 50 ppm a.i. respectively.

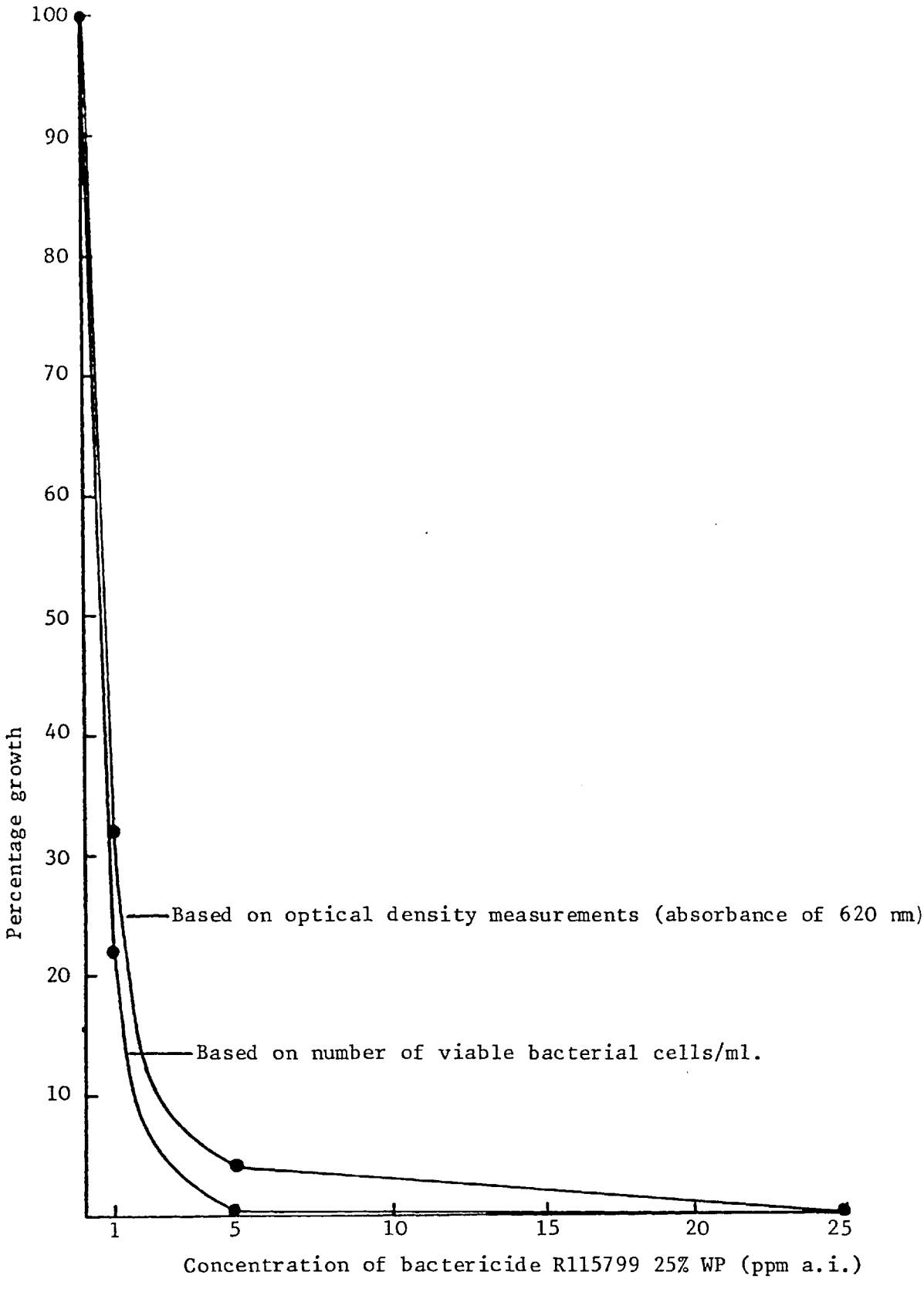


FIG.10. Relationship between various concentrations of bactericide R115799 25% a.i. WP and percentage of growth of *Xanthomonas oryzae* in 32-hour old liquid cultures.

8.3. Bactericide Screening in Relation to Rates of Application

8.3.1. Materials and Methods

The experiment was carried out in a glasshouse in conditions suitable for good growth of rice plants (refer Section 3.1.1 above). Rectangular plastic troughs (60.5 x 14.5 x 14 cm) were filled with a loamy clay soil (TABLE 8) to a depth of 10 cm. The soil was well-soaked with water before pre-selected seeds of a susceptible cultivar SMII were sowed directly at the rate of three seeds per point and a spacing of 12.5 x 6.5 cm. With this spacing each trough contained 8 hills of plants. The seeds were covered with a thin layer of similar soil which was allowed to moisten for 3-4 hours by uptake from below before seeds were watered lightly twice per day until the 5th day, when the germinated seedlings were flooded at the base. At 18 days after sowing the seedlings were reduced to 2 per hill giving a total of 16 plants in 8 hills per trough. Only one trough was used for each treatment. The seedlings were flooded with 1 cm of water at the base throughout the experiment.

The bactericides screened were Celdion 50% a.i. WP, Phenazine 10% a.i. WP, R115799 25% a.i. WP and R115799 1% a.i. G (APPENDIX 1). The wettable powder formulations (WP), thoroughly dissolved in water at the normal field spray volume of 1000 l/ha were sprayed on to the aerial parts of the plants by means of a laboratory spray gun (Shandon Scientific Co. Ltd., London) at the rates of 0, 0.1, 0.2 and 0.3% a.i. The granular formulation was broadcast by hand around the plants at the rates of 0, 1, 2 and 3 kg a.i./ha. The seedlings were treated with the various concentrations of bactericides 24 days after sowing (DAS) and incoulated with standard bacterial suspensions (refer Section 1.1 above) of a virulent strain (Isolate M) of *X. oryzae*, 2 days later, by the pin prick method (refer Section 3.1.1 above) on the first fully opened top leaves of all seedlings. The inoculated seedlings were observed daily for phytotoxicity symptoms and disease development which was assessed at 7, 10 and 13 days after inoculation (DAI) using the scale in TABLE 5.

8.3.2. Results

Two days after application only plants treated with Phenazine showed severe phytotoxicity symptoms at all concentrations compared to the control (0% a.i.) (TABLE 65). The percentage of leaves scorched increased with increase in concentration applied (TABLE 66). Even at the lowest concentration (0.1% a.i.) a highly significant (at the 1% level) proportion (86%) of leaves were scorched compared to the control. This was not significantly different (at the 5% level) from that given by 0.2% a.i. However, at 0.3% a.i. there was 100% scorching which was significantly higher (at either the 5% or 1% levels) than that given by 0.1 and 0.2% a.i. (TABLE 66). In view of the high phytotoxicity, Phenazine was not evaluated further against the disease.

The non-parametric Wilcoxon match-pairs signed-ranks test (SIEGEL, 1956) shows that Celdion 50% a.i. WP was not effective in controlling the disease even at 0.3% a.i. (TABLE 65). The mean disease scores of all concentrations were not significantly different (at the 5% level) from that of the control (PLATE 19) for all the three assessment times. By a similar test, both formulations of R115799 were shown to be effective even at the lowest concentration of 0.1% a.i. for wettable powder and 1 kg a.i./ha for granules. The mean disease scores for all concentrations at all the three assessment times were significantly lower (at either the 5% or 1% levels) than those of the controls.

The rate of increase in disease varied with the effectiveness of the bactericides and their rates of application. For an ineffective bactericide like Celdion 50% a.i. WP the rate of increase was relatively high and almost similar to that of the control (FIG. 11A), whereas for an effective bactericide, R115799 25% a.i. WP, the rate of increase decreased with increase in rates of application (FIG. 11B) which was more pronounced for its granular formulation (FIG. 11C), indicating that effective bactericide in granular formulation applied directly into the soil around the plants was superior to its wettable powder formulation applied as a spray.

TABLE 65. MEAN DISEASE SCORES[1] OF RICE CULTIVAR SMII AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF 3 BACTERICIDES AND INOCULATION WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* 2 DAYS AFTER TREATMENT

Bactericide/ Concentration (rate of application)	Disease assessment time (DAI)[2]		
	7 (9 DAA)[3]	10 (12 DAA)	13 (15 DAA)
Spray application			
1. Celdion 50% a.i. WP			
(a) 0% a.i.(control)	3.4 ± 0.5	4.3 ± 0.7	4.8 ± 0.4
(b) 0.1% a.i.	3.1 ± 0.4NS	4.2 ± 0.6NS	4.8 ± 0.4NS
(c) 0.2% a.i.	3.1 ± 0.4NS	4.3 ± 0.6NS	4.8 ± 0.4NS
(d) 0.3% a.i.	3.0 ± 0.5NS	3.9 ± 0.6NS	4.8 ± 0.4NS
2. Phenazine 10% a.i. WP			
(a) 0% a.i.(control)	NI[4]	NI	NI
(b) 0.1% a.i.	P[5]	P	P
(c) 0.2% a.i.	P	P	P
(d) 0.3% a.i.	P	P	P
3. R115799 25% a.i. WP			
(a) 0% a.i.(control)	2.8 ± 0.4	4.6 ± 0.6	4.9 ± 0.3
(b) 0.1% a.i.	1.4 ± 0.5**	3.3 ± 0.8**	4.3 ± 0.4*
(c) 0.2% a.i.	1.4 ± 0.5**	3.3 ± 1.0**	3.5 ± 0.8**
(d) 0.3% a.i.	0.9 ± 0.5**	2.7 ± 0.9**	3.4 ± 0.8**
Granular application			
4. R115799 1% a.i. G			
(a) 0 kg a.i./ha(control)	3.4 ± 0.5	4.4 ± 0.5	4.9 ± 0.3
(b) 1 kg a.i./ha	2.4 ± 0.7**	3.3 ± 0.6**	4.1 ± 0.7**
(c) 2 kg a.i./ha	1.3 ± 0.4**	1.5 ± 0.6**	2.1 ± 0.8**
(d) 3 kg a.i./ha	0.9 ± 0.2**	1.2 ± 0.4**	1.5 ± 0.5**

[1] : Mean of 16 disease scores based on a 0-5 scale of increasing disease (see TABLE 5). Results were analysed by the Wilcoxon match-pairs signed-ranks test (SIEGEL, 1956). NS = Not significant at the 5% level. * = Significant at the 5% level. ** = Significant at the 1% level. Every treatment was compared to the control.

[2] : DAI = Days after inoculation.

[3] : DAA = Days after bactericide application.

[4] : NI = Not inoculated.

[5] : P = Phytotoxicity (see TABLE 66).

TABLE 66. AVERAGE PERCENTAGE LEAVES OF RICE CULTIVAR SMII SCORCHED BY BACTERICIDE PHENAZINE 10% a.i. WP AT VARIOUS CONCENTRATIONS 2 DAYS AFTER SPRAYING

Concentration (rate of application)	Mean[1] % leaves scorched
1. 0% a.i. (control)	0 ± 0 a
2. 0.1% a.i.	86.1 ± 15.9 b
3. 0.2% a.i.	91.2 ± 13.5 b
4. 0.3% a.i.	100 ± 0 c

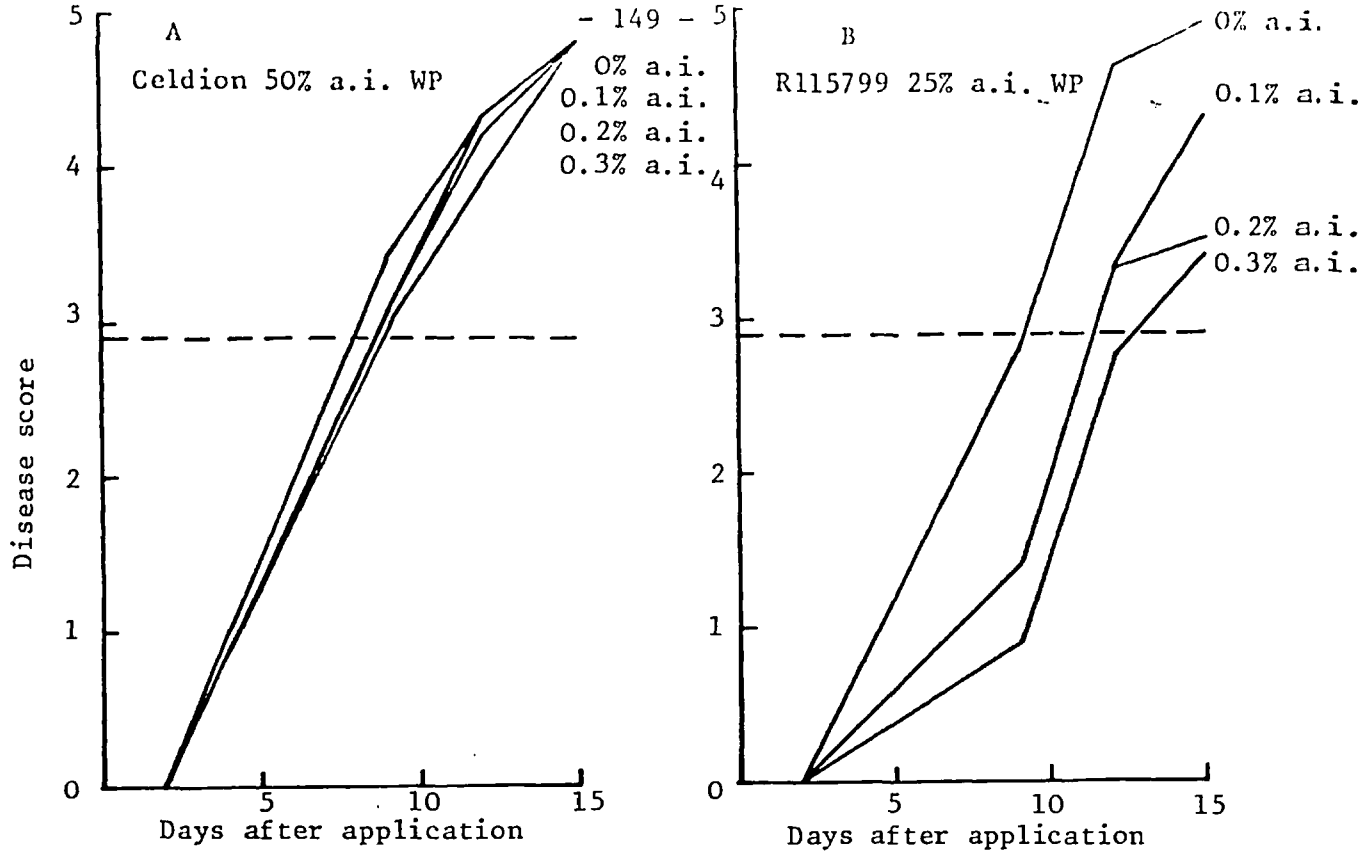
[1] : Mean per plant obtained from 16 plants. Means with common letters are not significantly different at the 5% level. Results were analysed by the non-parametric Wilcoxon Match-Pairs Signed-Ranks Test (SIEGEL, 1956).



PLATE 19. Typical severe bacterial blight symptoms produced on the control (untreated) plants 13 days after inoculation.



PLATE 20. Limited and restricted lesions with mottling effects characteristic of atypical symptoms of bacterial blight, produced on rice plants treated with bactericide R115799 1% a.i. G at the rate of 2 kg a.i./ha and above, at 13 days after inoculation (15 days after treatment).



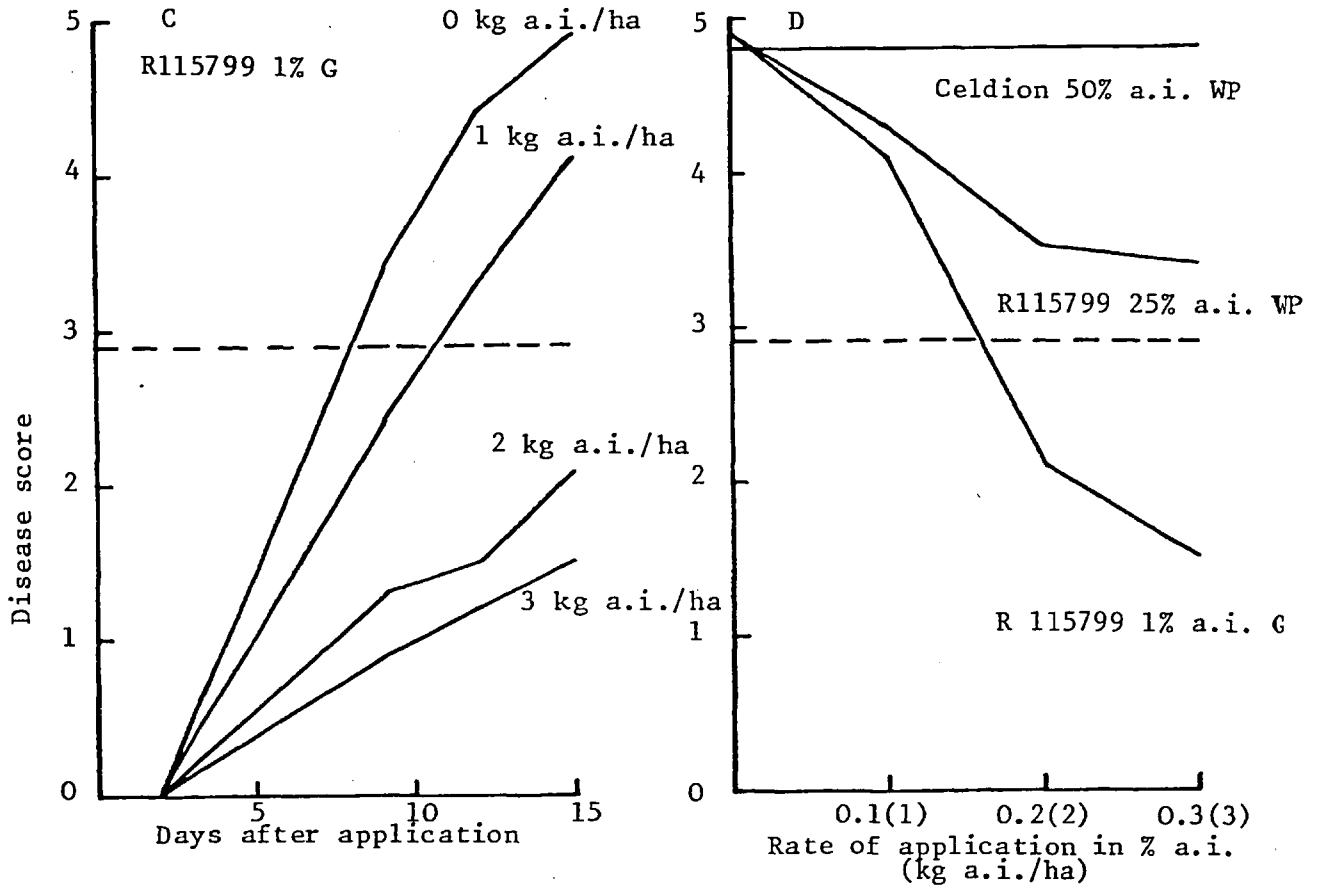


FIG.11A,B,C. Effects of different application rates of 3 bactericides on the development of bacterial blight disease (Celdion 50% a.i. WP & R115799 25% a.i. WP were applied by spraying; R115799 1% a.i. G by hand broadcasting).

FIG.11D. Effects of 3 bactericides in controlling bacterial blight disease at different rates of application 15 days after treatment (13 days after inoculation). Application methods were similar to FIG.11A,B,C.

Considering the effects of the various concentrations of bactericides on the disease at its advanced stage of development (13 days after inoculation) it can be seen (FIG. 11D) that while Celdion 50% a.i. WP was ineffective even at 0.3% a.i. (TABLE 65), R115799 25% a.i. WP, although effective at 0.1% a.i. (TABLE 65), was not totally effective as the disease was not controlled to below the intermediate score of 3 even at 0.3% a.i. The intermediate score of 2.9 is taken as the upper limit for effectiveness because lesions scored 3 are still typical bacterial blight symptoms capable of spreading and becoming susceptible lesions with time and changes in ecological factors. However, the granular form of R115799 again showed superiority over its wettable powder formulation as it was able to control the disease well below the upper limit (PLATE 20) at application rates of 2 kg a.i./ha and above (FIG. 11D).

8.4. Effects of Different Water Depths on the Effectiveness of Three Wettable Powder Bactericides Sprayed on to Plants

8.4.1. Materials and Methods

The materials and methods were similar to those in Section 8.3, except that 3 wettable powder bactericides, Celdion 50% a.i. WP, Phenazine 10% a.i. WP and R115799 25% a.i. WP (APPENDIX 1) were investigated at application rate of 0.2% a.i. with a spray volume of 1000 l/ha in relation to four water depths, 0, 1, 2 and 3 cm.

Seedlings were kept flooded at minimum levels until the time of application of bactericides when water depths were adjusted. Seedlings were sprayed with the bactericides 24 days after sowing and inoculated 2 days later. Plants at 0 cm water depth were watered twice daily. For others, water depths were kept at appropriate levels for a week after application of bactericides, after which water depth was kept at 1 cm.

8.4.2. Results and Discussion

Results in TABLE 67 confirm the severe phytotoxicity of Phenazine reported in Section 8.2. About 90% of the leaves treated with Phenazine were scorched 2 days after application for all water depths, the differences for depth being not significant at the 5% level (TABLE 68).

For Celdion 50% a.i. WP which had been found to be not effective against the pathogen (see Section 8.1) and the disease (see Section 8.3), water depth did not affect performance. The disease at 0 cm water depth, was not significantly different (at the 5% level) from those a different water depths for all the 3 disease assessment times (TABLE 67).

For R115799 25% a.i. WP, found to be lethal to the pathogen at 25 ppm (see Sections 8.1 & 8.2) and only slightly effective against the disease (see Section 8.3), decreasing water depth did not affect disease development at initial (7 DAI) and intermediate (10 DAI) stages, as their disease scores were not significantly different (at the 5% level) to each other (TABLE 67). However, at the advanced stage of disease development (13 DAI), the disease was increased significantly (at the 1% level) only when water depth increased up to 3 cm (TABLE 67). Even then, the disease (score 3.1) still exceeded the upper limit for effectiveness (score 2.9) which confirms results of previous experiment in Section 8.3.

8.5. Effects of Different Application Rates of Bactericide R115799 1% a.i. G Applied to Soil in Relation to Different Water Depths

8.5.1. Materials and Methods

The experiment in Section 8.4 was repeated here using different rates of bactericide R115799 1% a.i. G. All the four water depths (0, 1, 2 and 3 cm) were investigated for each of four application rates, 1, 2, 3 and 4 kg a.i./ha.

TABLE 67. MEAN DISEASE SCORES[1] OF RICE CULTIVAR SMII AFTER SPRAY TREATMENT WITH 3 BACTERICIDES AT 0.2% a.i. AT DIFFERENT WATER DEPTHS AND INOCULATION WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* 2 DAYS AFTER TREATMENT

Bactericide/ water depth	Disease assessment time (DAI)[2]		
	7 (9 DAA)[3]	10 (12 DAA)	13 (15 DAA)
1. Celdion 50% a.i. WP			
(a) 0 cm	3.4	4.3	4.8
(b) 1 cm	3.4 NS	4.5 NS	4.8 NS
(c) 2 cm	3.1 NS	4.3 NS	4.7 NS
(d) 3 cm	3.1 NS	4.3 NS	4.7 NS
2. Phenazine 10% a.i. WP			
(a) 0 cm	P[4]	P	P
(b) 1 cm	P	P	P
(c) 2 cm	P	P	P
(d) 3 cm	P	P	P
3. R115799 25% a.i. WP			
(a) 0 cm	1.4	2.4	3.1
(b) 1 cm	1.4 NS	2.4 NS	3.1 NS
(c) 2 cm	1.4 NS	2.5 NS	3.3 NS
(d) 3 cm	1.3 NS	2.9 NS	4.1 **

[1] : Mean of 16 disease scores based on a 0-5 scale of increasing disease (see TABLE 5). Results were analysed by the non-parametric Wilcoxon match-pairs signed-ranks test (SIEGEL, 1956). NS = Not significant at the 5% level. ** = Significant at the 1% level. Every treatment was compared to the control.

[2] : DAI = Days after inoculation.

[3] : DAA = Days after bactericide application.

[4] : P = Phytotoxicity (see TABLE 68).

TABLE 68. MEAN[1] PERCENTAGE OF LEAVES OF RICE CULTIVAR SMII SCORCHED BY BACTERICIDE PHENAZINE 10% a.i. WP AT DIFFERENT WATER DEPTHS 2 DAYS AFTER SPRAYING

Water depth	Mean % leaves scorched	
1. 0 cm	86.6	15.4 a
2. 1 cm	87.5	15.3 a
3. 2 cm	85.6	16.3 a
4. 3 cm	87.6	14.3 a

[1] : Mean of % leaves scorched per plant from 16 plants. Means with common letters are not significantly different at 5% level. Results were analysed by the non-parametric Wilcoxon match-pairs signed-ranks test (SIEGEL, 1956).

8.5.2. Results and Discussion

Results in TABLE 69 show that water depth is important in determining the effectiveness of the systemic granular bactericide, particularly when the rate of application is very low. At 1 kg a.i./ha it was more effective when the water depth was 0 cm than at 1, 2 and 3 cm, when assessed at 13 days after inoculation, as the amount of disease (score 2.9) was the upper limit for effectiveness and was significantly lower (at the 1% level) than those at other various water depths.

The results also show that water depth is very critical in determining the economic rate of application under flooded conditions. At 2 kg a.i./ha it was effective up to the water depth of 1 cm as the disease (score 2.6) was not significantly different from that at o cm (score 2.3) and both are well below the upper limit for effectiveness. At 2 cm and higher the disease was significantly higher (at either the 5% or 1% levels) (TABLE 69).

At higher rates of application (3 and 4 kg a.i./ha), lower water depths, although effective in enhancing the effectiveness of the bactericide, is relatively unimportant. At 3 kg a.i./ha, although 0 cm water depth produced a very significantly low (at the 1% level) disease (score 1.6), the disease produced up to 2 cm water depth (score 2.8) was still below the upper limit for effectiveness. And at 4 kg a.i./ha even disease at 3 cm water depth, although significantly higher (at the 1% level) was still acceptable being below the upper limit for effectiveness.

The overall results (FIG. 12A, B, C, D) generally show that the rate of increase in disease becomes progressively slower as water depth decreased and the rate of application increased. This suggests that an effective rate of application depends upon the depth of flooding in the field. Hence based on the upper limit of disease score (2.9) for effectiveness, the results of this study in FIG. 13 show the minimum rates of

TABLE 69. MEAN DISEASE SCORES[1] OF RICE CULTIVAR SMII AFTER TREATMENT WITH DIFFERENT APPLICATION RATES OF BACTERICIDE R115799 1% a.i. G AT DIFFERENT WATER DEPTHS AND INOCULATION WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* 2 DAYS AFTER APPLICATION

Water depth	Disease assessment time (DAI)[2]		
	7 (9 DAA)[3]	10 (12 DAA)	13 (15 DAA)
1. 1 kg a.i./ha			
(a) 0 cm	1.6 ± 0.7	2.5 ± 0.8	2.9 ± 0.7
(b) 1 cm	2.1 ± 0.7	3.4 ± 0.7	4.3 ± 0.8 **
(c) 2 cm	2.7 ± 0.5	3.7 ± 0.8	4.4 ± 0.6 **
(d) 3 cm	2.4 ± 0.5	3.7 ± 0.6	4.5 ± 0.5 **
2. 2 kg a.i./ha			
(a) 0 cm	1.2 ± 0.4	1.8 ± 0.5	2.3 ± 0.6
(b) 1 cm	1.6 ± 0.5	2.2 ± 0.5	2.6 ± 0.5 NS
(c) 2 cm	1.3 ± 0.6	2.2 ± 0.7	3.1 ± 0.9 *
(d) 3 cm	1.4 ± 0.5	2.6 ± 0.7	3.3 ± 0.6 **
3. 3 kg a.i./ha			
(a) 0 cm	0.9 ± 0.3	1.1 ± 0.3	1.6 ± 0.8
(b) 1 cm	1.2 ± 0.4	1.9 ± 0.6	2.6 ± 0.7 **
(c) 2 cm	1.2 ± 0.4	2.2 ± 0.8	2.8 ± 0.8 **
(d) 3 cm	1.4 ± 0.5	2.4 ± 0.5	3.3 ± 0.7 **
4. 4 kg a.i./ha			
(a) 0 cm	0.8 ± 0.4	1.0 ± 0.4	1.5 ± 0.6
(b) 1 cm	1.0 ± 0	1.2 ± 0.4	2.3 ± 0.8 **
(c) 2 cm	1.2 ± 0.4	1.6 ± 0.6	2.5 ± 0.5 **
(d) 3 cm	1.3 ± 0.6	1.6 ± 0.7	2.6 ± 0.5 **

[1] : Mean of 16 disease scores based on a 0-5 scale of increasing disease (see TABLE 5). Results were analysed by the non-parametric Wilcoxon match-pairs signed-ranks test (SIEGEL, 1956). NS = Not significant at the 5% level. * = Significant at the 5% level. ** = Significant at the 1% level. Every treatment was compared to the control.

[2] : DAI = Days after inoculation.

[3] : DAA = Days after bactericide application.

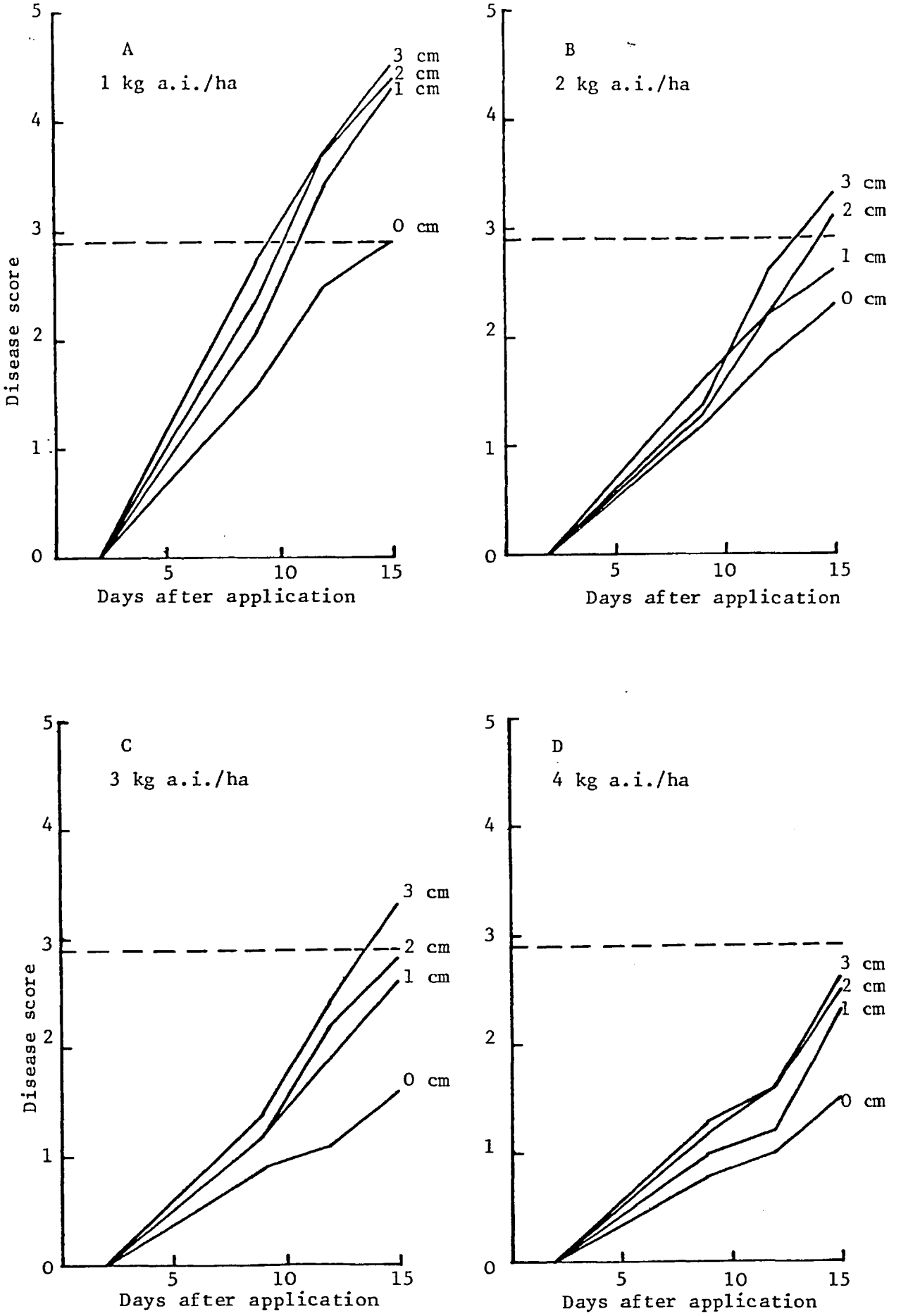


FIG.12A, B, C, D. Effects of different rates of application of Bactericide R115799 1% a.i. G on the development of bacterial blight disease under different water depths. (- - = upper limit of disease score (2.9) for effectiveness).

application of bactericide R115799 1% a.i. G likely to be effective in different water depth. The graph (FIG. 13) shows the relationship where the minimum effective rates of application increases proportionately with increase in the amount of water in the field.

Hence because of the existing widely differing field conditions especially on water depth, it would be highly erroneous to extrapolate results from small scale glasshouse experiments to large scale field experiments, let alone field recommendations.

8.6. Time of Application to Soil of Bactericide R115799 1% a.i. G without Fertilisers

8.6.1. Materials and Methods

Cultivar, soil, cultures, planting layout, inoculation method and method of disease assessment were as in Section 8.2.1. The following were different:

(i) 40-day old plants of cultivar SMII were used.

(ii) Bactericide R115799 1% a.i. G was applied to soil at 2 kg a.i./ha at different times relative to inoculation, 7 days before inoculation (DBI), 2 DBI, 2 days after inoculation (DAI), 7 DAI, and a control with no bactericide.

The disease was assessed at 13 DAI, using the scale in TABLE 5.

8.6.2. Results and Discussion

Without fertilizers, times of bactericide application relative to inoculation (TABLE 70) had a pronounced effect on control of the disease. The results suggest that the bactericide is best used preventively 7 days before inoculation (DBI) then giving a disease score of 1.6 which was significantly lower (at the 5% level) than the next best of 2.2 and 2 DBI. The effects of application at 2 DBI and 2 days after inoculation (DAI) were not significantly different (at the 5% level) and were well below the upper limit of disease

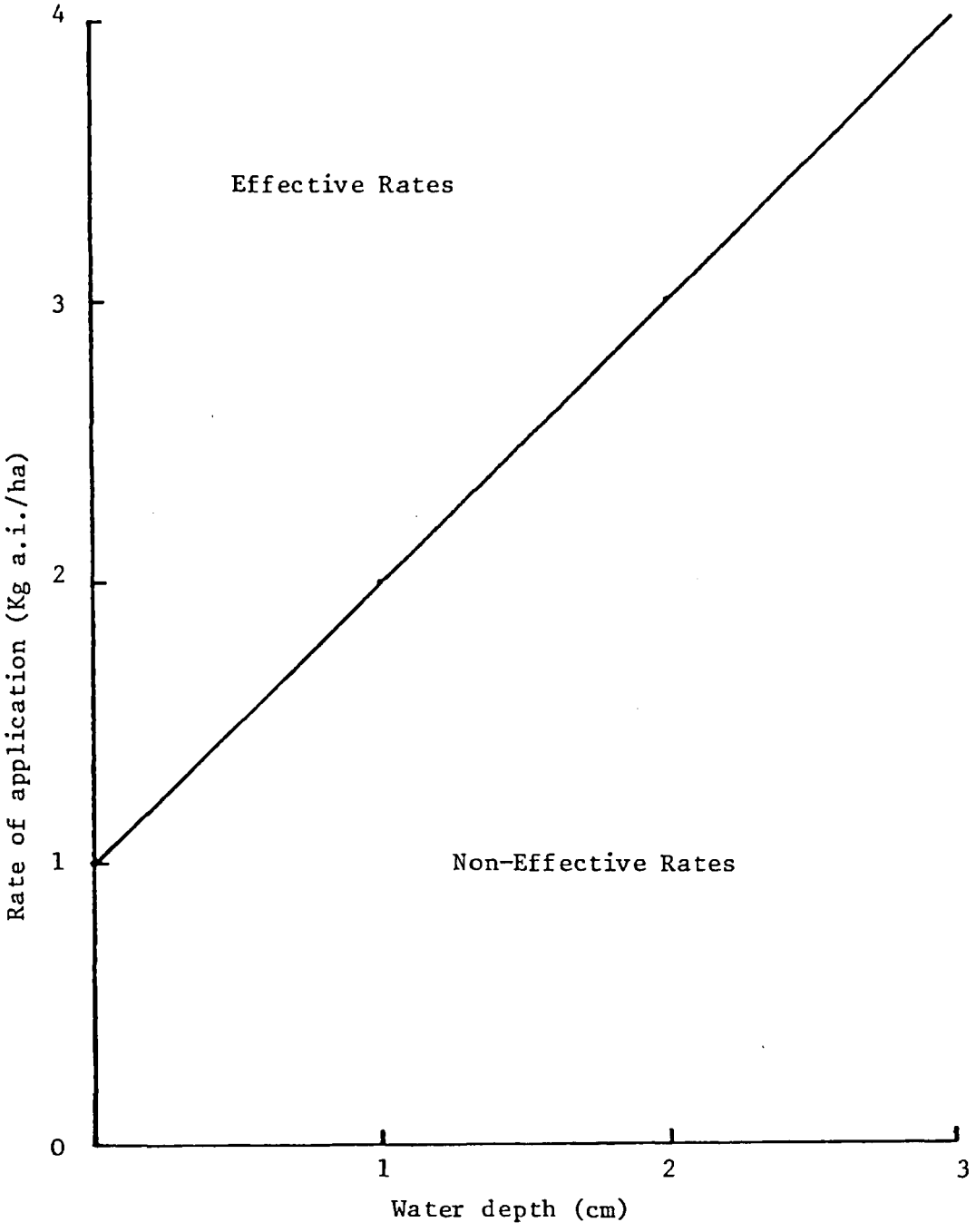


FIG.13. Graph of minimum effective application rates of bactericide R115799 1% a.i. G for different water depths based on the upper limit of disease score (2.9) for effectiveness.

score (2.9) for effectiveness (FIG. 14). This suggests that treatment at or near the time of infection would also be likely to reduce the disease significantly.

Treatment at the symptom appearance stage (7 DAI) gave significantly more disease (at the 1% level) than did treatments at the time of inoculation (2 DBI and 2 DAI) (TABLE 70). Disease was not significantly different (at the 5% level) from that in the control but nevertheless it exceeded that of the upper limit for effectiveness (FIG. 14). So, this was not considered to be a suitable time for application. Bactericide R115799 1% a.i. G therefore acts more efficiently as a preventive rather than a curative bactericide. Because of its protectant nature the persistence of the bactericide in the soil/water environment is important and was hence investigated further in Section 8.8 below, by applying bactericide at much earlier times before inoculation.

8.7. Effects of Different Nitrogen Levels on the Performance of Bactericide R115799 1% a.i. G Applied to Soil

8.7.1. Materials and Methods

Cultivar, soil, culture, planting layout, inoculation method and method of disease assessment were as in Section 8.2.1. The soil was treated with bactericide R115799 1% a.i. G at 2 kg a.i./ha together with fertilizers at different nitrogen levels, 0, 50, 100 and 200 kg N/ha, 21 days after sowing. The nitrogen was in the form of urea (46% N). All treatments received similar amounts of super-phosphate (50 kg P_2O_5/ha) and muriate of potash (40 kg K_2O/ha) which were applied at the same time. The plants were inoculated 3 times, 7 days after bactericide application (DAA), 12 DAA and 17 DAA, on the fully opened top leaves of all plants. For each time of inoculation, the leaves were assessed 3 times, 13 days after inoculation (DAI), 16 DAI and 19 DAI, using the scale in TABLE 5.

TABLE 70. MEAN DISEASE SCORES[1] ON RICE CULTIVAR SMII INOCULATED WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* AND TREATED WITH BACTERICIDE R115799 1% a.i. G AT DIFFERENT TIMES IN RELATION TO INOCULATION

Bactericide application time	Mean disease score
1. 7 DBI[2]	1.6 ± 0.5 a
2. 2 DBI	2.2 ± 0.4 b
3. 2 DAI[3]	2.3 ± 0.4 b
4. 7 DAI	3.4 ± 0.5 c
5. Control	4.1 ± 0.6 c

[1] : Mean of 16 disease scores based on a 0-5 scale of increasing disease (see TABLE 5) assessed at 13 DAI. Means with common letters are not significantly different at the 5% level. Those with different letters are significant at the 5% or 1% levels. Results were analysed by the non-parametric Friedman two-way analysis of variance by ranks (SIEGEL, 1956).

[2] : DBI = Days before inoculation.

[3] : DAI = Days after inoculation.

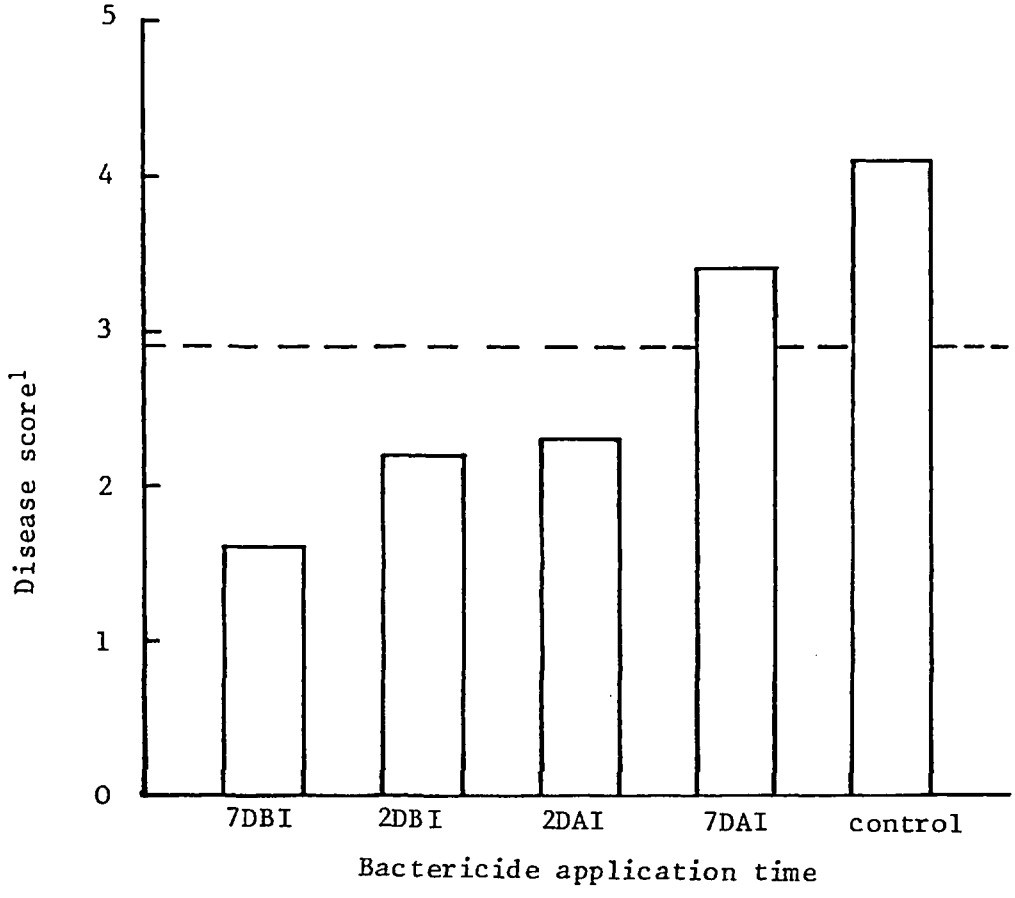


FIG.14. Effect of time of application of bactericide R115799 1% a.i. G in relation to time of inoculation on the development of bacterial blight disease (DBI = Days before inoculation; DAI = Days after inoculation. 1 = Assessed according to TABLE 5 at 13 DAI. - - = The upper limit of disease score (2.9) for effectiveness).

8.7.2. Results and Discussion

Nitrogen did not affect the disease significantly at 7 and 12 days after bactericide application (DAA) even when assessed 19 days after inoculation (DAI) (TABLE 71). However, at 17 DAA, the effects of nitrogen were significant (at the 5% level) at 16 DAI and 19 DAI (TABLE 71). Increasing the nitrogen level in the soil brought about more disease, there being no significant difference between 0 and 50 kg N/ha and between 100 and 200 kg N/ha (at the 5% level). Increase from 100 kg N/ha onwards increased the disease significantly (at the 5% level) to levels above the upper limit of disease score (2.9) for effectiveness, whereas levels below 50 kg N/ha did not seem to increase the disease. This suggests the need to use higher than normal rates of application of the bactericide for effective control of the disease in areas where large amounts of nitrogen is used.

8.8. Persistence of Bactericide R115799 1% a.i. G Applied to Soil

8.8.1. Materials and Methods

The experiment was conducted in a randomised complete block design with 2 replicates. Cultivar, soil, cultures, planting layout, inoculation method, and method of disease assessment were as in Section 8.2.1. The plants were fertilized with 100 kg N/ha in the form of urea, 50 kg P_2O_5/ha in the form of superphosphate and 40 kg K_2O/ha in the form of muriate of potash 20 days after sowing. The soil was treated with bactericide R115799 1% a.i. G at 2 kg a.i./ha at different times such that first and second inoculations of plants of all treatments were synchronised at 48 and 61 days after sowing respectively as follows:

TABLE 71. MEAN DISEASE SCORES[1] ON SUSCEPTIBLE RICE CULTIVAR SMII INOCULATED WITH A VIRULENT STRAIN OF *XANTHOMONAS ORYZAE* AT DIFFERENT TIMES AFTER TREATMENT WITH BACTERICIDE R115799 1% a.i. G (2 kg a.i./ha) AT VARIOUS NITROGEN FERTILIZER LEVELS, WITH RESULTS OF F-TEST AND DUNCAN's MULTIPLE RANGE TEST.

Nitrogen fertilizer level (kg N/ha)	Unprotected control	Inoculation time								
		7 DAA[2] (28 DAS)[3]			12 DAA (33 DAS)			17 DAA (38 DAS)		
		13 DAI[4]	16 DAI	19 DAI	13 DAI	16 DAI	19 DAI	13 DAI	16 DAI	19 DAI
0	0	1.6	2.7	3.6	1.7	2.3	3.2	1.5	1.7 a	2.7 a
50	0	1.9	2.8	3.4	1.6	2.3	3.3	1.5	1.6 a	2.7 a
100	0	2.6	3.2	3.7	1.8	2.7	3.4	1.6	1.9 ab	3.3 b
200	0	2.1	3.2	3.7	1.8	2.6	3.3	1.7	2.3 b	3.3 b
F-test										
Replicate		NS	NS	NS	NS	NS	NS	NS	NS	NS
Treatment		NS	NS	NS	NS	NS	NS	NS	*	*
Grand mean		2.0	3.0	3.6	1.7	2.5	3.3	1.6	1.9	3.0
Standard deviation		0.5	0.3	0.2	0.2	0.2	0.2	0.2	0.3	0.3
CV[5] (%)		25	10	7	15	4	5	16	8	5

[1] : Mean of 8 disease scores from 2 replicates, based on a 0-5 scale of increasing disease (see TABLE 5). NS = Not significant at the 5% level. * = Significant at the 5% level. Means with common letters are not significantly different at the 5% level.

[2] : DAA = Days after bactericide application.

[3] : DAS = Days after sowing.

[4] : DAI = Days after inoculation.

[5] : CV = Coefficient of variation.

Treatment	Time of bactericide application	
	1st inoculation	2nd inoculation
1	28 days before inoculation (DBI)	41 DBI
2	21 DBI	34 "
3	14 "	27 "
4	7 "	20 "
5	2 "	15 "
6	2 days after inoculation (DAI)	11 "
7	7 DAI	6 "
8	Control (no bactericide)	Control (no bactericide)

The water level in the troughs was kept at 1 cm throughout the experiment. All inoculated leaves were assessed at 13, 16 and 19 days after inoculation using the scale in TABLE 5.

8.8.2. Results and Discussion

There was no significant difference between the 2 replicates. The treatments showed significant effects (at the 5% and 1% levels) for all the 3 successive times of assessment (TABLE 72). The results confirm that the bactericide applied at 2 kg a.i./ha acted as a preventive bactericide, because it was not effective when applied 7 days after inoculation, the disease scores at the 3 assessment times being not significantly different (at the 5% and 1% levels) from that of the unprotected control. However, when applied as near as 2 days before and 2 days after inoculation, the disease was significantly reduced (at the 5% and 1% levels) compared to the unprotected control, which confirms similar results in Section 8.6. The bactericide remained effective within the infected leaves from 2 days up to 28 days after application, their disease scores being significantly less (at the 5% and 1% levels) than that of the unprotected control. However, at advanced stage of disease development (19 days after inoculation) the disease of plants treated at 21 to 28 days before inoculation (score 3.0), although significantly less (at the 1% level) than that of the unprotected control, appeared to be typical disease lesions capable of spreading. As such it can only be concluded that within the infected

TABLE 72. MEAN DISEASE SCORES[1] OF DIFFERENT TIMES OF BACTERICIDE (R115799 1% a.i. G at 2 kg a.i./ha) APPLICATION (1st INOCULATION) FOR THE CONTROL OF BACTERIAL BLIGHT DISEASE, ASSESSED AT 3 DIFFERENT TIMES, WITH RESULTS OF F-TEST AND COMPARISON BETWEEN THE DISEASE SCORES OF EACH OF THE 7 APPLICATION TIMES AND THE CONTROL USING THE LSD TEST[2]

Treatment (Time of bactericide application)[3]	Mean disease score		
	13 DAI	16 DAI	19 DAI
T_1 (28 DBI)	1.1 **	2.0 *	3.0 **
T_2 (21 DBI)	1.2 **	2.3 *	3.0 **
T_3 (14 DBI)	1.1 **	1.6 **	2.5 **
T_4 (7 DBI)	1.1 **	1.9 *	2.3 **
T_5 (2 DBI)	1.0 **	2.0 *	2.4 **
T_6 (2 DAI)	1.1 **	1.9 *	2.6 **
T_7 (7 DAI)	2.4 NS	3.1 NS	3.8 NS
T_8 (Control)	2.3	3.3	4.0
F-test			
Replicate	NS	NS	NS
Treatment	**	*	**
Standard deviation	0.6	0.6	0.6
Coefficient of variation	10.7%	18.0%	8.1%
LSD 5%	0.4	1.0	0.6
LSD 1%	0.5	1.5	0.9

[1] : Mean of 16 disease scores from 2 replicates, based on a 0-5 scale of increasing disease (see TABLE 5).

[2] : LSD = Least significant difference test. NS = Not significant at the 5% level. * = Significant at the 5% level. ** = Significant at the 1% level.

[3] : DBI = Days before inoculation. DAI = Days after inoculation.

leaves, the bactericide could persist and hence protect the leaves effectively when applied preventively within 2 weeks before infection.

The results of 2nd inoculation on fresh fully opened top leaves 13 days after the 1st inoculation (TABLE 73) show that the bactericide was very persistent in the soil/water environment. Even when applied 41 days before inoculation, it was still effective when assessed at 13 and 16 days after inoculation (DAI), disease scores being significantly lower (at the 1% level) than that of the unprotected control. However, at advanced stage of disease development (19 DAI), it persisted only up to 34 days after application. Even then, the disease score of 3.0 still would not guarantee an effective control of the disease at it was still a typical disease lesion capable of spreading. It would therefore be safe to conclude that the effective persistence limit of the bactericide in the soil/water environment was about 4 weeks after application.

TABLE 73. MEAN DISEASE SCORES[1] OF DIFFERENT TIMES OF BACTERICIDE (R115799 1% a.i. G at 2 kg a.i./ha) APPLICATION (2nd INOCULATION) FOR THE CONTROL OF BACTERIAL BLIGHT DISEASE, ASSESSED AT 3 DIFFERENT TIMES, WITH RESULTS OF F-TEST AND COMPARISON BETWEEN THE DISEASE SCORES OF EACH OF THE 7 APPLICATION TIMES AND THE CONTROL USING THE LSD TEST[2]

Treatment (Time of bactericide application)[3]	Mean disease score		
	13 DAI	16 DAI	19 DAI
T_1 (41 DBI)	1.4 **	2.5 **	3.1 NS
T_2 (34 DBI)	1.7 **	2.5 **	3.0 *
T_3 (27 DBI)	1.3 **	2.0 **	2.9 *
T_4 (20 DBI)	1.0 **	2.2 **	2.7 *
T_5 (15 DBI)	0.9 **	1.7 **	2.3 **
T_6 (11 DBI)	0.6 **	1.4 **	2.1 **
T_7 (6 DBI)	0.8 **	1.7 **	2.5 **
T_8 (Control)	3.2	3.8	4.2
F-test			
Replicate	NS	NS	NS
Treatment	**	**	*
Standard deviation	0.8	0.7	0.7
Coefficient of variation	17.3%	12.4%	15.8%
LSD 5%	0.5	0.7	1.1
LSD 1%	0.8	1.0	1.6

[1] : Mean of 16 disease scores from 2 replicates, based on a 0-5 scale of increasing disease (see TABLE 5).

[2] : LSD = Least significant difference test. NS = Not significant at the 5% level. * = Significant at the 5% level. ** = Significant at the 1% level.

[3] : DBI = Days before inoculation. DAI = Days after inoculation.

GENERAL DISCUSSION

The widely used Wakimoto's (1960) potato semisynthetic agar was shown to be still the best medium for copious surface growth of *Xanthomonas oryzae*. As such it was employed in most subsequent studies. However, its modified synthetic form without potato decoction but with addition of $FeSO_4.7\ H_2O$ shown by OU (1972) and KARGANILLA *et al* (1973) to be the best for single colony growth, was employed in studies involving platings and viable bacterial cell counts.

The variations in the resistance of rice cultivars in relation to variations in the virulence of isolates of *X. oryzae* suggest a vertical host-parasite relationship where cultivars showed differential (vertical) resistance and isolates differentiated into strains differing in virulence. Such a relationship according to VAN DER PLANK (1968) is of particular importance in breeding for resistance to disease.

The study emphasised primarily efficient and rapid techniques in strain typing and screening for resistance as part of the programme in breeding for resistance to the disease. Such techniques have been worked out in a preliminary manner for field, glasshouse and laboratory conditions. The mass pin prick and the guntacker methods of inoculation for seedlings and adult plants respectively are worthy of comments as they caused the least amount of physical damage to the plants and created well-placed inoculation points which resulted in well-formed lesions very suitable for assessment using the proposed disease assessment scale. Efficiency could be achieved by the use of the pin prick apparatus either for mass or single leaf inoculations at seedlings, maximum tillering and the flag leaf stages. This was facilitated by the simple 0-5 disease assessment scale based on easily recognised lesion types. Results could also be obtained rapidly by using the *in vitro* detached leaf culture, the technique of which has also been satisfactorily worked out. The technique was along lines adopted by WOLFE (1969) and RUSSELL *et al* (1975) where soft agar (0.5% ionagar) was used with 150 ppm of benzimidazole. However my studies showed that while a 150 ppm solution of benzimidazole was satisfactory

for a liquid medium, in soft agar addition of 0.25 concentration of HEWITT's (1952) Long Ashton nutrient solution performed better for rice leaves. HEWITT's (1966) 'low nitrogen' Long Ashton nutrient solution delayed leaf senescence even longer. The best method of inoculation for detached leaf culture (the scissors - basal dip method) was different from that of the best *in vivo* method (the mass pin prick method). It is obvious that the leaf segments in detached leaf culture required greater exposure to the inoculum in terms of greater number of severed vascular bundles and time of direct contact between inoculum and leaf segments, for good disease reaction. The *in vitro* method tended to give slightly greater disease reactions on the differential cultivars. This was mainly because infection *in vitro*, did not give typical disease lesions and assessment of disease reactions was based entirely on length of lesions rather than lesion types. However, despite these differences in disease expression, results when used for strain typing were strickingly similar. With the aid of these efficient and rapid techniques, large number of isolates obtained from different ecological environments could be tested on established set of differential cultivars to identify one or more strains with the widest spectrum of virulence which could then be used for screening promising breeding lines and new cultivars for disease resistance.

From past experience it was noted that a virulent strain may lose virulence or even die (KIRALY *et al.*, 1974) after constant successive sub-culturing over a period of time. Loss of virulence, according to FUJII (1976), can be due to increased proportions of avirulent mutants formed within original pure cultures. Similar indications were obtained from present studies. This would adversely affect disease resistance evaluations and hence justified investigation into simple and practical methods of maintaining and preserving the virulence of *X. oryzae* cultures for long periods. Such techniques should help maintain the original virulence of the strain used during the period between isolation of the pathogen, typing and immediate screening programmes for at least two seasons. Although freeze-drying or lyophilization is the most effective method for long term storage of bacteria (KIRALY *et al.*, 1974) the method may be impracticable because of the lack of facilities and also, from past experience, that *X. oryzae* preserved in this manner could not be recovered.

Results of studies on strain typing carried out at different times, simple culture maintenance techniques in the laboratory and the transmission and dissemination of the disease in the field, confirmed that the virulence of artificially cultured strains could, in the long run, become progressively weaker compared to its wild types. This strongly indicates the sensitiveness of *X. oryzae* and its vulnerability under artificial conditions.

Some common cultural practices like the use of seeds from severely infected plants as planting materials and cutting of leaf tips of seedlings before transplanting, were found to be able to initiate disease in the field. The initiation of disease is perhaps one of the important primary factors in the development of disease epidemic, the occurrence of which then depends on favourable ecological and environmental pre-disposing factors. Therefore in order to prevent disease epidemic, it is necessary initially to discourage the use of these and other cultural practices which might serve as primary factors favouring disease epidemics.

Chemical control of the disease has not achieved any measurable success despite constant efforts directed towards this end. This is partly because bactericides developed did not perform well under normal field conditions. This was probably due to failure to carry out bactericide screening trials under actual field ecological conditions and agronomic practices with which rice is normally cultivated in the tropics. The volume of water in the rice field and the amount of nitrogen fertilizer applied had been shown to affect significantly the effectiveness of bactericide, with the former tending to dilute the effective active ingredient applied while the latter tending to enhance disease (HO & LIM, 1977c, 1979). Using the maximum effective water depth of 1 cm and nitrogen fertilizer level not exceeding 50 kg N/ha, the new experimental bactericide R115799 1% a.i. G has been shown to be very effective against the disease from 2 kg a.i./ha and higher. It is obvious therefore that the rate of application of the bactericide would have to increase with increase in water depth and the amount of nitrogen fertilizer applied. Although its effective persistence in the soil/water environment was about four weeks after application, the

best time of application for effective control of the disease was about a week before infection which suggests the preventive nature of the bactericide. Its granular formulation facilitates manual field application and its protectant nature makes it suitable to be applied together with fertilizers at active to maximum tillering stages, during which bacterial infection of plants normally begins. It is recommended that the bactericide could be incorporated into granular fertilizers in the future to facilitate further effective control of the disease through fertilizer applications.

Effective control of the disease by preventive means depends very much on early detection of the disease or better still, of the pathogen in the plants or their environment at the pre-symptom stage which justifies investigations into methods of early detection of pathogen in the plants and environment. However as this is in the realm of disease forecasting, it was not dealt with in this study.

Development of effective bactericides, has always been hampered to a great extent by unacceptable phytoxicity and mammalian toxicity levels (HO, 1977; EGLI & STURM, 1980). The development of R115799 1% a.i. G as an effective non-phytotoxic bactericide against *X. oryzae* does not guarantee its commercial availability at the moment as it has still to undergo several other toxicity tests. Even if these obstacles were overcome, the high costs of such bactericides would certainly become a major deterrent to its widespread use by rice farmers in the tropics. Hence large-scale use of bactericides in the tropics would ultimately be confined to government agencies under their subsidy schemes which often operate only when epidemics occur. As such availability of effective bactericides would only guarantee an effective interim control measure pending change to new resistant cultivars in the following seasons. However, as pathogen tends to develop resistance to effective bactericides especially antibiotic-based bactericides (EGLI & STURM, 1980) and overcome cultivar resistance (VAN DER PLANK, 1963) with time, it is always safe and economical for rice farmers to integrate cultural preventive measures mentioned above with adoption of resistant cultivars and chemical control.

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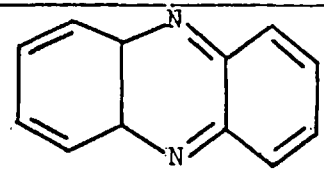
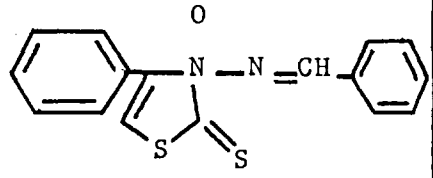
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APPENDIX

APPENDIX 1. TECHNICAL INFORMATION OF THE 3 BACTERICIDES UNDER STUDY

Trade name	Common name	Compound code no.	Formulation	Chemical name	Structural formula	Molecular wt.
1. Phenazin, Phenadine	Phenazine	Nil	Wettable powder (10% a.i.)	5-monoxi-phenazine		196.2
2. Celdion, Selzion	Fentiazon	Nil	Wettable powder (50% a.i.)	3-benzylideneamino -4-phenyl-1, 3-thiazoline-2-thione		296.4
3. N.A.	N.A.	R115799 (P2)	(i) Wettable powder (25% a.i.) (ii) Granular (1% a.i.)	Glyoxilic acid thiosemicarbazone "	N.A.	N.A.

N.A. = Not available.