DISCUSSION
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1. Isolates: Character and virulence

With the limited number of isolates of *Lanthomonas oryzae* obtained from different sources in this country and examined here from many angles it seems reasonable to assume that these isolates showed considerable variations in their *in vitro* physiological characteristics and pathogenic potential on susceptible varieties of *Oryza sativa*. Beyond these observations, it would seem hazardous to generalise on this pathogenic species of *Lanthomonas* and its variability in nature.

2. Infection

It is well known that bacteria enter their hosts through natural openings, nectaries and fresh wounds. Early in this investigation, several attempts were made to infect rice plants by spraying a heavy load of inoculum to the foliage followed by incubation in humid chambers for 24 to 48 h.

Infection under these conditions was erratic. Better infection was, however, obtained when the inoculated rice plants were subjected to water congestion by post-inoculation humidity treatment and flooding (experiment 1.4). According to Hildebrand (1942), even a single bacterium can initiate
the infection if it is placed under proper conditions inside the host. When the bacterium is placed on/inside the host, it must find immediately optimum surroundings and enough nutrients to multiply before succumbing to the host defensive reactions. The water congestion by pre-inoculation humidity and flooding treatments would naturally saturate the conductive system in the host with water and nutrients. When bacterium is sprayed under such conditions it finds a suitable environment to multiply and then enters through the natural openings, particularly through hydathodes. There are recent reports (Shekawat and Srivastava, 1971) on the blight infection that indicate entry of the pathogen through hydathodes rather than through stomata. This is in contrast to what is obtaining in the bacterial leaf streak infection. In the present investigation the best method of inoculation of rice plants was by injury (pin prick method) in which 100% infection was obtained. Other forms of mechanical injury can also give uniform infection (experiments 1.4 and 1.5). In nature, predisposing factors such as high humidity, flooding and natural wounds to rice plants during transplantation or cultivation are supposed to help in the spread of the disease.
3. Symptomatology

In natural infection, as well as in artificial spray inoculation, lesion-initiation is along the edges of leaf blades from where the pathogen seems to enter the host. The presence of a large number of hydathodes along the rice leaf margin has been demonstrated by Tabei and Muke (1960). Further, the lesions enlarge lengthwise faster than breadthwise. This can be explained by the anatomy of rice leaves which have parallel venation and secondly the bacteria are mainly confined to the vascular bundles. Thus, infection and lesion development are closely linked with the morphology and anatomy of the rice plant.

The bacterial mass can be readily seen under the microscope when an infected leaf is cut and mounted in water. Sometimes the bacterial oozing can be seen for hours after the first injury. The oozing can be noted only from the cut end of the vascular bundles (experiment 1.5). This shows the vascular nature of the disease. Similarly, the bacterial mass emerges out as a column in water if left undisturbed in a test tube. This oozing phenomenon has been useful in disease diagnosis. Wilting of leaves can occur in the seedling stage infection as also when symptoms appear in mature plants. Like in other typical wilt diseases, rapid water loss from the leaves characterizes
this disease. Occlusion of the bacterial mass and/or toxin production in the xylem elements seem to hinder water uptake. The water potential of the diseased leaf decreased before the symptoms became visible (experiment 1.5). Thus, as the disease progresses, the water content of the diseased leaves decreased rapidly.

Loss of pigment alongside the lesion is another main event. Quantitative estimation of chlorophyll in the lesion area as also in the entire infected lamina showed rapid fall in the amount of pigments in the diseased leaves.

The role of IAA and its accumulation in diseased plants has been studied in several plant wilt diseases. In the present investigation (experiment 2.4) it has been seen that all the isolates could synthesise and release IAA in vitro, when the medium was augmented with tryptophan. (A. oryzae is incapable of synthesising the indole structure). It is interesting to note that tryptophan has been reported from rice plants (Cagampang et al., 1971). Therefore, it seems reasonable to assume that A. oryzae can synthesise IAA in the rice host if tryptophan or any other indole precursor would be available in the host. Further work, however, is needed in this area to understand the exact role of IAA produced by A. oryzae in the blight disease of rice.
4. *In vitro* wilt inducing factors

In the present studies cell free culture filtrates of virulent isolates of *X. oryzae* were found to be viscous, (experiment 2.1). There are several reports on the production of high molecular weight compounds (polysaccharides and glycopeptides) by pathogenic bacteria, and they have been assigned a causal role in wilts (Husain and Kelman, 1958; Rai and Strobel, 1967; Sutton and Williams, 1970). The culture filtrate of *X. oryzae* could bring about wilting of cut leaves of rice as well as excised seedlings.

This wilt inducing factor in the culture filtrate could also cause wilting in excised shoots of cotton and tomato besides excised rice leaves (experiment 2.2). Steaming of the culture filtrate does not seem to destroy the wilt inducing ability. However, such induced wilting could be reversed upon transferring excised leaves/shoots to water. Among the two fractions (F-1 and I-2) obtained from alcoholic precipitation of culture filtrate, tested for wilt inducing property, only I-2 fraction acted as a wilting agent. However, both the fractions did not induce wilting of intact seedlings (experiment 2.3). The dialysate of culture filtrates failed to induce wilt in rice leaves (experiment 2.1). These observations suggested the presence
of a thermostable, non-specific wilt inducing factor which could be a high molecular weight compound present in the culture broth of *A. oryzae*.

The F-2 fraction has been shown to be a protein-free polysaccharide (experiment 3.2). By paper chromatography the purified extracellular polysaccharide was shown to contain D-glucose, D-mannose, D-glucuronic acid and glucuronolactone (experiment 3.4), similar to the one identified from the culture filtrates of *A. oryzae* by Misaki et al. (1962). CTAB-precipitate method was found to be more convenient to isolate the polysaccharide over the alcoholic precipitation method. The purified polysaccharide was tested for protein as a possible contaminant and found to be free from it. In the present studies the purified extracellular polysaccharide has been shown to bring about wilting of excised rice leaves (experiment 3.3), analogous to the action of culture filtrate on the host as stated in experiment 2.1.

The intrinsic viscosity of the toxin was very high suggesting molecular weight of the order of a million (experiment 3.6). Furthermore, the toxin moves as a single band in disc electrophoresis (experiment 3.5) slower than gum karaya (molecular weight 9,500,000). The migration as a single band seemed to indicate the possibility of the toxin comprising of a single molecular species. Kuo et al., (1970) working with their isolates of *A. oryzae*, reported more than
four kinds of molecular species from the extracellular polysaccharide fraction. In studies here, with polysaccharide as antigen, two precipitin lines (immunoserological) were obtained in comparison with toxins (experiment 5.2; Plate XIXb). These results indicate the multiple molecular nature of the extracellular polysaccharide fraction of *X. oryzae* (It would be premature to draw any conclusion on the nature of molecular species in the polysaccharide).

Infrared spectrum of the toxin (experiment 5.8) was typical of a carbohydrate and it could be stated that all the toxins examined here are essentially carbohydrates in nature.

The production of slime by bacteria has been shown to be controlled by several conditions (Goodman et al., 1967). The genetic factor of the bacterium and the environmental factors are of prime considerations in inducing slime production in bacteria. Husain and Kelman (1958) have demonstrated that some mutants of *Pseudomonas solanacearum* lost the slime producing ability along with virulence. In the present investigation a mutant of *X. oryzae* (M19) was obtained similarly by culturing a highly virulent isolate (M12) in 4-5 month old tryptone broth culture. This mutant lost the polysaccharide producing ability as also its agressive virulence. The mutant could, however, infect the rice plants but would
produce only mild attenuated symptoms (experiment 4.1).

The ability to produce polysaccharide by different isolates could be correlated with the virulence of the isolates in the present investigation (experiments 1.3 and 4.1). Similar findings were reported by Corey et al., (1957) in bean plants inoculated with *A. phaseoli* and Husain and Kelman (1958) in the wilt of tomato caused by *P. solanacearum*. There is, however, a report by Satoh (1966) that virulence is not correlatable with slime production in *A. oryzae* isolates with which he was experimenting. There is also a report by Soto and Okabe (1967) of mutants in culture with smaller, translucent, deep yellow colonies but with very weak pathogenicity. These could be non-polysaccharide producing types as the description of colony morphology resembles the non-polysaccharide producing mutant (M19) in this study. Thus, the genetic factor seems to be one of the basis in slime production in bacteria. Furthermore, it was found that polysaccharide production was greatly influenced by environmental conditions such as hydrogen ion concentration, amount of carbon source, concentration of inorganic salts in the medium and temperature (experiments 4.2, 4.3, 4.4 and 4.5). Anderson and Rogers (1963) have shown that in members of Enterobacteriaceae the effect of electrolyte concentration on slime production was profound and increased when the salt concentration in the medium was in between
0.4 to 0.5 M. Similarly, the production of polysaccharide in these studies with X. oryzae isolates reached an optimum level at salt concentration in between 0.3 to 0.5 M whether used individually or collectively as chlorides or sulphates of NH$_4^+$, K+, Na+ or Mg$^{++}$. The explanation offered for this by Goodman et al., (1967) is that it is an osmotic effect which activated the slime production pathway in bacteria and the results presented here take us to a similar conclusion.

5. In vivo production of slime

Polysaccharides and glycopeptides produced extracellularly in vitro by a variety of wilt inducing pathogens and their presence in diseased plants has been demonstrated (Husain and Kelman, 1958; Strobel, 1970; Sutton and Williams, 1970). In the present investigation the presence of slime produced by X. oryzae was demonstrated by immunoserosology (experiment 5.2), disc electrophoresis (experiment 5.3), infrared absorbance (experiment 5.4) and specific optical rotation (ORD-Spectrum) techniques (experiment 5.5). The presence of slime, produced by X. oryzae in vivo, has been demonstrated in experiment 5.1 by treating cut leaves of rice with tracheal fluids of diseased leaves.

By using the intragel cross absorption test in immunoserosology (experiment 5.3), the antigenic similarity
between the purified polysaccharide from culture filtrate of *X. oryzae* and that from infected rice leaves was established. This confirmed the presence of extracellular polysaccharide produced by *X. oryzae* in rice leaves infected by *X. oryzae*. The formation of two precipitin lines in the immunodiffusion test (Pl. XIXa) suggests that either the polysaccharide migrates through the agar as two different molecular forms or that the bacteria, both in *vitro* and *in vivo*, produced two different antigenic polysaccharides. Sutton and Williams (1970) have also made similar observations in the black rot of cabbage. Further, the polysaccharide appears to be unaffected by the healthy leaf extract, by the detection of two precipitin lines with antigens 1 and 3 (Pl. XIXb).

In disc electrophoresis (experiment 5.3) the toxin from both sources (from culture as well as from infected leaves) move as a single band to a uniform distance of 1.6 cm from the origin. Further, the mixture of toxins from both sources moved as a single band with the same electrophoretic mobility (experiment 5.3; Pl. XIX). The detection of a single band and migration of the band to a constant distance suggested a possible homogeneity of the two samples. In infrared spectra (experiment 5.4) and optical rotatory dispersion (experiment 5.5) both the samples were
identical. Therefore, by these evidences one may draw conclusion that the toxin produced in the diseased plant and the toxin produced in culture are more or less similar in chemical composition, structure and configuration.

It has been shown that the virulent isolates and the mutant differ in the amount of polysaccharide they produce in vitro (experiment 4.1). The rate of polysaccharide production by the two isolates (highly virulent M12, and weakly virulent M51) and the mutant (M19 non-polysaccharide producing and weakly virulent) was the same in vitro and in vivo (experiment 5.5).

The amount of the toxin necessary to induce wilt of leaves (experiment 5.7) depended on the age of the particular leaf. The younger the leaves, smaller the amount of toxin needed to wilt. It is also a fact that, the time taken to wilt is less in younger leaves than in older ones. This could be because the younger leaves are unable to prevent water loss compared to the older leaves and consequently water deficiency sets in earlier in the younger than in the older leaves.

6. Host nutrition and disease

The intensity of the leaf blight disease is known to be enhanced by heavy application of fertilizer (Tagami and Misumi, 1962). Excess nitrogen, phosphorus, silicon,
magnesium (IRRI, 1966) and potassium (Kim and Cho, 1970) are known to stimulate disease intensity. In the present investigation, it has been shown that addition of inorganic salts to the culture media (experiment 4.5) influences not only the in vitro polysaccharide producing ability but also the relative growth of *L. oryzae*. To test this finding in vivo, rice plants were fed with increased amounts of salts (NH₄NO₃, KCl, Na₂HPO₄ and MgCl₂) and inoculated with H12 (highly virulent) isolate and M19 (weakly virulent mutant). The intensity of the disease was found to be related to salt concentration supplied to the host (experiment 5.7, Part I).

The soluble polysaccharide fraction was estimated from healthy and infected plants (with H12 and M19) supplied with increased concentration of major elements (experiment 5.7 Part II). It was found that increase in the concentration of nutrient salts to the rice plants increased the amount of soluble polysaccharide produced in vivo when infected by the highly virulent isolate H12. On the other hand, there was no increase of soluble polysaccharide in plants infected with the non-polysaccharide producing non-wilt inducing mutant M19. These observations indicate the role of slime of *L. oryzae* in enhancing disease intensity with high fertilizer application.
Generally, pathological wilting due to in vivo production of phytotoxic polysaccharides and glycopeptides has been ascribed to increased viscosity of tracheal fluid. According to Dimond (1970) the viscosity could not be the sole cause of wilting, since viscosity must double to reduce the rate of flow to one half, if pressure remained unchanged. For such large molecules to be transported and then to induce wilting, the molecular weight of the metabolite is of prime consideration. Hodgson et al. (1949) have shown that large molecules remain entrapped in conductive elements and induce loss of turgor of stem and petioles. Lower size range molecules accumulate in the margin of leaves where they produce localized withering and drying. Strobel and his co-workers (Strobel and Hess, 1969; Strobel, 1970; Rai and Strobel, 1971) have demonstrated the transport of glycopeptides of Corynebacterium in this manner and they have further shown that destroy structural integrity of cellular, chloroplast and mitochondrial membranes.

Dimond (1970) concluded that the polysaccharides and glycopeptides produced by pathogens in the host can affect the water economy of plants in two ways. A portion of them may accumulate in pit membranes of vessels which act as ultrafilters. Such entrapment will reduce lateral and longitudinal flow of water through the system. Secondly, smaller molecules transported to leaves disrupt water
transfer from veins and accumulate in membranes of parenchymatous cells where they cause damage which appears as marginal drying.

Thus, the slime produced by *A. oryzae* is not without any significant role in inducing the wilt syndrome in rice plants. In these experiments, the extracellular polysaccharide of *A. oryzae* has been obtained from diseased rice plants and the part played by it in the disease syndrome has been demonstrated. To classify the extracellular polysaccharide of *A. oryzae* under vivotoxins needs further experimentation on the chemical characterization of the toxin and its unmistakable identity from *in vivo* isolations.
SUMMARY
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Some aspects of the bacterial leaf blight of rice disease caused by *Xanthomonas oryzae* have received attention in this thesis. The highlights of the findings are summarized as follows:

1. Variability and virulence of different isolates of *X. oryzae* collected from different localities were found to differ in some of their physiological characters and virulence.

2. Pre-disposing factors like high humidity (especially pre-inoculation humidity treatment) flooding and mechanical injury to plants were found to increase the degree of infection. Pin-prick method of inoculation of the rice plant was found to be the most reliable method of inducing successful infection.

3. Heavy loss of chlorophyll pigments and water were the main physiological disturbances in the diseased plants.

4. The toxic factor(s) in cultures of *X. oryzae* have been demonstrated.

All the five isolates employed in this study were found to synthesize the auxin, indole-3-acetic acid, *in vitro* when tryptophane was supplied. This indicated a possible role in altering the water economy of diseased plants.

A thermostable, non-specific, alcohol precipitable high molecular weight substance was detected in the culture
filtrates of _A. oryzae_, which caused wilting of excised rice leaves and excised seedlings.

5. Some of the properties of the alcohol precipitable substance have been studied and found to be a high molecular polysaccharide made up of glucose, mannose and glucuronic acid. Isolation and purification methods have been standardized to some extent but much more has to be done in this area.

6. Genetic as well as environmental factors were found to be responsible for the production of _in vitro_ extracellular polysaccharide (toxin). All the isolates varied in the amount of toxin production according to their virulence. A mutant strain of _A. oryzae_ which had lost the ability of polysaccharide production _in vitro_ has been obtained and included for studies with virulent strains. _In vitro_, effect of pH, temperature, sugar concentration and osmotic effects due to salt concentration in media were found to have a marked effect on toxin production.

7. Presence of the toxin in active form in diseased leaves has been demonstrated. The soluble polysaccharide fraction from the transeal fluid of diseased leaves was obtained and confirmed to be identical with the extracellular polysaccharide of _A. oryzae_ produced in cultures by immuno-serological cross adsorption tests, disc electrophoretic and spectral analysis.
8. Increased supply of nutrient salts (macroelements) to the rice plants was found to enhance the wilting of diseased rice leaves. This has been partly ascribed to the \textit{in vivo} increased toxin production under high salt concentration in the diseased plants. The role of the extracellular polysaccharide in inducing wilting of rice leaves has been discussed.
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