BACTERIAL LEAF BLIGHT OF RICE PLANT. III. Phytotoxic polysaccharides produced by *Xanthomonas oryzae*[1]

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Abtsract

A toxic slime substance capable of causing wilting on rice cuttings has been isolated from cultures of *Xanthomonas oryzae*. At the concentration of 10 ppm the purified toxin causes the rice cuttings to wilt in one hour. The toxin is nonspecific. It is heat resistant and water soluble polysaccharides. It could be separated into four polysaccharide fractions when purified with Sephadex G-200 column chromatography. The estimated molecular weights of the four fractions were $\geqslant$200,000, 153,000, 147,000 and 29,200 respectively. The first three fractions are antigenic and phytotoxic, whereas fraction 4 lacks these properties. The sugar residues of the crude toxin and fraction I are mannose and glucose.

Introduction

One of the most destructive damage of rice plants caused by *Xanthomonas oryzae*, a pathogen of bacterial leaf blight, is wilting of seedlings. In Japan high rate of wilting has been observed, when rice seedlings were immersed in the water contaminated with *X. oryzae*, (Sekitani and Hisahara, 1958). Since infected seedlings wilt and die rapidly without showing any other symptoms, the damage is very often neglected in the fields. The mode of action of this pathogen have not been definitely determined. Two theories have been proposed to explain the wilting mechanism of this type of diseases, e.g.,: (1) occlusion of water conducting tissues by bacterial mass (2) the production of a systemic toxin by the pathogen that disturbs the osmotic system of the leaves.

Recently, Tabei (1968) based on histological studies of the tissues infected

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by *X. oryzae* suggested that the wilting of rice leaves is due to the plugging of xylem vessels by the secondary colonization of infecting bacteria. However, accumulated evidences strongly suggest that this type of wilting symptom is caused by the phytotoxic polysaccharides produced by phytopathogenic bacteria (Ferder and Ark 1951; Hodgson *et al.* 1947; Hodgson *et al.* 1949; Strobel 1967.). Same kind of polysaccharides were also demonstrated in the infected plant tissues (Rai and Strobel 1969; Spenser and Gorin 1961).

X. oryzae produces large amount of slime substances in culture medium. When the cuttings of rice seedling were placed in the supernatant from cultures of this organism they wilted rapidly. In this work, the slime subtances were further purified and their biological, chemical and physical properties were studied.

Materials and Methods

Bacterium strain, medium and cultivation: Xanthomonas oryzae strain 500 was obtained from the culture collection in our Institute. They were maintained on PS agar medium at 0°C. The composition of PS medium has previously been described (Kuo *et al.* 1967). For production of toxic polysaccharides, the cultures were grown in one-liter Erlenmeyer flasks containing 250 ml of PS liquid medium and were incubated at 25°–30°C on rotary shaker for one week.

Purification of toxic polysaccharides:— The crude toxic polysaccharide was prepared by centrifugation of the cultures at 5000×g. After centrifugation the supernatant was evaporated to one fifth of the original volume and the polysaccharide was precipitated by adding 3 volumes of ethanol. The precipitate was harvested by centrifugation, redissolved in distilled water and the crude polysaccharide solution was treated with Amberlite 1 R 120 and Dowex 1. Resins were removed by centrifugation, and the supernatant was saved and evaporated to a small volume, then the polysaccharide was reprecipitated with ethanol and dried. Further purification was carried out by placing 1 mg of crude polysaccharide preparation on a 1.5 × 20 cm column of Sephadex G–200 and eluted with H_2O. Polysaccharide in each fraction was detected by the method described by Dubois *et al.* (1956) and the ability of causing the wilting of rice cuttings in each fraction assayed.

Bioassay:— The biological activities of polysaccharides were determined by the time required for a cutting to begin wilting. Uniform two-week old rice seedlings were removed from soil and thoroughly washed with tap water, then the cuttings were severed under sterile tap water. In order to prevent the spontaneous wilting of rice cuttings in distilled water, a solution containing 1.4 mM $MgSO_4$, 22 mM KH_2PO_4, 10 mM NaCl (MKN solution), was used, The cuttings were immersed in the MKN solution for 2 hr before they were

placed into various test solutions. Five cutting were placed in each testing solution, and the MKN solution was used as control. Cuttings in the test solutions were held in a growth chamber with 6,000–8,000 lux light at 30°C and at a relative humidity of approximately 70 percent. At the end of the designated test periods, the cutting were examined for symptom of wilting. All experiments on cuttings were repeated at least 3 times.

Specificity of toxin:— Specificity of the crude toxin was determined by treating two-week old plant cuttings of several species under conditions mentioned above. The cuttings were treated in different concentrations of toxin solution (1–1000 ppm). Wilting was recorded when the margines of leaves became flaccid.

Serological Analysis of toxin:— Antiserum of *X. oryzae* strain 500 was prepared by the method described previously (Lin *et al.*, 1969). Ring precipitation test was carried out in capillary tubes. The antiserum of strain 500 in a two fold dilution series were mixed with the crude and the further purified toxion fractions respectively. Normal serum and saline buffer were used as controls.

Acid Analysis:— Ten ml of 1.0 N sulfuric acid was added to a 20 ml test tube containing 10 mg of toxin. The mixture was hydrolyzed in sealed ampules at 100°C for 8 hr, then cooled at room temperature and neutrolized with excess barium hydroxide. The supernatant was concentrated with evaporator.

Analysis of sugar residue:— Separation and identification of sugar residues were done by one-dimensional descending paper chromatography on Whatman No 1 filter paper in the following solvents systems: (1) n-butanol: acetic acid: water (4:1:5 v/v) (2) ethyl acetate: pyridine: water (36:10:11 v/v) (3) n-butanol: acetic acid: saturated boric acid solution (9:1:1 v/v). Sugars were detected with benzidine and p-anisidine HCl (Herbert *et al.* 1965).

Molecular weight estimation:— Column chromatography with Sephadex G-200 was used for molecular weight estimation of polysaccharides (Granath, 1965). The molcular weights of polysaccharides were estimated according to the formula: $\frac{Ve}{Vt}$ = 3.20–0.58 log molecular weight, where Ve equals to the volume at which the sample is eluted from the column minus the void volume of the column, and Vt equals to the total volume of the column.

Results

Effect of culture filtrate and crude toxin on rice cuttings:— The cuttings of rice seedling placed in the culture filtrate of *X. oryzae* exhibited wilting symptom after one hour and were completely wilted at the end of 16 hours. The first symptom is an inward curling of the leaf margine, followed by the complete

loss of turgor and shriveling of the leaves. Some times necrosis of margine could be observed when cuttings were placed in low concentration of toxin for a longer period of time. No wilting developed in cuttings when they were placed in MKN solution. The different concentrations of crude toxin were also tested. As indicated in Fig. 1, at the concentration of 1 ppm the rice cuttings wilted after 16 hours. After the wilting was examined, the basal 2 cm of each stem was trimmed off and the cuttings transferred to MKN solution so that possible recovery of turgor could be checked. The result showed that once the wilting was detected the leaves never recovered from wilting.

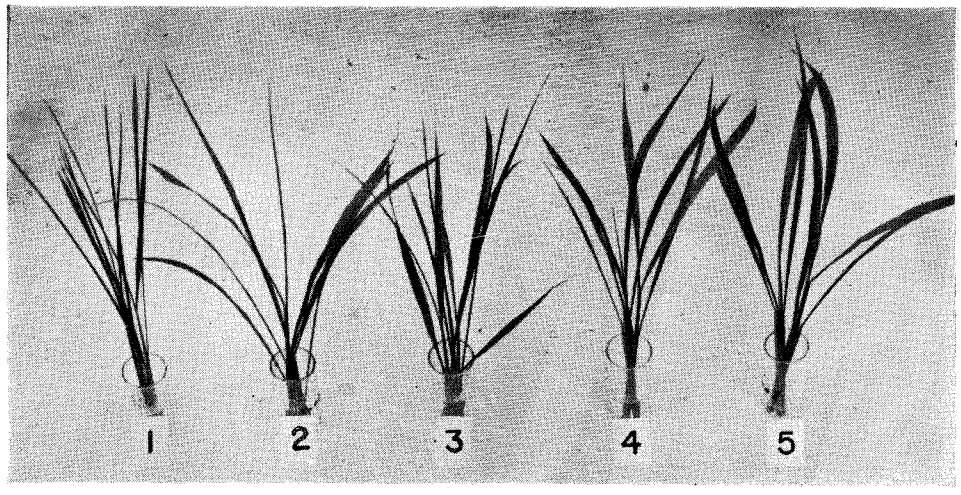

Fig. I. Effect of different concentrations of partialy purified toxin on rice cuttings. (1) 1000 ppm, (2) 100 ppm. (3) 10 ppm. (4) 1 ppm. (5) control. The wilting was examined after 16 hr.

The cuttings taken from mature plants wilted much more readily than did those from young seedlings. The cuttings from two-week old plants placed in solutions containing 100 ppm crude toxin wilted after three hours, whereas the cuttings from two-month old plants placed in the same concentration of toxin solution wilted after 30 minutes. An aliquot of the culture filtrate was dialyzed against distilled water to remove any toxic compounds of low molecular weight. No wilting was observed on rice cuttings placed in dialyzates of culture filtrate, while cuttings in the dialyzed materials wilted as rapidly as those in the culture filtrate.

Stability of crude toxin:— The effect of heat on crude toxin was determined. The crude toxin in MKN solution was heated in boiling water for 10 minutes then diluted (10 to 1000 ppm); and their wilting-inducing abilites compared with that of nonheated toxin. It was found that there were no difference between the heated and nonheated crude toxins. The stability of crude toxin

in weak acid and alkali was also examined. The crude toxin dissolved in 1 N HCl or 1 N NaOH solution were kept in room temperature for 24 hours, then neutrolized to pH 7.0 and dialyzed against MKN solution. After that the wilting-inducing abilities were compared with the nontreated toxin with various concentrations. Again no significant difference was observed.

Host specificity:— Host specificity of the crude toxin was determined by treating the cuttings of two-week old seedlings of spinach, lettuce, mung bean, barley, tomato, wheat, oat, watermelon, corn, soybean, mustard, broad bean, cowpea, and turnip with different concentrations of toxin solution. As indicated in Figure 2, rice plants, mustard, soybean, watermelon, broad bean, oat, turnip and spinach were the most sensitive ones, barley, wheat, mung bean were intermediate, while lettuce, tomato and corn were resistant ones.

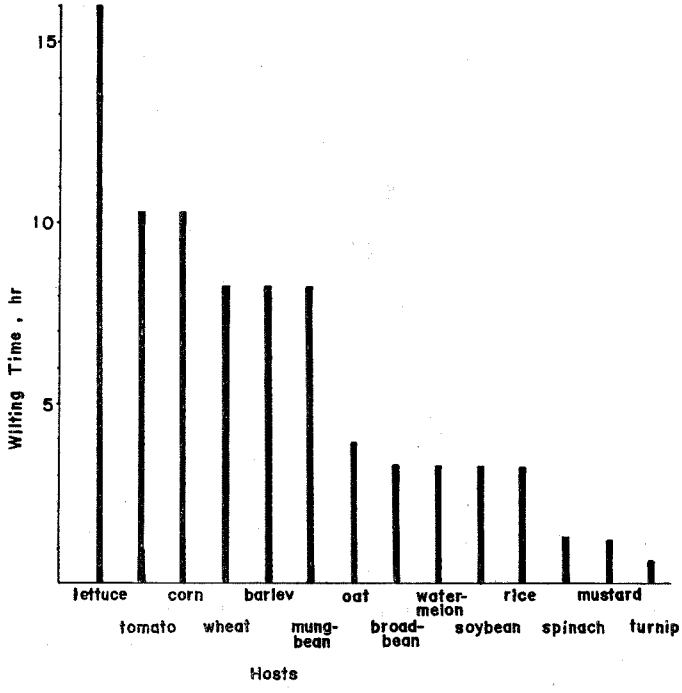

Fig. 2. Host specificeity of the partially purified toxin of *Xanthomonas oryzae*. Toxin concentration used was 100 ppm.

Purified toxic polysaccharides:— The average yield of crude polysaccharide was 2 g/liter. After passing through Sephadex column, approximately 70% of polysaccharide preparation was recovered as toxic polysaccharide. A typical elution curve of the separation of polysaccharides is shown in Figure 3. The ratio of these four fractions was fraction I 86%, fraction II 5%, fraction III

5% and fraction IV 4%. The specific toxicities of crude toxin and four purified toxin fractions were also compared. As indicated in Table 1, the specific toxicity was increased after purification. At 10 ug/ml of polysaccharide concentration, the crude toxin required 4 hours to cause the wilting of rice cuttings whereas fraction I, and II, required only 1 hr. However, fraction III was less toxic and no toxicity was detected from fraction IV.

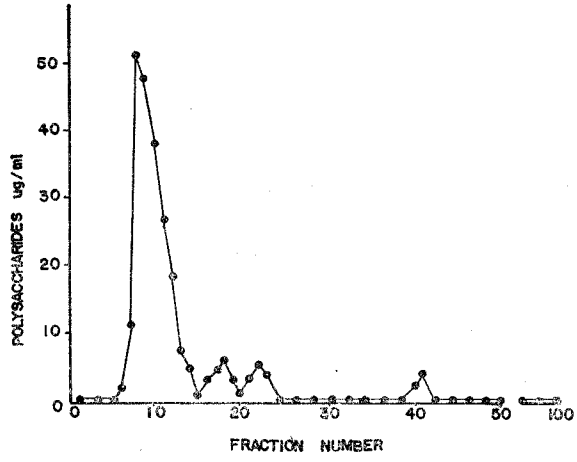

Fig. 3. The separation of toxic polysaccharides by Sephadex column chromatography. 1 mg of a crude preparation was placed on a Sephadex G-200 column and eluted with distilled water. Fractions (1 ml) were collected and polysaccharides were quantitatively measured by the method of Dubois *et al.* (1956) The total mg of polysaccharide in each tube is plotted against the tube number. Toxicity of each fraction was also assayed by the rice cuttings.

Table 1. *Comparison of the toxicities of the crude toxin and purified polysaccharide fractions.*

Polysaccharide factions	Specific toxicity* Hours
Crude preparation	4
Fraction I	1
Fraction II	1
Fraction III	8
Fraction VI	0

* Specific toxicity: the time required for the wilting of rice cuttings at 10 ppm polysaccharide concentration.

Molecular Weight:— Fraction I eluted at void volume of the column hence its molecular weight is 200,000 or greater. The estimated molecular weights of

the fraction II, III, IV are 152,000, 147,000, and 29,200 respectively.

Antigenic properties of toxic polysaccharides:— The results of the ring precipitation test between toxic polysaccharides and antiserum of *X. oryzae* showed that these polysaccharide fractions were antigenic. The crude toxin and fraction I yielded a precipitin reaction up to a 8 fold and 2 fold dilution of the antiserum respectively, whereas fraction II, III reacted only with concentrated antiserum.

Sugar residues of toxic polysaccharide: Since fraction I possessed about 86% of the total polysaccharide recovered from Sephadex column, it was used for sugar residue analysis. Only two spots were detected on paper chromatography, and were indentical to glucose and mannose in three solvent system. The ratio of glucose: mannose was 54:46.

Discussion

The most commonly accepted theory for wilting caused by polysaccharides produced by plant pathogenic bacteria is the plugging of the vessels in the stem (Buddenhagen and Kelman 1964). Recently Strobel (1967) has isolated a physiologically active polysaccharide from the culture of *Corynebacterium sepedonium*, the amount of the toxic polysaccharide taken up by a 7.5 cm tomato leaf prior to wilting was only 50 μg. which seemed to be too small an amount to cause plugging. Thus he suggested that the mechanism of wilting was not the plugging of xylem vessels. The polysaccharide isolated from *Xanthomonas oryzae* was also very toxic, only 10 ppm of toxin was required to cause the wilting of rice cutting in 1 hours. Although no direct comparison was made between toxins produced by *X. oryzae* and *C. sepedonium*, from the data of quantitative requirement of toxic polysaccharide to cause the wilting of respective host plants, the *X. oryzae* polysaccharide seemed more toxic than that of *C. sepedonicum*. Our evidence prevalently supported Strobel's suggestion and an explaination other than plugging seems to be required.

The toxic polysoccharide is nonspecific, but those plants tested showed different degrees of sensitivity. Among 14 kinds of plant tested, turnip, spinach and rice plant are the most sensitive ones. At the concentration of 1 ppm the toxin is still capable of causeing wilting of these plant cuttings. Lettuce, tomato, and corn were the resistant ones, they would not show any symptom even at 10 ppm of toxic polysaccharide solution. The effect of toxin on monocotyledons and dicotyledons seems different. When rice, wheat, barley and corn were tested, the wilting was irreversible, however, when tomato, watermelon, mustard and mung bean were tested, the cuttings became turgid after they were transfered to water.

Futher purification of the polysaccharide showed that more one polysaccharides were present in crude toxin preparation. Although only four polysaccharide fractions were obtained by Sphedex G-200 column chromatography, there might actually be more. Since fraction I came out in front of eluent, it is generally considered to be a group of polysaccharides with molecular weight larger than 200,000. Acid hydrolysis followed by chromatography of crude polysaccharide preparation revealed that two reducing compounds were present, they are glucose and mannose in a ratio of 55:46. The composition of the polysaccharide obtained is much simpler than that of polysaccharides isolated from other bacterial cultures.

水稻白葉枯病之研究 III.
病原細菌所產生之多醣類毒物質

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自水稻白葉枯病病原細菌之培養液中可分離出能使水稻萎凋之多醣類毒物質。此毒物經酒精沈澱及 Sephadex G-200 之 column 純化後能產生分子量大小不同之四種多醣類物質。其分子量各爲 ≥200,000，153,000，147,000，29,200，前三種不但具有毒性而且具有抗原性 (antigenic)。最後一種則二者都缺如。經純化之毒物質，在10 ppm 之濃度下能使水稻切枝枯萎。此類毒物質對寄主植物無特異性，但水稻系較敏感之一種。對熱之抵抗性頗强。經强酸水解後得悉此多糖類物質係由相等量之甘露糖及葡萄糖所構成。

Literature Cited

BUDDENHAGEN, I, and A. KELMAN. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Ann. Rev. Phytopathology **2**: 203-230, 1964.

DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH. Colorimetric method for determination of sugars and related substances. Analy. Chem. **28**: 350-356, 1956.

FEDER, W. A., and P. A. ARK. Wilt inducing polysacchairdes derived from crown gall, bean blight and soft-rot bacteria. Phytopathology, **41**: 804-808, 1951.

GRANATH, K. A. Gel filtration, fractionation of dextrin, Vol. V. p. 20-28. In V. R. Whistler (ed). Methods in carbohydrate chemistry. Academic Press, New York. 1965.

HERBRRT. L., M. M. GRAINGER and A. N. SIAKOTOS. Polysaccharide and glycoprotein formation in the Cockroach. I. Identity and titer of bound monos. J. Bio. Chem **240** 594-602 1965.

HODGSON, R., A. J. RIKER and W. H. PETERSON. A wilt-inducing toxic substance from crown-gall bacteria, Phytopathology, **37**: 301-318, 1947.

HODGSON, R., W. H. PETERSON and A. J. Riker. The toxicity of polysaccharides and other large molecules to tomato cuttings. Phytopathology, **39**: 47-62, 1949.

KUO, T. T., T. C. HUANG, R. Y. WU and C. M. YANG. Characterization of three bacteriophages of *Xanthomonas oryzae* (Uyeda et. Isiyama) Dowson. Bot. Bull. Acad. Sinica, **8**: 246-254, 1967.

LIN, B. C., C. C. LI. and T. T. KUO. Bacterial leaf blight of rice, 1. Serological relationships between virulent strain and weakly virulent strain of *Xanthomonas oryzae* Bot. Bull. Acad. Sinica, **10**: 130-133, 1969.

RAI. P. V. and G. A. STROBEL. Phytotoxic glycopeptides produced by *Carynebacterium michiganense*. II. Biological properties. Phytopatology, **59**: 53-57, 1969.

SEKITANI, N. M. and Y. M. HISAHARA. Studies on the infection of bacterial leaf blight on rice seedling, the amount of inoculum and inoculation method. Ann. Phytopathol. Soc. Japan **23**: 9, 1958.

SPENCER, J. F. T. and P. A. J. GORIN. The occurrence in the host plant of physiologically active gums produced by *Corynebacterium insidiosum* and *C. sepedonicum*. Can. J. Microbiol. **7**: 185-188, 1961.

STROBEL, G. A. Purification and properties of a phytotoxic polysaccharide produced by *Corynebacterium sepedonicum*. Plant Physiol. **42**: 1433-1441, 1967.

TABEI, H. Anatonical studies of rice plant affected with bacterial leaf blight, with special reference to wilt symptom. Ann. Phytopothol. Soc. Japan **34**: 137-139, 1968.