Static Suppression of Tomato Bacterial Wilt by Bacterial Coagulation Using a New Functional Polymer That Coagulates Bacterial Cells and Is Highly Biodegradable

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Tomato bacterial wilt by *Ralstonia solanacearum* was suppressed by coagulation of bacterial cells without disinfection using a copolymer of methyl methacrylate with *N*-benzyl-4-vinylpyridinium chloride in a molar ratio of 3:1 (PMMA-co-BVP) as a polymeric coagulant for bacterial cells. When 10 mg/kg of PMMA-co-BVP was added to soil before transplanting of tomato seedlings, and 2 mg/kg was supplemented once a week after transplanting, a 51% reduction of appearance and a 54% reduction of index of symptoms were observed. PMMA-co-BVP did not exhibit bactericidal activity against *R. solanacearum*, and coagulation of the bacterial cells appeared to reduce the opportunity for infectious contact of roots of tomato with cells of *R. solanacearum*, and resulted in disease suppression. PMMA-co-BVP was shown to be highly biodegradable, and the half-life was 5.1 d when treated with activated sludge in soil.

Key words: tomato bacterial wilt; *Ralstonia solanacearum*; biodegradable synthetic polymer; coagulation of bacterial cells; poly(methyl methacrylate-co-*N*-benzyl-4-vinylpyridinium chloride)

Control of soil-borne plant diseases is a difficult subject in agriculture. The conventional method for controlling these diseases is soil disinfection by fumigation with methyl bromide, chloropicrin, or other related chemicals as fungicides. But methyl bromide will be prohibited from use before 2005 because of its property to destroy the ozone layer. Chloropicrin is not desirable because of its severe toxicity. Therefore, an alternative method to control these diseases is urgently required, and biological control has received increasing attention, for example, in the control of tomato bacterial wilt. In our research group, we are attempting to develop a new green chemical method for controlling soil-borne plant diseases taking protection of the natural environment carefully into consideration. For this purpose, we avoid chemical fungicides, because indiscriminate disinfection of microorganisms in soil is not desirable for protection of the environment. We also avoid chemical materials having poor biodegradability, because they remain unchanged in the natural environment for a long period.

We have previously reported control of tomato bacterial wilt by *Ralstonia solanacearum* without disinfection using an equimolar copolymer of *N*-benzyl-4-vinylpyridinium chloride (BVP) with styrene (PBVP-co-ST) that captured bacterial cells alive on the surface and was highly biodegradable. Tomato bacterial wilt was controlled by the addition of sawdust coated with PBVP-co-ST prior to transplanting of tomato seedlings. In this study, we attempted to develop another methodology for controlling tomato bacterial wilt by coagulation of pathogenic bacterial cells in the soil.

In a previous report from this laboratory, we described coagulation of various microbial cells by poly(*N*-benzyl-4-vinylpyridinium chloride) (PBVP). But this polymeric coagulant is not suitable for the purpose of the present work, because PBVP exhibits strong antimicrobial activity, and we have attempted to use a copolymer of methyl methacrylate with *N*-benzyl-4-vinylpyridinium chloride (PMMA-co-BVP). We intended to exclude the antimicrobial activity of PBVP, keeping the characteristic of polymeric coagulant for microbial cells, by incorporation of an appropriate amount of methyl methacrylate into the polymer chain. We expected biodegradability for PMMA-co-BVP with confidence, because poly(methyl methacrylate) became biodegradable by incorporation of a small amount of *N*-benzyl-4-vinylpyridinium chloride into the polymer chain.
Materials and Methods

PMMA-co-BVP. Copolymer of methyl methacrylate with 4-vinylpyridine in a molar ratio of 3:1 (PMMA-co-BVP) was prepared by free radical copolymerization of methyl methacrylate with 4-vinylpyridine using 2,2'-azobisisobutyronitrile as an initiator in ethanol, and was converted to PMMA-co-BVP by a reaction with benzyl chloride in ethanol according to the procedure described in a previous report. Chemical analysis showed that PMMA-co-BVP contained 74.6 mol% of methyl methacrylate, 24.3 mol% of N-benzyl-4-vinylpyridinium chloride, and 1.1 mol% of 4-vinylpyridine. The weight average molecular weight determined by GPC analysis was 81,000.

*R. solanacearum*. Commercial products of triphenyl tetrazolium chloride (TTC), polymyxin B sulfate, chloromycetin, polypeptone, casamino acid, crystal violet, and other chemicals were used without further purification. *R. solanacearum* strain OE1-1 obtained from Dr. R. Ishikawa (2) of Takeda Chemical Industries, Ltd., Osaka, Japan, was used. Cells were precultured at 30°C for 3 days on an agar plate containing TTC medium prepared by dissolving polypeptone (10 g), casamino acid (1.0 g), sucrose (6.0 g), TTC (50 mg), and agar (16 g) in 1,000 ml sterilized distilled water. Pathogenic germs were distinguished by the formation of fluidal white colonies, and were cultured in a growth medium prepared by dissolving polypeptone (10 g), casamino acid (1.0 g), sucrose (6.0 g), TTC (50 mg), and agar (16 g) in 1,000 ml sterilized distilled water. The soil thus washed was sterilized by autoclaving at 121°C for 180 min. After 2 days, the autoclaving was repeated once again just before the experiments in biological degradation of PMMA-co-BVP.

In this study, artificial sewage (13) was used to assist biological degradation of PMMA-co-BVP. It was prepared according to a recipe in the literature. Peptone (6.0 g), meat extract (4.0 g), urea (1.0 g), sodium chloride (0.30 g), potassium chloride (0.14 g), calcium chloride (0.14 g), magnesium sulfate (0.10 g), and disodium hydrogenphosphate (1.0 g) were dissolved in 1,000 ml distilled water, and pH was adjusted to 8.5. The chemical oxygen demand (COD) of this undiluted solution was about 10,000 mg/l. The solution was diluted with an appropriate amount of distilled water to prepare artificial sewage of prescribed COD concentration.

Biological degradation of PMMA-co-BVP was performed by treatment with activated sludge in soil. A polymer sample (100 mg) was dissolved in a mixed solvent of toluene with ethanol in a volume ratio of 7:3 (10 ml), and the polymer solution was mixed with the purified soil (50 g). Thus, the content of the polymer sample in the dried soil was 2.0 g/kg. The mixture was then placed in a desiccator, and the solvent was removed by drying under reduced pressure to constant weight.

Washed activated sludge (710 mg in wet weight corresponding to 50 mg in dry weight) and artificial sewage (1.0 ml) with a COD concentration of 10,000 mg/l were mixed with the dried soil (50 g) containing 100 mg of the polymer sample. Since 1.0 ml of the artificial sewage contained 10 mg of COD, and weight of the polymer sample was 100 mg, and the amount of organic materials in the artificial sewage added to 1 g of polymer sample was 100 mg as COD. The total amount of water in the test soil was set to 180 g/kg. The mixture was allowed to stand at room temperature.

After a prescribed time, the remaining polymer sample was recovered by Soxhlet extraction using a mixed solvent of toluene with ethanol in a volume ratio of 7:3 for 30 hours. Fine soil particles contained in the extractive were removed by centrifugation three times at 2,000 g. The supernatant was placed in a rotary evaporator, and the mixed solvent was removed by evaporation. Ethyl acetate was added to the residue, and the recovered polymer was precipitated. The precipitated polymer was isolated and dried to a constant weight under reduced pressure.
Tomato. Seeds of tomato (Lycopersicon esculentum Mill. cv. Momotaro) were purchased from Takii Seed Corporation, Kyoto, Japan. The seeds were sowed on wet sterilized absorbent cotton that was placed in a laboratory dish. The dish was allowed to stand in a growth chamber for about 7 d. After this procedure, young seedlings with two leaves were obtained and submitted for the pot test. The operation conditions of the growth chamber are described below.

Suppression of tomato bacterial wilt by PMMA-co-BVP. Test soil for the experiments in disease control was obtained from a farm of the Kyoto Institute of Technology, and was mixed with that obtained from the shore of Lake Biwa in a weight ratio of 4:1, and 30 wt % of vermiculite was added to the mixed test soil. The mixture was sterilized by autoclaving at 121 °C for 180 min. After 2 d, the autoclaving was repeated once again. Harvested cells of R. solanacearum were mixed with test soil in a planter and kept in a growth chamber. Young tomato plants at the two-leaf stage were transplanted into the test soil containing R. solanacearum and kept in a growth chamber for 42 d at 25 °C with a 12-h photoperiod using fluorescent lamps. During the residual 12 h, the growth chambers were kept at 15 °C in the dark. Relative humidity was not controlled, and fluctuated between 75 and 85%. An aqueous solution of PMMA-co-BVP was added to the test soil just before transplanting, and 1/5 of the initial amount of PMMA-co-BVP was supplemented once a week afterwards. The amount of water in the test soil was kept at about 120 g/kg during cultivation. Once a week, symptoms of individual plants were investigated and classified into five categories. The extent of disease was evaluated based on the degree of the symptoms.

The population of R. solanacearum in the soil was measured as follows. Test soil (1.0 g) was sampled and added to sterilized distilled water (100 ml). After 10 min, the supernatant was transferred to agar plates containing the TTC medium described above for measurement of viable cell counts. A 0.1-ml portion of the supernatant was removed and quickly mixed with 0.9 ml of distilled water, and then decimal serial dilutions were prepared from this by adding 0.1 ml into 0.9 ml of sterilized distilled water and mixing. From these dilutions, the surviving R. solanacearum was counted on the TTC medium by the spread-plate method. After inoculation, the plates were incubated at 30 °C and kept in a growth chamber for about 7 d. After this procedure, young seedlings with two leaves were obtained and submitted for the pot test. The operation conditions of the growth chamber are described below.

Results and Discussion

Coagulation of R. solanacearum using PMMA-co-BVP as a polymeric coagulant

Prior to disease control, coagulation of R. solanacearum by PMMA-co-BVP was investigated. In a glass tube, an aqueous solution of PMMA-co-BVP was added to a suspension of R. solanacearum and the mixture was stirred using an automatic mixer for 10 min. After stirring, the mixture was allowed to stand at room temperature.

No formation of visually observable bacterial flocks was detected even when PMMA-co-BVP was added to the suspension of R. solanacearum, contrary to the cases of other bacteria such as Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium, Serratia marcescens, Arthrobacter atrocyaneus, Bacillus subtilis, and Staphylococcus aureus.9

However, coagulation of cells of R. solanacearum exerted by PMMA-co-BVP was confirmed based on the time course of decrease in population of R. solanacearum in the supernatant layer of the mixture (Fig. 1). In the absence of PMMA-co-BVP, no change in the supernatant population was observed (Fig. 1, closed circles). The coagulation appeared to be accomplished within 1 h, and the supernatant population reduced to 1/90, 1/180, and 1/230 when the concentration of PMMA-co-BVP was 62.5, 125, and 250 mg/l respectively. The addition of more than 200 mg/l of PMMA-co-BVP was desirable for effective coagulation of R. solanacearum (Fig. 2). No formation of bacterial flocks was visually observed in the case of Pseudomonas aeruginosa,9 similarly to the case of R. solanacearum. Therefore, we estimated that PMMA-co-BVP functioned as a polymeric coagulant even for R. solanacearum and P. aeruginosa, although the visible turbidity of the bacterial flocks was low in this case. In the case of capture of microbial cells by cross-linked PBVP and insoluble linear copolymers of PBVP, the capturing interaction was independent of the viability of the microorganisms,14 probably because the capturing interaction was derived from some physicochemical force rather than from biological activity. Therefore,
bacterial coagulation was assumed to be independent on the viability of bacterial cells. We did not examine the coagulation of dead cells of *R. solanacearum*, because dead cells are not important in disease control.

**Degradation of PMMA-co-BVP by treatment with activated sludge in soil**

Degradation of PMMA-co-BVP by activated sludge was carried out in soil. Figure 3 shows the time course of weight reduction during biological treatment. In control experiments carried out in the absence of activated sludge, the polymer sample was quantitatively recovered from test soil, and the weight reduction shown in Fig. 3 was considered a result of biological degradation of PMMA-co-BVP by activated sludge. The time course of weight reduction (Fig. 3) followed first-order kinetics, and the half-life of PMMA-co-BVP was 5.1 d under the experimental conditions. This half-life suggests that the amount of residual PMMA-co-BVP would be reduced to 1/1000 within 2 months of biological treatment. Although biodegradation of PMMA-co-BVP in the natural environment may require a much prolonged period, it is not necessary to be afraid of severe persistency in the case of PMMA-co-BVP in the natural environment.

**Suppression of tomato bacterial wilt using PMMA-co-BVP as coagulant for bacteria**

We expected control of tomato bacterial wilt by reduction of infectious contact between roots of tomato and cells of *R. solanacearum* due to coagulation of the cells by PMMA-co-BVP. The symptoms of individual plant were investigated once a week and classified into five categories, as follows: degree 0, no perceivable symptom was recognized; degree 1, symptoms were limited to the tip of plant; degree 2, the whole body of the plant drooped but still stood; degree 3, the plant fell but the body was still partly greenish and watery; degree 4, the plant withered and the whole body was dry.

The extent of disease was evaluated based on the percentage of symptoms, i.e., the percentage of plants showing any degree of symptoms. The extent of disease control was evaluated based on reduction in percentage of symptoms.

In addition, an index of symptoms was defined as follows, taking degree of symptoms of each plant into consideration:

\[
\text{Index of symptoms} = \frac{B + 2C + 3D + 4E}{A + B + C + D + E} \times 100
\]

Here, A, B, C, D, and E are the number of plants that show degree of symptoms 0, 1, 2, 3, and 4 respectively. In the case in which all test plants fall, the index is 100. On the other hand, when no symptom of disease is observed for any test plant, the index is 0. The effect of disease control was evaluated based on reduction in the index of symptoms.

The time course of percentage of symptoms and index of symptoms during cultivation are shown in Figs. 4 and 5 respectively. Cross marks in Figs. 4 and 5 show the results of control experiments performed in the absence of *R. solanacearum* and PMMA-co-BVP. All test plants grew favorably, and this shows that the experimental conditions were satisfactory for cultivation of tomato. Closed circles in Figs. 4 and 5 show the results obtained in the presence of *R. solanacearum* but the absence of PMMA-co-BVP. The percentage of symptoms reached 80% and the index of symptoms increased to 70 over 7 weeks. The results show that the experimental conditions were appropriate for investigation of tomato bacterial wilt caused by *R. solanacearum*. 
The open triangles in Figs. 4 and 5 show the results obtained where 5 mg/kg (corresponding to 41.7 mg/l for water in soil) of PMMA-co-BVP was added to soil before transplanting, and 1 mg/kg was supplemented once a week after transplanting. Control of the disease was not significant in this case. The open circles in Figs. 4 and 5 show the results obtained where 10 mg/kg (corresponding to 83.3 mg/l for water in soil) of PMMA-co-BVP was added to the soil before transplanting, and 2 mg/kg was supplemented once a week after transplanting. Control of the disease was not significant in this case.

The most effective suppression of disease among the means examined in this study was obtained by the addition of 10 mg/kg of PMMA-co-BVP to the soil (corresponding to 83.3 mg/l for water in the soil) before transplanting, and supplementation of 2 mg/kg once a week after transplanting (Figs. 4 and 5, open circles). On the other hand, Fig. 2 shows that the addition of more than 250 mg/l of PMMA-co-BVP is desirable for effective coagulation of cells of R. solanacearum suspended in water. The addition of too much PMMA-co-BVP is not desirable for controlling tomato bacterial wilt.

Figure 6 shows the population of R. solanacearum in the soil during cultivation. Closed circles represent the results obtained in the absence of PMMA-co-BVP. Open triangles, open circles, and open squares respectively represent the results obtained by the addition of 5, 10, and 15 mg/kg of PMMA-co-BVP before transplanting and supplementation of 1, 2, and 3 mg/kg once a week after transplanting, respectively. Figure 6 shows that PMMA-co-BVP did not exhibit bactericidal activity after transplanting. Figures 4 and 5 show a 51% reduction in appearance and a 54% reduction in the index of symptoms respectively under the conditions. The open squares in Figs. 4 and 5 show the results obtained where 15 mg/kg (corresponding to 125 mg/l for water in soil) of PMMA-co-BVP was added to the soil before transplanting, and 3 mg/kg was supplemented once a week after transplanting. Control of the disease was not significant in this case.

The open triangles in Figs. 4 and 5 show the results obtained where 5 mg/kg (corresponding to 41.7 mg/l for water in soil) of PMMA-co-BVP was added to soil before transplanting, and 1 mg/kg was supplemented once a week after transplanting. Control of the disease was not significant in this case. The open circles in Figs. 4 and 5 show the results obtained where 10 mg/kg (corresponding to 83.3 mg/l for water in soil) of PMMA-co-BVP was added to the soil before transplanting, and 2 mg/kg was supplemented once a week after transplanting. Thirty-five plants were used in each experiment.

The open triangles in Figs. 4 and 5 show the results obtained where 5 mg/kg (corresponding to 41.7 mg/l for water in soil) of PMMA-co-BVP was added to soil before transplanting, and 1 mg/kg was supplemented once a week after transplanting. Control of the disease was not significant in this case. The open circles in Figs. 4 and 5 show the results obtained where 10 mg/kg (corresponding to 83.3 mg/l for water in soil) of PMMA-co-BVP was added to the soil before transplanting, and 2 mg/kg was supplemented once a week after transplanting. Thirty-five test plants were used for each experiment.
against \textit{R. solanacearum} under these conditions. Suppression of tomato bacterial wilt by PMMA-co-BVP can be attributed to the reduction of infectious contact between roots of tomato and cells of \textit{R. solanacearum} due to coagulation of the bacterial cells by PMMA-co-BVP.

When 10 mg/kg of PMMA-co-BVP was added to the soil (83.3 mg/l for water in soil) followed by supplementation of 2 mg/kg once a week, the population of \textit{R. solanacearum} in the soil (Fig. 6, open circles) was 3.6 to 4.7 times larger than that obtained in the absence of PMMA-co-BVP (Fig. 6, closed circles). As can be seen in Fig. 2, however, the supernatant population was reduced to about 1/120 by coagulation of cells of \textit{R. solanacearum} by PMMA-co-BVP when 83.3 mg/l of PMMA-co-BVP was added to the suspension. These results suggest that the population of free cells of \textit{R. solanacearum} was 1/27 to 1/35 of that obtained in the absence of PMMA-co-BVP. The disease control shown by open circles in Figs. 4 and 5 can be explained in terms of coagulation of \textit{R. solanacearum} by PMMA-co-BVP in the soil.

As reported previously, the rate of sedimentation of microbial cell flocks formed by microbial coagulation using PBVP depended largely on the type of microorganisms, but all examined microorganisms showed coagulation by PBVP. Electrostatic interaction between the positive charge of PBVP, a cationic polymer, and the negative charge of the microbial cell surface appeared to play an important role in microbial coagulation. The negative charge of the microbial cell surface depends largely on the type of microorganisms. Therefore, it seems difficult to expect selective coagulation of pathogenic bacteria by PMMA-co-BVP.

When 15 mg/kg of PMMA-co-BVP was added to the soil (125 mg/l for water in soil) before transplanting followed by supplementation of 3 mg/kg once a week after transplanting, the population of \textit{R. solanacearum} in the soil (Fig. 6, open squares) was close to that obtained in the absence of PMMA-co-BVP (Fig. 6, closed circles). Figure 2 suggests that the supernatant population was reduced to about 1/160 by coagulation of cells of \textit{R. solanacearum} by PMMA-co-BVP when 125 mg/l of PMMA-co-BVP was added to the suspension, but disease control was not satisfactory under these conditions (Figs. 4 and 5, open squares). The addition of too much PMMA-co-BVP is not desirable for controlling tomato bacterial wilt. A possible reason is the unfavorable influence of PMMA-co-BVP on the growth of tomato. Further research is required on this subject.

We proposed a novel method to control tomato bacterial wilt taking protection of the natural environment into consideration. The most effective control of the disease was obtained by the addition of 10 mg/kg of PMMA-co-BVP prior to transplanting and supplementation of 2 mg/kg once a week after transplanting (Figs. 4 and 5, open circles), which resulted in a 51% reduction in appearance and a 54% reduction in the index of symptoms. This disease suppression was explained in terms of a reduction in infectious contact between roots of tomato and cells of \textit{R. solanacearum} due to coagulation of bacterial cells by PMMA-co-BVP. Although the rate of reduction of symptoms was not very satisfactory, the new methodology might be significant for the control of tomato bacterial wilt. Further extensive research is required for a reliable and definitive conclusion on the extent of static suppression of soil-borne plant disease by microbial coagulation before practical application of this novel methodology. We hope for further development of this new type of green chemical methodology for the protection of plants from soilborne diseases.

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References


