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Lectures by the Winners of the Society Fellowship

Ecology and Biocontrol of Soft Rot of Chinese Cabbage†

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1. Ecology of soft rot bacterium

Bacterial soft rot, caused by *Erwinia carotovora* subsp. *carotovora* (Ecc), is one of the most destructive diseases of Chinese cabbage [*Brassica campestris* L. (pekinensis group)] in Japan. The pathogen is widely distributed in agricultural areas as well as in uncultivated land. In 1949 the soft rot pathogen caused catastrophic losses of Chinese cabbage in Miyagi Prefecture. The next year in our laboratory, Tsuyama and Sakamoto initiated studies on the behavior of soft rot bacteria in the rhizosphere and non-rhizosphere soils, as well as on or in Chinese cabbage plants in order to control the disease. Since 1961, we have continued and developed their work.

(1) Growth in rhizosphere of crop plants and weeds

Twenty-eight species of crop plants belonging to 10 families were grown in an experimental field. On the 67th and 82nd days after seeding, the rhizosphere population of each plant was determined. Soft rot bacteria were found in the rhizosphere soil of Chinese cabbage, Bitaminna, teosinte, Chinese leek and tomato. These organisms were not detected in seven species of leguminous crop plants, but a large population bacteria was observed in the rhizosphere of cruciferous plants. Similar observations were made on 15 species of weeds which grew naturally in the field after the harvest of Chinese cabbage. The soft rot bacteria grew selectively in the rhizosphere of cowthistle, pigweed, purslane and spiderwort at a level of 2.6×10^3 to 1.2×10^5 cfu per g of fresh root.

(2) Multiplication in rhizosphere of various plants grown aseptically

The fact that soft rot bacteria were found in the rhizosphere of a restricted number of plant species led to the idea that other plants might not be exuding nutrients from their roots to support the growth of the organisms. To test this, 25 species of crop and flowering plants belonging to 11 families were grown aseptically in large test tubes containing 25 ml of modified Hoagland's nutrient agar in the greenhouse. After culturing the seedlings for 5 days, 0.5 ml of Ecc suspension (1.2×10^5 cfu/ml) was added to each tube, and the population estimated 7 and 10 days after inoculation. Every plant stimulated bacterial multiplication, suggesting that each of the plants exuded nutrients necessary for the soft rot pathogen. However, when grown in the field, some factors other than nutrients may affect enrichment of the pathogen in the rhizospere.

(3) Variation of rhizosphere microflora with plant growth

We examined the change in rhizosphere microflora in relation to the growth stages of Chinese cabbage. Plants were seeded in the field on August 9 and September 5, 1967, and the microbial population was monitored throughout growth. The microbial population peaked at the end of the leaf-wrapping stage. Rhizosphere effect was most pronounced in the case of dye-tolerant bacteria and less so in the case of actinomycetes. The proportion of dye-tolerant to total bacteria was markedly high (56%) at an early growth stage, then decreased gradually to 15% as the plants grew. Regardless of the sowing date, soft rot bacteria were found in the rhizosphere 45 to 48 days later. The organisms then preferentially increased in number as the plant grew. Finally, they amounted to 10% of the number of dye-tolerant bacteria. These results indicated that a high populaton of soft rot bacteria in the rhizosphere of the plant is closely connected to the development of the host plant.

(4) Seasonal variation

Soil in contact with or near the petioles of Chinese cabbage plant is known as "phyllosphere soil". Initial infection usually occurs on the outer petioles in contact with this soil when the Chinese cabbage plant has reached its wrapping stage. Exudates from aerial plant parts, therefore, will affect the microbial population in the phyllosphere soil. To examine the fluctuation, soil samples were periodically taken from the rhizosphere and phyllosphere of Chinese cabbage as well as at some

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distance from the plant (non-rhizosphere) in 1970. Soft rot bacteria appeared in soil samples from the rhizosphere and phyllosphere by mid-June and the population levels ranged from 10^5 to 10^7 cfu per g of dried soil. These levels were maintained over the next 6 months. By January of the next year, soft rot bacteria had disappeared in all the samples. On the other hand, soft rot bacteria in non-rhizosphere samples were either absent or at a very low population throughout the year, reaching a maximum of 10^2 cfu per g of dried soil. From this evidence and that mentioned previously, the soft rot bacteria are enriched and remain at a higher population level in the presence of Chinese cabbage or related crops. Furthermore, seasonal fluctuations of these organisms in agricultural areas are determined mainly by the cultivation of such crops.

(5) Seasonal development of disease

Incidence of soft rot was monitored throughout the year by bi-weekly planting of Chinese cabbage in the field from February 20 to September 2, 1970 and periodically observing the disease development. For early spring plantings, seedlings were raised inside a greenhouse. The disease was prevalent from early summer to fall and developed rapidly especially under hot and humid conditions. Chinese cabbage planted after August 19 was rarely affected by this disease. The development of soft rot was not directly related to the pathogen population in soil. Other factors that influence the host-parasite relationship need further investigation.

(6) Occurrence of soft rot bacteria in stem

The population of soft rot bacteria in surface-sterilized stems of Chinese cabbage was estimated. Bacterial population levels ranged from 10^3 to 10^7 cfu per stem in almost all the healthy stems obtained from 10 individuals of each of the six cultivars differing in degrees of resistance. High population levels were more frequently observed in the stems of susceptible cultivars than in resistant ones.

(7) Survival in fallow soil

Chinese cabbage was continuously cultivated from 1977 to 1984 in the field at Katahira Campus, Tohoku University. The campus is in an urban area, devoid of farm land. Bacterial soft rot occurred in 1979, and the prevalence and severity of the disease increased with successive croppings. After the fall cropping of Chinese cabbage at the end of October 1984, the field was left fallow and covered with an opaque plastic film. Surface-sterilized seeds of Chinese cabbage were again sown in each experimental plot inside the vinylhouse in April 1986 to 1989. Soft rot bacteria were isolated every year from rotted leaf petioles of Chinese cabbage. The selected taxonomic characteristics of 374 isolates were similar to those of Ecc. The findings indicated that Ecc survived for at least 5 years in the bare, fallow soil. However, the saprophytic ability differed among the isolates as determined by bacteriocin typing.

2. Biocontrol of soft rot disease of Chinese cabbage

Despite the economic importance of soft rot disease, no effective control method has been established, although copper-bactericides and antibiotics are widely used. After bacteriocin typing of 484 strains of Ecc, we used bacteriocins as biological agents to control soft rot. The main motivation is that some strains produce bacteriocin(s) which kill a wide spectrum of Ecc strains, but are also highly resistant to the bacteriocins produced by about 1000 other Ecc strains tested. These traits are absolutely fundamental for a biocontrol agent. Four strains were finally designated as candidates. Avirulent mutant strains were derived from the candidate strains using ethyl methanesulfonate (EMS) as a mutagen. The bacteriocin(s) activity of the mutants against other Ecc strains was determined. The avirulent mutants showed similar bacteriocin response to that of parent strains used as biocontrol agents.

(1) Suppression of lesion development

Freshly cut petioles of Chinese cabbage were wounded with a bundle of 10 fine needles, inoculated simultaneously with an Ecc suspension (2×10^6 cfu/ml) and biocontrol agent in the ratio of *ca* 1 : 1 and incubated in a moist chamber at 25°C for 24 hr. Soft rot lesion development was strongly suppressed. Lesion development was completely inhibited when the biocontrol agent was applied 3 hr before the pathogenic strain. The suppression of lesion development by biocontrol agents is highly correlated with the sensitivity of Ecc strains to the bacteriocins produced by the biocontrol agents. We therefore propose that the suppression mechanism is through the production of bacteriocin(s) by the biocontrol agent.

(2) Induction of bacteriocin production

A drop (20 μl) of mixed bacterial suspension (4×10^6 cfu/ml) of the Ecc pathogenic strain and the biocontrol agent was placed on a nonwounded petiole of Chinese cabbage and incubated in a moist chamber at 25°C for 2 days. The two populations increased similarly. However, the pathogenic populations decreased remarkably when an equal volume of sterile Chinese cabbage juice was added to the inoculum or when the inoculation site was wounded with a bundle of needles. Chinese cabbage juice is known to induce both pectinase and bacteriocin production in Ecc. Glucose and fructose in the juice is the inducer of bacteriocin.

(3) Behavior of biocontrol agent in the field

One or 2 days after foliar application of biocontrol agents, their populations decreased markedly from 10^{6-7} to 10^{3-4} (cfu/g fresh weight) levels, but no further decrease in population was observed thereafter. The biocontrol agents multiplied simultaneously with the pathogen at the wrapping stage of Chinese cabbage, giving the agents an advantage over agricultural chemicals.

(4) Efficacy trials

Biological control tests during 1991 to 1997 in several fields in Japan resulted in protection between 50 to 75%, indicating that the biocontrol agents are effective against soft rot disease. Furthermore, protection was improved considerably by using a mixture of two compatible biocontrol agents. Copper bactericide-resistant mutants, derived from the biocontrol agents and integrated into the copper-bactericide sprays reduced soft rot in the field. Based on these results, a new disease control program will incorporate the mutants into copper bactericide sprays and use fewer applications of chemicals.

(5) Cloning and expression of Ecc gene encoding low-molecular-weight bacteriocin

Two kinds of bacteriocins are produced by Ecc ; namely, low- and high-molecular-weight bacteriocins. The low-molecular-weight bacteriocin, carocin H1, is comprised of two structural genes, *caroH1K* and caroH1K, which encode the killing protein (CaroH1K) and the immunity protein (CaroH1I). The killing protein has a nuclease activity. A putative sequence similar to the consensus *E. coli* cyclic AMP receptor protein binding site (CAP site, −312 bp) upstream of the start codon was found, which supports our observation that bacteriocin can be induced by glucose.