Isolation of *Pseudomonas cepacia* D-202: an Available Biocontrol Agent to the Storage Rot of Sugar Beet

Shu ISHIKURI*, Hirokatsu UCHINO* and Katsuichi KANZAWA*

Key words: biological control, storage rot, sugar beet, *Botrytis cinerea*, *Penicillium expansum*, *Pseudomonas cepacia*.

In Japan, sugar beet (*Beta vulgaris* L.) is cultivated only in Hokkaido. The total area for cultivating the sugar beets is about 72,000 ha and the total amount of the harvest is about 3.9 billion kg. The roots of sugar beet are harvested between middle October and early November. About two third of the crop are stored outdoors in piles covered with plastic sheets. And some of them are stored up to late March to await processing.

During the storage period, the reduction of the sugar content of roots occurs partly by the normal respiration of beet roots but mainly by the storage rot^{1,2)}. The disease is caused mainly by *Botrytis cinerea* and/or *Penicillium expansum* in Japan³⁾. However the prevention of the disease during the storage period is an important problem, the application of chemical fungicide to harvested crops is illegal in Japan, and also the tolerant varieties are not registered. Therefore we manage to keep the temperature of the storage piles between 4 and 6°C in order to prevent the disease. For a relatively short storage period, this method works well, but with the lapse of storage period especially after middle January, the occurrence of storage rot is often observed.

As a new method for preventing the storage rot of beet roots, we investigated the biological control of the pathogens. In this paper, we report the isolation process and antifungal activities of the bacterium against *B. cinerea* and *P. expansum*; which was identified as *Pseudomonas cepacia*.

Isolation of bacteria from soils. We collected 133 soil samples from fields of various crops around Obihiro city in November 1987. For the isolation of bacteria, we applied egg albumin and DNB (100-fold diluted nutrient broth)⁴⁾ agar media. For the assay of the antifungal activities of the bacteria, we used two fungi, *B. cinerea* (58-SR-105) and *P. expansum* (57-SR-61) as indices; both of them were isolated from rotted beet roots³⁾. The spores of *B. cinerea* and *P. expansum* were collected from the two weeks old plate culture on Clark's medium⁵⁾ and potato-dextrose agar, respectively.

After suitable dilutions with distilled water, the soil suspension was inoculated to 10 agar plates for each medium. The plates were incubated for two weeks at 27°C. Spore suspension (10⁵ conidia/ml) of *B. cinerea* was sprayed onto respective five of them, *P. expansum* (10⁵ conidia/ml) was also sprayed onto respective five plates. Plates were incubated for three days at 27°C. After incubation, the bacteria which showed inhibition zone against *B. cinerea* or *P. expansum* were picked up and maintained on egg albumin or DNB agar slants.

We obtained 3,013 isolates of antifungal bacteria; 1,526 isolates were obtained from plates sprayed with the spores of *B. cinerea*, and 1,487 isolates were obtained from that of *P. expansum*.

Isolation of bacteria from beet roots. We collected beet roots from six storage yards around Obihiro city in January 1988. For each yard, we collected two of the healthy roots and that of rotted ones. In the case of healthy roots, the soil on the roots containing the peridermal tissues was dispersed by the blender and plated. And in the other case, in addition the same method, rotted tissue was also plated. The media applied, and incubation conditions were the same as soil samples.

* Research Center, Nippon Beet Sugar Mfg. Co., Ltd., Obihiro, Hokkaido 080, Japan 日本甜菜製糖(株)総合研究所

We obtained 101 isolates of antifungal bacteria; 55 isolates were obtained against *B. cinerea*, and 46 isolates were obtained against *P. expansum*.

Preliminary screenings. First screening was carried out on the agar medium as follows. All bacterial isolates were cultured in the peptone-yeast extract liquid medium (peptone 0.5%, yeast extract 0.3%, nutrient extract 0.3%, glucose 1.0%, pH 7.0-7.2) for five days at 25°C. Forty μ l of cultural suspension was applied to the paper disk (8 mm in diameter) and the paper disk was put on the center of potato-dextrose agar plate. For each isolate, we prepared two plates and sprayed the spores of *B. cinerea* and *P. expansum*, respectively. Plates were incubated for three days at 25°C, and the area of inhibition zone was measured. We repeated this experiment for four times and selected 61 isolates; 27 isolates inhibited the growth of *B. cinerea*, and 34 isolates inhibited *B. cinerea* and *P. expansum*.

Second screening was carried out on the pieces of beet roots. For each isolate, we prepared two pieces of beet roots (5 cm square with 1 cm thick). On the surface of the piece of beet roots, 0.8 ml of bacterial suspension was applied. We gave the bruise to make depression on the center of piece and applied 40 μ l of spore suspension (10^7 conidia/ml) of *B. cinerea* and that of *P. expansum*, respectively. These pieces were kept in the petri dishes and incubated for two weeks at 8°C. By this experiment we selected 18 isolates; all of them inhibited the growth of *B. cinerea* and *P. expansum*.

Screenings on beet roots. The storage rot of beet roots was reported to occur by the natural infection from bruised tissue during the storage period at outdoors in piles^{1,6)}. To make a similar experimental condition, we used split or bruised beet roots.

Third screening was carried out with the sections of beet roots split in four. For each isolate, we prepared five sections and applied about 5 ml of bacterial suspension to each section by dipping. These sections were stored in the plastic bag to keep wet and stored for two months at 8°C. By this experiment we selected five isolates (D-19.2, D-189.2, D-193.1, D-197.2 and D-202) which showed the successful reduction of storage rot. These are the isolates from non-rhizosphere soil with DNB medium.

Final screening was carried out with the whole beet roots which were bruised by the same mechanism of soil separator of beet harvester. For each isolate, we prepared 120 bruised beet roots (cv. Monoace). And we applied about 20 ml of bacterial suspension to each beet root by dipping. These beet roots were kept in the plastic bag to keep wet and stored for 105 days at 8°C. Compared with the stored beet roots without bacterial application, all the five isolates showed successful reduction of storage rot (Table 1 and Plate I). Among them, the most effective isolate D-202 was selected, which was obtained from a field cultivated sugar beets.

Identification of bacterium. The isolate D-202 was classified according to Palleroni⁷⁾. Table 2 shows the morphological and biochemical characteristics of the isolate and *Pseudomonas cepacia* IFO-14595. As shown in Table 2, the isolate showed almost the same characteristics to *Pseudomonas cepacia* IFO-14595 and was identified as *Pseudomonas cepacia* with the following results; Gram-negative

Table 1. Effect of the antifungal bacteria on the control of storage rot of beet roots

Isolates	Storage Rot	
	Average Index ^{a)}	Occurrence Ratio ^{b)} (%)
D-19.2	0.26	23.3
D-189.2	0.23	21.7
D-193.1	0.24	23.3
D-197.2	0.19	25.0
D-202	0.13	13.3
Control ^{c)}	0.50	41.7

a) Average Index = $\frac{\text{Sum of the indices of each beet root}}{\text{Total number of beet roots}}$, where the indices of storage rot are defined as follows;

0: No symptom was observed.

1, 2, 3, 4 and 5: About 10, 30, 50, 80 and 100% volume of beet roots were rotted, respectively.

b) Occurrence ratio = $\frac{\text{Number of rotted beet roots}}{\text{Total number of beet roots}} \times 100(\%)$.

c) Beet roots without bacterial application.

Table 2. Morphological and biochemical characteristics of the isolate

Characteristics	D-202	IFO-14595 ^{a)}
Cell shape	Short rods	Short rods
Cell diameter, μm	0.7-1.1	0.7-1.0
Cell length, μm	1.7-3.1	1.4-2.3
Gram stain	—	—
Motility	+	+
Flagella	Polar > 1	Polar > 1
PHB accumulation	+	+
Arginin dihydrolase	—	—
Catalase reaction	+	+
Denitrification	—	—
Gerlatin liquefaction	+	+
H ₂ S production	—	—
Indole production	—	—
Lysin decarboxylase	+	+
Nitrate reduction	+	—
OF-test	Oxidative	Oxidative
Ornithine decarboxylase	+	+
Oxidase reaction	+	+
Vogel-Proskauer	—	—
Urease	+	+
Utilization of: Arabinose, 2,3- Butylene glycol, Citrate, Glucose, Fructose, Malate, Mannitol, Sucrose, Xylose, Levulinate	+	+
Adonitol, Inositol, Rhamnose		
	—	—

a) IFO-14595: *Pseudomonas cepacia*.

aerobic rod, motile by several polar flagella, Oxidase-positive, Catalase-positive, Oxidative OF test, PHB is accumulated as carbon reserve material, Arginine dihydrolase present, utilization of levulinate and 2, 3-Butylen glycol.

This strain, *Pseudomonas cepacia* D-202, has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology Japan, under the accession number FERMP-11559.

The inhibition mechanisms of *P. cepacia* D-202 against *B. cinerea* and *P. expansum* are now under investigation.

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和 文 摘 要

石栗 秀・内野浩克・神沢克一：テンサイ貯蔵腐敗病の微生物防除：実用株 *Pseudomonas cepacia* D-202 の分離

テンサイ貯蔵腐敗病を拮抗微生物を用いて防除するために、土壌および貯蔵中のテンサイ根から主たる病原菌である *Botrytis cinerea* および *Penicillium expansum* に拮抗作用を示す細菌を 3,114 株分離した。寒天平板並びにテンサイ根を用いて、これらの病原菌の生育を最も強く抑えた 1 株 (D-202) を選択した。本菌株による貯蔵腐敗病の低下は、テンサイ個体を用いた実験により確認された。本菌株について細菌学的な検討を行ったところ、*Pseudomonas cepacia* と同定された。

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Plate I

Biological control of storage rot of sugar beets by *P. cepacia* D-202.

A: The roots applied by *P. cepacia* D-202,

B: Storage rot on beet roots without bacterial application. Dark brown rotted tissues were observed.