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Biological control of bacterial soft rot in Chinese cabbage by *Lactobacillus plantarum* strain BY under field conditions

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HIGHLIGHTS

- Lactic acid bacteria (LAB) were screened for the ability to control plant diseases.
- *Lactobacillus plantarum* strain BY suppressed bacterial soft rot on various crops.
- BY populations persisted especially on wounded plant leaves.
- BY showed antibacterial activity against soft rot pathogen.
- First report on application of LAB for biological control under field conditions.

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ABSTRACT

Bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* is one of the most important diseases of Chinese cabbage. To develop a microbial pesticide that is not only safe but also easily acceptable by consumers, we screened strains of lactic acid bacteria for biocontrol potential. One of 1166 isolates, *Lactobacillus plantarum* strain BY significantly reduced soft rot disease severity in Chinese cabbage in six different fields in Japan. This isolate also suppressed disease in onion, potato, and tomato. BY populations persisted on wounded Chinese cabbage leaves after spraying. Moreover, BY inhibits pathogen growth in an antibiosis assay. These results strongly suggest that BY inhibits the invasion of the pathogen at wounded sites on leaves and its proliferation in host tissues. This is the first report indicating the application of lactic acid bacteria for biological control of a plant disease under field conditions.

1. Introduction

Bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) is one of the most important diseases of Chinese cabbage in many countries, including Japan (Kikumoto, 2000). The disease symptoms first appear as small water-soaked lesions along the basal midribs of the outer leaves, which are usually in contact with the soil, and then rapidly progress towards the cabbage head. The infected area becomes soft and mushy with an offensive odor. Eventually the leaves, stems, and roots are entirely decomposed by the pathogen. Bacterial soft rot occurs worldwide in a wide variety of ornamental plants and economically important vegetable crops (Toth et al., 2003). Moreover, this disease can occur not only in the field, but also after harvest during transit, storage, or marketing (Bhat et al., 2010).

Chemical pesticides are generally effective for controlling plant diseases. However, the use of synthetic chemical pesticides tends to be considered undesirable because of concerns about the potential environmental effects of residues (Gill and Garg, 2014). Therefore, efficient and safe ways to control diseases are needed as alternatives to chemical pesticides. Previous studies have shown that some *Pseudomonas*, *Bacillus*, and nonpathogenic *Pectobacterium* strains can act as biological control agents against soft rot pathogens (Cronin et al., 1997; Kyeremeh et al., 2000; Sharga and Lyon, 1998; Takahara et al., 1993). Among these agents, a nonpathogenic *Pectobacterium* formulation was registered as a microbial pesticide in 1997, and has been used to control soft rot diseases in Japan.

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Lactic acid bacteria (LAB) are relatively common microorganisms that are found in various fermented foods, and recently there has been increasing interest in their use as probiotics. Probiotics are living microorganisms that have health-promoting effects that might be exerted through improvement of the intestinal microflora and proposed modulation of immune system function (Nagano et al., 2000). Moreover, recent attention has been directed towards the use of these bacteria for biopreservation, or the use of microorganisms to preserve foods (García et al., 2010). LAB are regarded as safe for use in foods because of the long history of their use to preserve foods, and because some LAB species have "generally regarded as safe" status in some countries, their use in biopreservation can sometimes be accelerated. The suppressive effects of LAB against pathogenic microorganisms might be due to competition for nutrients and colonization sites, or antibiosis *via* the production of various antimicrobial compounds including lactic acid (Ghanbari et al., 2013). The safety of some species for use in food and their disease-suppressive effects could also make some LAB suitable for the biological control of plant diseases.

The goal of the present study was to analyze LAB strains for the development of a safe microbial pesticide. Here, LAB isolates were screened for use as biological control agents. First, LAB isolates were screened for the ability to suppress bacterial soft rot caused by Chinese cabbage under field conditions. Subsequently, the disease-suppressive effects of formulations prepared from selected LAB isolates were confirmed at various field locations in Japan. Finally, the population dynamics on Chinese cabbage plants and antimicrobial activities against soft rot pathogen were also investigated for one LAB isolate.

2. Materials and methods

2.1. Bacterial strains and growth media

LAB isolates were collected from various fermented foods (such as fermented milk or pickled vegetables). Samples homogenized with sterile distilled water (SDW) were plated out on Man, Rogosa, and Sharpe (MRS) agar medium (Difco Laboratories, Detroit, MI) containing 0.5% $CaCO_3$, and then incubated at 30 °C for 3 d (Lim and Im, 2009). Colonies surrounded by clear zones were collected as LAB isolates, and stored at −80 °C in 10% skim milk containing 1% sodium L-glutamate monohydrate. The type strains of *Lactobacillus* species provided by the Japan Collection of Microorganisms (RIKEN BRC, part of the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan) include *Lactobacillus plantarum* JCM1149, *L. brevis* JCM1059, *L. acidophilus* JCM1132, and *L. casei* JCM1134. LAB cultures were maintained on MRS agar medium at 30 °C. *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) strain MAFF302818, provided by the National Institute of Agrobiological Science (NIAS) Genebank (Ibaraki, Japan), was used as a plant pathogenic bacterial strain. A nonpathogenic Pcc strain, npPcc1-1, was isolated in pure culture from the commercial biocontrol formulation (BIOKEEPER®, Central Glass Co., Ltd., Tokyo, Japan). These bacterial cultures were maintained on King's B agar medium at 28 °C.

2.2. Screening of candidate biocontrol isolates

LAB cultures incubated in MRS broth (Difco, Detroit, MI, USA) at 30 °C for 2 d were centrifuged at 5000 rpm for 5 min. The cell pellets were resuspended in SDW and centrifuged a total of three times. The resultant cell suspensions were adjusted to approximately 10^9 CFU/ml by measurement of optical density at 600 nm. The pathogenic Pcc strain MAFF302818 incubated on potato dextrose agar (PDA, Difco, Detroit, MI, USA) plates at 28 °C for 24 h was also suspended in SDW. The cell pellet was harvested by centrifugation at 5000 rpm for 5 min, and washed three times with SDW, and then cell suspensions were adjusted to approximately 10^9 CFU/l.

The first screening was performed as follows. The Pcc cell suspension was mixed with LAB isolate cell suspension at a ratio of 1:100 for use as an inoculation solution. Eight leaf disks (1 cm in diameter) of commercial cabbage were submerged in the inoculation solution at 24 °C for 1 h, and then the solution was blotted from the leaf disks with paper towels. The disks were placed in 9-cm petri dishes and incubated at 28 °C for 24 h. The extent of soft rot on each disk was then evaluated according to the following disease scoring system (Takahara et al., 1993): 0 = no lesions; 1 = spreading lesions covering up to half the disk area; 2 = spreading lesions covering more than half the disk area; and 3 = full lesions. Disease severity index (DSI) and protective value (PV) were then calculated using the following formulas, respectively: DSI = [Σ (the disease score of each disk)/(3 × the total number of disks)] × 100, PV = (1 − DSI in treatment/DSI in control) × 100.

The second screening was performed as follows. A mixture of Pcc and LAB cells prepared as described above was inoculated onto adaxial midribs of outer leaves of Chinese cabbage (one-month-old seedlings of Chinese cabbage, cv. 'Muso') by puncturing the midrib using a bundle of 10 sewing needles (1 mm in diameter) to a depth of 1 mm. After the inoculated plants had grown in a greenhouse maintained at 20–28 °C for 10 d, disease symptoms were evaluated using a disease scoring system as follows (Takahara et al., 1993): 0 = no infection or mere darkening of tissue; 1 = less than 1 cm rot zone diameter; and 2 = rot on midrib of diameter greater than 1 cm. The nonpathogenic Pcc formulation was used as a control. Inoculation was performed twice on three midribs per plant (five plants per treatment).

The third screening was conducted in two independent fields at the Biotechnology Research Department (Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center, in the town of Seika, Kyoto, Japan) during Oct. to Nov. 2006. To prepare the pathogen-infested fields, Chinese cabbage plants grown in the field were inoculated with the pathogenic Pcc strain MAFF302818 on 22 May 2006, and were tilled into the soil using a rotary tiller on 2 Jun. 2006. Field 1 contained 18 plants per plot with two replications and Field 2 contained eight plants per plot with three replications per treatment. Seeds of Chinese cabbage (cv. 'Muso') were sown in plastic pots on 31 Aug. 2006, and seedlings were transplanted into the infested fields spaced 40 cm apart in double rows 1.2 m apart on 21 Sep. 2006. LAB isolate suspensions (approximately 10^7 CFU/ml) and the nonpathogenic Pcc formulation (approximately 10^7 CFU/ml) were applied by foliar spray at 2 d before transplanting (5 ml/pot), and were reapplied at 7-d intervals after transplanting (15 L/a) a total of nine times. On harvest day (21 Nov. 2006), the disease symptoms of each plant were recorded using the following disease scoring system: 0 = no symptoms; 1 = rot on part of outer leaves; 2 = rot on outer leaves and part of head; and 3 = rot on most of head. Marketable yield of Chinese cabbage in each treatment was recorded as grams fresh weight per plant.

2.3. Disease-control effects of LAB formulations

LAB isolates cultured in MRS liquid medium were collected at log phase, and then freeze-dried. The dried cells were ground and formulated with clay into wettable powder. The cell density of each formulation was adjusted to 1×10^{10} CFU/g. The biological control efficacy of each LAB formulation was investigated in various fields of Chinese cabbage in Kanagawa, Miyagi, Fukushima, Ibaraki, Nara, and Kyoto Prefectures in Japan in 2007–2008. The efficacies of LAB formulations on soft rot disease of other plants were also investigated in the field of the Biotechnology Research