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Characterization and chemical control of soft rot disease caused by *Pantoea* sp. strain PPE7 in *Pleurotus eryngii* mushroom crops

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Abstract The bacterial pathogen *Pantoea* sp. strain PPE7 causes severe soft rot disease in the king oyster mushroom, *Pleurotus eryngii*. Typical symptoms of the disease include a dark-brown water drop at early stages of infection, ultimately leading to soft rot accompanied with an offensive odour. In this study, we showed that inoculation of *Flammulina velutipes* and *Agaricus bisporus* mushrooms with *Pantoea* sp. strain PPE7 also resulted in severe disease, including water-soaked lesions and soft rot symptoms. Treatment with chlorine solution (175 to 700 ppm active chlorine) significantly reduced the incidence of soft rot symptoms in *Pleurotus eryngii*. However, exposure to concentrations greater than 350 ppm caused harmful effects such as bleaching on the cap of the fruit body. Calcium hypochlorite solutions with 175 ppm active chlorine were effective for reducing soft rot disease of *Pleurotus eryngii* without affecting the mushroom yield.

Keywords Bacterial pathogen · Calcium hypochlorite · *Pantoea* sp. strain PPE7 · *Pleurotus eryngii* · Soft rot disease

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Pleurotus eryngii was originally cultivated in northern Italy and Switzerland, where it is known locally as *cardoncello* (Ohga and Royse 2004), but is now commonly cultivated in Europe, the Middle East, and North America, as well as in parts of Asia. Cultivation of *P. eryngii* on an industrial scale began in Korea in 1996, and by 2012, production of *P. eryngii* was estimated to be 50,605 t (Ministry for Food, Agriculture, Forestry and Fisheries 2013). To date, a number of fungal and bacterial diseases such as dry bubble disease on *Agaricus bitorquis* by *Verticillium fungicola* var. *fungicola,* internal stipe necrosis on *Agaricus bitorquis* by *Ewingella americana,* and bacterial soft rot on *Flammulina velutipe*s by *Erwinia carotovora* subsp. *carotovora.* causing severe damage to mushroom has been reported as they are involved in the major steps of the cultivation process (Inglis and Burden 1996; Okamoto et al. 1999; Gea et al. 2003). Lincoln et al. (1999) reported that *Janthinobacterium agaricidamnosum* infection in *A. bisporus* results in a novel soft rot disease. Especially, blotch diseases have been frequently reported in cultivated mushrooms.

These are mainly due to *Pseudomonas tolaasii,* causing brown blotch disease, and *Pseudomonas gingeri*, responsible for ginger blotch disease (Tolaas 1915; Paine 1919; Wong et al. 1982; Flippi et al. 2002). The gram negative bacterium *Pantoea* sp. has been reported as causal agent of soft rot disease with symptoms of water-soaked lesions on the stipes and pileus of *P. eryngii* (Kim et al. 2007). A number of different methods have been developed to control bacterial diseases in the cultivation of mushrooms. Environmental controls, including low relative humidity, temperature (van de Geijn 1981), carbon dioxide level, and the cleaning of cultivation rooms play important roles in diminishing the spread of disease. Nair and Bradley (1980) emphasized the importance of keeping mushroom caps dry by regulating the temperature, relative humidity, and ventilation to prevent bacterial propagation. Biological control methods using antagonistic microorganisms and specific phages have also been studied. Fermor et al. (1991) showed that the application of antagonistic bacteria to mushroom beds resulted in significant control of *P. tolaasii* infection, with a consistent reduction (at least 50 %) in bacterial blotch disease occurrence in mushroom crops following treatment with selected antagonists. Kim et al. (2011) reported that bacteriophage could block the brown blotch disease on *Pleurotus ostreatus* by *Pseudomonas tolaasii*. However, biological controls have not always been able to prevent and control this disease. Several compounds, including antibiotics (Geels 1995), have been examined to control bacterial disease in cultivated mushrooms, although none of these is fully effective or safe for human consumption. Despite these shortcomings, compounds containing active chlorine are, at the present, the most commonly utilized chemicals for bacterial disease control. Although many secondary effects are associated with their use, low concentrations of chlorine have no detrimental effects on the yield or quality of mushrooms and pose no health hazards to consumers or farm workers (Ayers and Lambert 1955). Barden (1987) reported that addition of 0.5 % calcium chloride to the irrigation water significantly reduced the bacterial growth rate during postharvest storage. Royse and Wuest (1980) reported that routine watering with chlorinated water considerably reduced the severity and incidence of brown blotch disease of *A. bisporus*. The successive treatment of activated chlorine dioxide reduced bacterial blotch incidence on *A. bisporus* (Geels et al. 1991). In the present study, we describe the characterization of bacterial soft rot disease and the effectiveness of calcium hypochlorite treatments in controlling this disease in *P. eryngii* artificially inoculated with *Pantoea* sp. strain PPE7.

Infected mushroom samples were collected from mushroom farms located in Gyeong-nam province in Republic Korea. Several pieces with lesions from stipes and caps were crushed with a sterile pestle, and suspended with 10 ml of sterile distilled water. Then the suspensions were plated onto LB (Luria-Bertani) agar medium (Difco, Detroit, MI, USA) by a decimal dilution and incubated at 28 °C for 24 h.

The typical symptoms of soft rot disease include a dark brown water drop in the early stages of infection, followed by the development of water-soaked lesions on the stipe and cap of mushrooms within 8 days after the mushrooms are transferred to the cultivation room. The lesions expand gradually and constitute a viscous, mucus-like fluid, finally leading to a mushy soft rot accompanied by an offensive odour in the plastic bottle during growth (Fig. 1).

When the several pieces with lesions were macerated with a sterile pestle, bacteria with pale yellow pigment were consistently isolated and appeared as dominant on the plates. Bacterial colonies were pale yellow pigmented in colour, convex, circular with entire margins, and shiny texture (Fig. 2). Cells were Gram-negative, round ended rod-shaped, 1.0-1.4×0.4-0.5 μm in size, and had an optimum growth temperature and pH with 25–30 °C and 6.0, respectively.

To analysis the causal bacterium, a 1.5 kb fragment of the 16S rDNA was amplified using the universal primer pairs (Forward, 5′-CGGAGAGTTTGATCCTGG-3′ and Reverse, 5′-TACGGCTACCTTGTTACGAC-3′). The GenBank databases were used for similarity searches using the BLAST program (National Center for Biotechnology Information, U.S. National Institute of Health, Bethesda, MD). The result of the partial 16S rDNA sequence had been deposited into the GenBank database with accession number AY501386 named as *Pantoea* sp. PPE7.

Bacteria isolated from diseased mushrooms were used for pathogenicity tests. Bacterial suspensions were prepared from 24 h Luria-Bertani broth cultures to inoculate with the mycelium of *P. eryngii. P. eryngii* strain KNR2312 was provided by Gyeong-Nam Agricultural Research and Extension Service in Gyeong-Nam province, Republic Korea. *P. eryngii* was maintained on Mushroom Complete Medium (MCM: 2 g Yeast

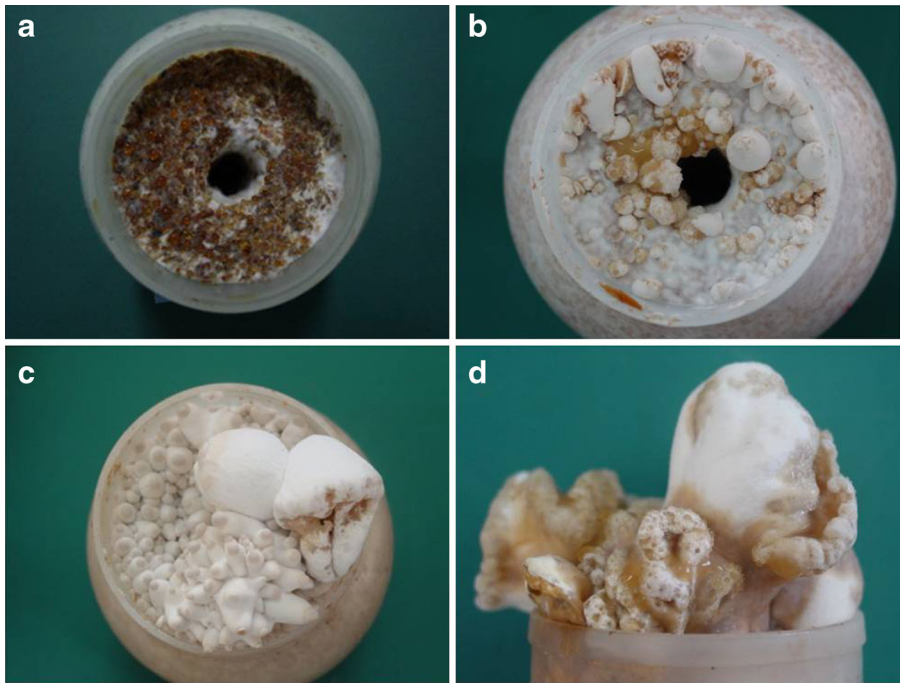

Fig. 1 Symptoms of soft rot disease of *P. eryngii* collected from the mushroom farms. The pathogen induced a dark brown water drop in early stages of infection, which developed into a viscous fluid on fruit body. **a** Regeneration stage after removing the inoculated spawn, **b** Primordia formation stage, **c** Growing stage, **d** Harvesting stage

Extract, 2 g Peptone,0.5 g $MgSO_4$, 0.46 g KH_2PO_4,1 g K_2HPO_4, and 12 g Glucose per litre of medium) agar plate at 25 °C and MCM agar slants at 4 °C for stock cultures. The substrate for the growth and fruiting of *P. eryngii* consisted of 55 % of a 2:1 sawdust mixture of pine tree (*Pinus rigida*) and poplar (*Populus euramericana*), 25 % wheat bran, and 20 % rice bran (v/v, in terms of dry weight). The final moisture content of the substrate was 65 %. Approximately 550 g of substrate was packed into 850-ml polypropylene bottles and sterilized at 121 °C for 90 min. Inoculated bottles with 10 ml of the liquid spawn were taken to an incubation room, where the temperature and humidity were maintained at 22–24 °C and 65–68 % for 35 days. When the substrate was colonized by the mycelium of mushroom, the spawn was removed from the surface and inoculated with 10 ml of bacterial suspensions (approximately 1×10^6 cfu/ml) using a pipette. Inoculated bottles were incubated in a cultivation room at 15 °C and relative humidity 90–95 %. The symptoms of disease development were examined for up to 20 days.

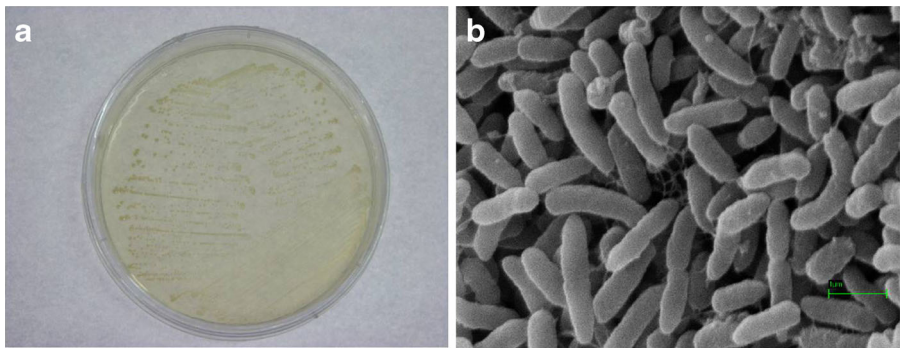

Fig. 2 Colonies grown on LB agar media (**a**) and scanning electron micrographs (b, 50,000 ×) of isolated strain, *Pantoea* sp. strain PPE7. Bacterial suspensions (approximately 1×10^6 cfu/ml) were prepared from LB broth cultures for 24 h. Bar indicates 1 μm

Early symptoms of the disease included a dark-brown water drop that developed on the hypha and primordia of the mushrooms inoculated with the causal bacteria after 5 to 7 days. After 13 days, water-soaked lesions had developed on the cap and stipes, and normal growth of the mushrooms was inhibited. The diseased mushrooms had an offensive odour and the infection soon developed into a severe soft rot that was similar to the symptoms of the disease that developed under natural conditions (Fig. 3). Bacteria isolated from the diseased mushroom were shown to be identical to the inoculated strain, *Pantoea* sp. strain PPE7, used in these pathogenicity tests. These results showed that the disease symptoms could be reproduced and the bacteria re-isolated, thus satisfying Koch's postulates.

The infection ability of causal bacteria was studied on other three types of musrhoom (*Pleurotus ostreatus, Flammulina velutipes, and Agaricus bisporus*). Healthy fruit body of *F. velutipes*, stipe section of *P. ostreatus*, and pileus of *A. bisporus* were transferred to Petri dishes on sterile Whatman No. 1 filter soaking with 5 ml distilled water. One ml of bacterial suspension (approximately 1×10^6 cfu/ml) was inoculated by a pipette to healthy fruit body or stipe section. The sterilized water was used as control. Inoculated mushrooms were incubated for 7 days in a cultivation room of laboratory conditions at 16–17 °C and relative humidity 80–92 %. Assays were repeated at least three times and the results were scored after 5 days.

After 2 to 3 days, changes in colour appeared at inoculation sites on all mushrooms, which developed into water-soaked lesions and soft rot disease after 5 days (Fig. 4). Especially, *F. velutipes* and *A. bisporus* were severely damaged by *Pantoea* sp. strain PPE7.

Concentrations of calcium hypochlorite that inhibited growth of *Pantoea* sp. strain PPE7 were determined as follows. First, a solution of calcium hypochlorite (AC70%; Duksan Co. Ltd., Ansan, Korea) was made at a concentration of 20 %. Solutions of calcium hypochlorite with active chlorine concentrations of 14, 140, 175, 233, 350, 700, and 1,400 ppm were prepared by means of a dilution method of 100 or 200 times, and 500 μl of each dilution was mixed with 500 μl of *Pantoea* sp. strain PPE7 suspension (1×10^6 cfu/ml). The final concentration of calcium hypochlorite was 7, 70, 87.5, 116.7, 175, 350, and 700 ppm. The mixture was incubated at 25 °C for 10 min, and 100 μl of each sample was plated on LB agar medium. 100 μl of

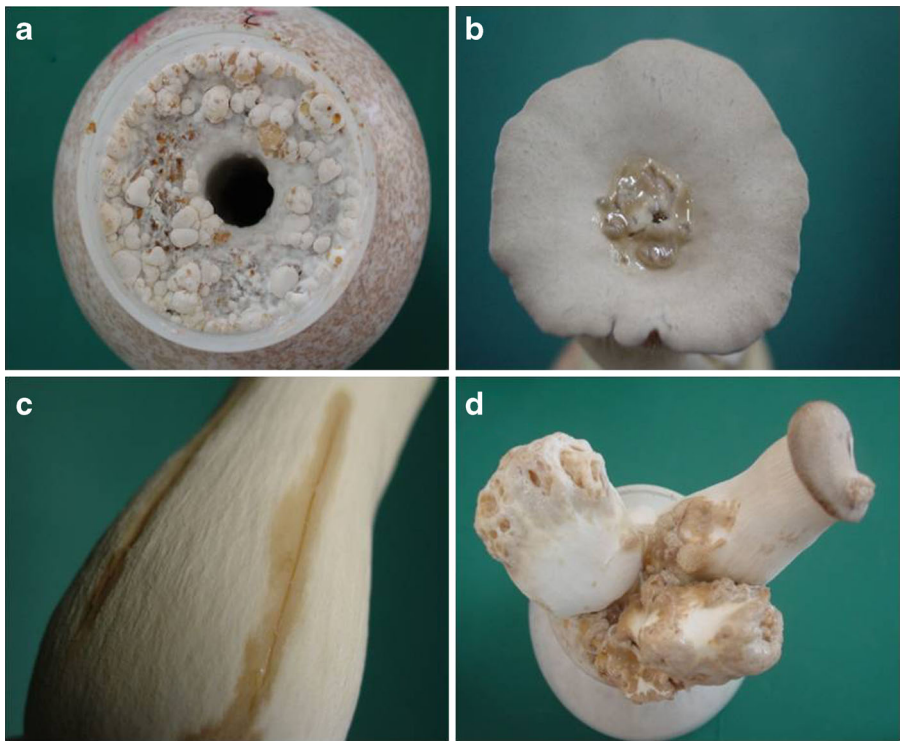

Fig. 3 Symptoms of soft rot disease of *P. eryngii* after artificial inoculation with *Pantoea* sp. strain PPE7. Symptoms caused by the artificial infection were similar to those in naturally occurring disease . **a** Primordia, **b** Cap, **c** Stipe, **d** Fruit body

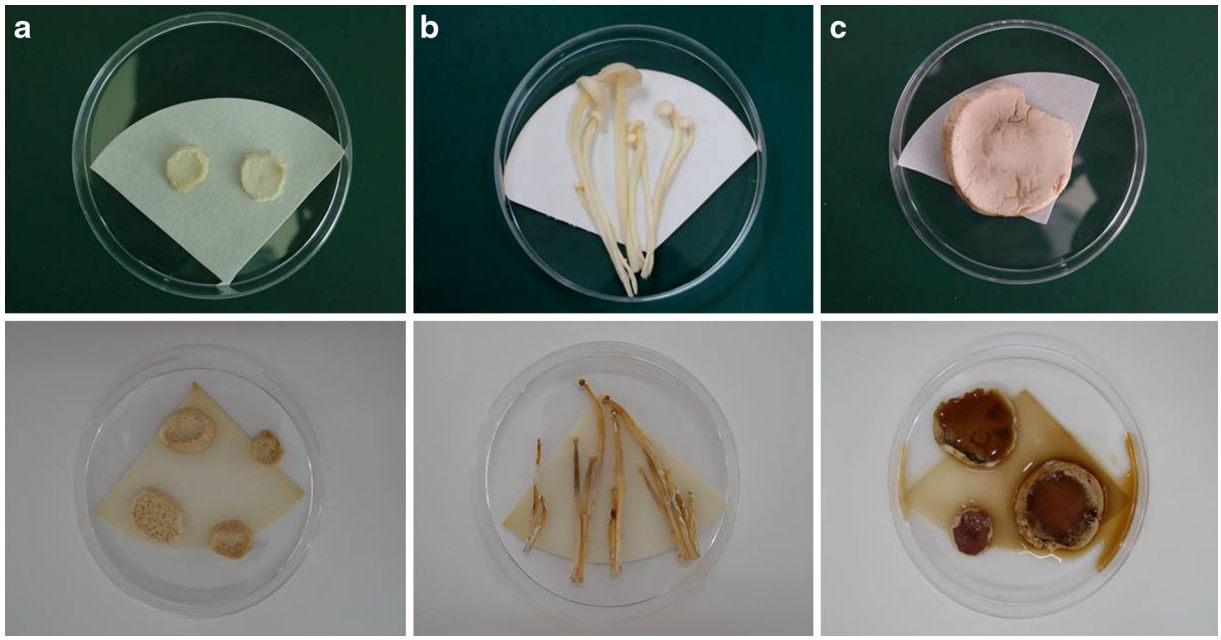

Fig. 4 Symptoms of edible mushrooms inoculated with sterilized water (*upper*) and *Pantoea* sp. strain PPE7 (*bottom*). After 2 to 3 days, changes in colour appeared at inoculation sites in all mushrooms, and finally developed into water-soaked lesions and soft rot disease. **a** *P. ostreatus*, **b** *F. velutipes*, **c** *A. bisporus*

Pantoea sp. strain PPE7 suspension (1×10^6 cfu/ml) without calcium hypochlorite were used as control. Viable cells on LB agar medium were examined by naked eyes after incubation at 28 °C for 24 h. Inhibitory concentrations were defined as those that completely inhibited bacterial growth.

Pantoea sp. strain PPE7 was inhibited at final concentrations of active chlorine greater than 87.5 ppm (Table 1). Wong and Preece (1985) showed that 5 ppm of free active chlorine was sufficient to inhibit growth of *Pseudomonas tolaasii* within seconds of exposure. Shin et al. (1994) also reported that 40 ppm of sodium hypochlorite was the minimal inhibitory concentration for *Pseudomonas agarici*. However, these results showed that a higher concentration of active chlorine was needed to inhibit the growth of *Pantoea* sp. strain PPE7.

To determine the effect of calcium hypochlorite on mycelium growth of *P. eryngii*, solutions of calcium hypochlorite with active chlorine concentrations of 14, 140, 175, 233, 350, 700, and 1,400 ppm were prepared by a dilution method. Volumes of each solution were added to molten sterile MCM media before pouring, giving active chlorine concentrations 7, 70, 87.5, 116.7, 175, 350, and 700 ppm. An agar plug (5 mm diameter) of *P. eryngii* was placed in the centre of an MCM plate containing calcium hypochlorite and incubated at 25 °C. The sterilized MCM media without calcium hypochlorite were used as control. Mycelial growth diameter across two diameters was measured for each plate after 7 days. The effects of treatments on mycelial growth were examined for 20 days.

When the substrate in the bottles was colonized, the spawn was removed by a scratching method and then immediately treated with 10 ml mixture consisting of 5 ml calcium hypochlorite and 5 ml bacterial suspension (1×10^6 cfu/ml) to determine the effect of calcium hypochlorite on infected mushrooms. The final concentration of calcium hypochlorite was 7, 70, 87.5, 116.7, 175, 350, and 700 ppm. The control was inoculated with 10 ml bacterial suspension (1×10^6 cfu/ml).

At levels below active chlorine 175 ppm, calcium hypochlorite did not affect the mycelia growth of *P. eryngii,* but mycelium growth was inhibited at above 350 ppm (Table 1). These results showed that watering with concentrations of active chlorine above 350 ppm can affect the mycelia growth after removing the spawn by a scratching method. To confirm the effect of calcium hypochlorite *in vivo*, the spawn was removed by a scratching method and then immediately treated with 10 ml mixture consisting of 5 ml calcium hypochlorite containing several concentrations of active chlorine (14, 140, 175, 233, 350, 700, and 1,400 ppm) and 5 ml

Table 1 Effect of calcium hypochlorite on *Pantoea* sp. strain PPE7 viability and the mycelial growth of *P. eryngii*

Concentration (ppm) [a]	Viability of pathogen [b]	Mycelial growth of mushroom (mm/20 days) [c]
Control	+	86.7±0.3
7.0	+	86.0±1.0
70.0	+/−	86.3±0.7
87.5	−	85.7±1.3
116.5	−	86.0±1.0
175.0	−	85.8±1.2
350.0	−	83.3±1.5
700.0	−	44.3±0.6

[a] Indicates use of the supernatant of calcium hypochlorite solution (20 g calcium hypochlorite suspended in 100 mL distilled water)

[b] +, Survival; +/−, Trace; −, None

[c] Values are mean ± S.D

bacterial suspension (1×10^6 cfu/ml). We found that a watering treatment with active chlorine of 175 to 700 ppm controlled soft rot disease during cultivation of *P. eryngii*. However, treatment with 350 and 700 ppm active chlorine caused harmful effects such as bleaching of the sporophores and cap of the fruit body (Table 2).

Two king oyster mushroom farms were chosen to determine the effect of calcium hypochlorite on controlling bacterial soft rot disease in field. One had been suffering seriously from bacterial disease and yield loss for 6 months. The other had estimated around 70 % yield loss due to bacterial soft rot disease. When the substrate in the bottles was colonized, the spawn was removed from the surface by a scratching method and then water containing active chlorine 175 ppm of calcium hypochlorite was immediately sprayed to the media of 1,000 bottles (850-ml) in a scratching room. The bottles treated with calcium hypochlorite were then transferred to a cultivation room at 16–17 °C and relative humidity 80–92 % for 20 days. Fruiting was induced by maintaining a low temperature (about 15 °C) and high humidity (approximately 90–95 %). Tap water was used as the control. Effect of calcium hypochlorite was assessed based on yields of mushroom and incidence of soft rot disease. The disease incidence and mushroom yield were expressed as a percentage.

Table 2 The ability of calcium hypochlorite to control bacterial soft rot disease in *P. eryngii*

Treatments [a] (ppm)	Inhibitory effect against soft rot disease [b]	Harmful effect on mushroom[c]
Control	−	N.I.
7.0	−	N.I.
70.0	−	N.I.
87.5	−	N.I.
116.5	−	N.I.
175.0	+	−
350.0	+	+/−
700.0	+	++

[a] Indicates use of the supernatant of calcium hypochlorite solution (20 g calcium hypochlorite suspended in 100 ml distilled water)

[b] +, Complete control; −, No effect

[c] ++, Moderate; +, Weak; +/−, Trace; −, None; N.I.; None investigation

In mushroom farms where *P. eryngii* were afflicted with soft rot disease, treatment with 175 ppm active chlorine significantly reduced disease incidence and improved yield (Table 3).

In the present study, we demonstrated that watering treatments with 175 to 700 ppm active chlorine controlled soft rot disease during cultivation of *P. eryngii*. These results showed the difference between the minimum inhibitory concentration (>87.5 ppm) *in vitro* and the actual concentration (>175 ppm) required to control bacterial disease in *P. eryngii*. However, treatment with active chlorine at concentrations over 350 ppm caused harmful effects, including bleaching of the sporophores and the fruit body cap. Because this concentration is higher than the previously reported for bacterial control, it might be inferred from our data that *Pantoea* sp. strain PPE7 is more resistant to chemical treatment than *Pseudomonas* species (Wong and Preece 1985; Shin et al. 1994). This might be due to the extracellular polysaccharides produced by *Pantoea* sp. that protect against external stress and antimicrobial chemicals.

Table 3 Disease incidence and mushroom yield after treatment of infected *P. eryngii* with chlorinated water containing 175 ppm active chlorine

Mushroom farm	Disease incidence (%)		Yield (kg/1,000 bottles)	
	Treated	Non-treated	Treated	Non-treated
A	5.3±0.2b [a]	31.0±0.8 a	92.5±1.5a	61.7±2.9b
B	6.3±0.3b	75.1±0.5a	95.3±2.4a	23.8±0.8b

[a] Values are mean ± SD. Mean followed by different letters are significantly different (*t*-test, $p<0.05$)

(Wehland et al. 1999; Kim et al. 2007). The results obtained in this study demonstrated that bacterial disease could be significantly reduced by watering treatments with calcium hypochlorite, without a decrease in crop yield. Based on these results, it seems that 350 ppm active chlorine would be required to manage severe soft rot in mushroom farms, and that 175 ppm active chlorine may be sufficient to control the weak and mild bacterial disease effectively. In addition, the observed pathogenic ability of *Pantoea* sp. strain PPE7 in other edible mushrooms should necessitate the design of control measures for bacterial disease in these species.

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