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Effective Heat Treatment Techniques for Control of Mung Bean Sprout Rot, Incorporable into Commercial Mass Production

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Seedlot disinfection techniques to control mung bean sprout rot caused by *Colletoricum acutatum* and *C. gloeosporioides* were evaluated for commercial production scheme. Soaking seedlots in propolis (100 X) and ethanol (20% for 30 min) appeared promising with control values of 85.5 and 80.8 respectively, but still resulted in up to 20% rot incidence. None of the *C. acutatum* conidia survived through hot water immersion treatment (HWT) for 10 min at temperatures of 55, 60 and 65°C, whereas the effective range of the dry heat treatment (DHT) was 60-65°C. Tolerance of mung bean seedlot, as estimated by hypocotyl elongation and root growth, was lower for HWT than for DHT. Germination and growth of sprouts were excellent over the range of 55-65°C at 5°C intervals, except for HWT at 65°C for 5 min. At this marginal condition, heat damage appeared so that approximately 2% of seeds failed to sprout to normal germling and retarded sprouts were less than 5% with coarse wrinkled hypocotyls. These results suggested that DHT would be more feasible to disinfect mung bean seedlots for commercial sprout production. Heat treatment at above ranges was highly effective in eliminating the epiphytic bacterial strains associated with marketed sprout rot samples. HWT of seedlot at 55 and 60°C for 5 min resulted in successful control of mung bean sprout rot incidence with marketable sprout quality. DHT at 60 and 65°C for 30 min also gave good results through the small-scale sprouting system. Therefore, we optimized DHT scheme at 60 and 65°C for 30 min, considering the practical value of seedlot disinfection with high precision and accuracy. This was further proved to be a feasible and reliable method against anthracnose incidence and those bacterial strains associated with marketed sprout rot samples as well, through factory scale mung bean sprout production system.

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Current domestic market of mung bean sprout is estimated to be 170 billion won per annum. Since it shows a better quality compared to other sprouts, its consumption is expected to increase domestically and abroad as well. However, it is highly prone to decay so that food sanitation is a major limiting factor for mass production up to consumption. Han and Lee (1995) identified *Colletotrichum truncatum* and *C. gloeosporioides* from mung bean plants for the first time in Korea. They also found that seed infestation rate was higher for *C. truncatum* and lower for *C. gloeosporioides*.

We previously identified *C. acutatum* from mung bean sprout rot samples on market (Kim et al., 2003) and recently some bacterial strains as well. Unfortunately, most related works have focused on soybean sprout rot, including *Erwinia carotovora* (Park et al., 1997a) and *Pseudomonas putida* biovar A. (Park et al., 1997b) as pathogens. Intermittent application of acidic water at pH 3 (PBS butter 0.01 M 200 g^{-1}) during sprout cultivation or soybean seedlot treatment with acetic acid at 0.5% against bacterial sprout rot (Park and Choi, 1995) was suggested, but not likely be feasible or cost-effective. Alternative approaches are the use of crude herbal extract (Lee et al., 2003) against bacteria and fungi, and calcium salts against *C. gloeosporioides* and *C. acutatum* (Biggs, 1999). However, control methods reported so far only provide pathogen control at a given point in the sprouting process. Recently, Weiss and Hammes (2005) worked out temperature/time regimes against bacterial contaminants on mung bean, alfalfa and radish seeds for sprout production. Here, we attempted to establish the heat treatment as a potential seedlot disinfection technique implementable to

commercial scale mung bean sprout production.

Material and Methods

Fungal and bacterial strains and culture condition. Microbes associated with mung bean sprout rot were isolated from samples from Jinju and Sacheon, Korea. Fungal and bacterial strains (Table 1) were cultured on Potato Dextrose Agar (PDA) and/or Tryptic Soy Agar (TSA) at 27°C, which were identified by morphological characteristics and MIDI gas chromatography, respectively. Large quantities of bacterial cells or spore on PDA were collected by washing surface on PDA plate with 5 ml of ultra pure water and filtering with sterile cheesecloth. The suspensions were centrifuged for 5 min at 8,000 rpm, and resuspended in 0.1 M $MgSO_4$ for bacterial cells or in sterile water for fungal spores, which were inoculated on seedlot.

Mung bean seed inoculation and pathogenicity test. Mung bean seedlots (*Vigna radiata* cv. Zhong Lu 1) were submerged in bacterial or fungal conidia suspension (10^6-10^7 CFU/ml, 10^3-10^4 spores/ml, respectively) for 5 min and dried at ambient temperature on a clean bench for 1 h. The inoculated seeds were subjected to small scale production procedure or a commercial mung bean sprout cultivation protocol to confirm pathogenicity.

Treatment of propolis and ethanol. Those seedlots inoculated with *C. acutatum* as described above were subjected to the propolis (100-fold diluted solution for 30 min) and ethanol (20% and 40%, for 30 min) to determine the efficacies and evaluate the sprout growth in 6 days.

Thermotherapy *in vitro*. E-tubes of 950 μl distilled water were placed at 5°C intervals from 50 to 65°C in hot block (Thermobath ALB128, FINEPCR, Korea), to which 50 μl of conidial suspension, adjusted to 4×10^3 spores/ml, were added in E- tube. After the indicated time period from 5 to 30 min at 5 min intervals, 50 μl of the corresponding sample were enriched in PDA for evaluating the lethal effect of HWT. For DHT, filter paper discs (0.8 cm in diameter) were immersed in conidial suspensions, prepared as above, and air dried under sterile conditions in clean bench, which were transferred to drying oven adjusted to desired temperatures as above. After the corresponding lapse of time, each filter discs was also transferred individually to 1ml distilled water in E-tube, and vortexed, 50 μl aliquots were enriched in PDA for evaluating lethal effect of DHT.

Thermotherapy *in vivo*. Hereafter, temperature regimes for seedlot treatment were carried out from 55 to 65°C at 5°C intervals: 5 min and 30 min for HWT and DHT, respectively. After heat treatment as above, the heat sensitivity of mung bean seedlots without inoculation were monitored for 3 days in 10 L plastic container with underwatering system in laboratory through the small scale production procedure, skipping imbibition and aeration compared to mass production system, and inspected for germinability defects and deformity of sprouts. In order to optimize disinfection scheme, seedlots inoculated with conidia were exposed to heat treatment condition as above, and was subjected to sprouting for 5 days, followed by inspection for necrotic spot and sprout growth.

Lethality of sprout rot associated epiphytic bacteria. Seedlots were artificially inoculated by each of the above bacterial strains and were subjected to the heat treatment scheme as described above. Disinfected seed samples were screened for lethal effects of heat treatment.

Evaluation of HWT and DHW as seedlot disinfection technique incorporable to standard cultivation procedure. Finally, heat treatment regimes were validated so seedlot

Table 1. Fungal and bacterial isolates associated with mungbean sprout rot and their pathogenicity

Isolate	Location	Fungus/Bacterium	Pathogenicity[a]
Colletotricum acutatum	Jinju	Fungus	+
Corynebacterium aquaticum	Jinju	Bacterium	–
Corynebacterium aquaticum	Sacheon	Bacterium	–
Pseudomonas fluorescence	Jinju	Bacterium	–
Microbacterium liquefaciens	Jinju	Bacterium	–
Colletotricum gloeosporioides	Jinju[b]	Fungus	+

Fungal and bacterial strains associated with mungbean sprout rot was isolated from samples from Jinju and Sacheon and cultured on PDA and/or TSA at 27°C, which were identified by morphological characteristics and MIDI gas chromatography. Mungbean seedlots were submerged in bacterial and or conidial suspension ($10^{6\text{-}7}$ CFU/ml, $10^{3\text{-}4}$ spores/ml, respectively) for 5 min and dried at ambient temperature in clean bench for 1 h. The artificially infested seeds were subjected to small scale production procedure to confirm pathogenicity.

[a]–, avirulent; +, highly pathogenic.

[b]isolatid from soybean, but proved to be pathogenic to mungbean sprout in this study.

disinfection process by scale-up experiments was established by our research team (Kang et al., 2004a, b, c, d): disinfection, imbibition (5 hr at 20°C), aeration (4 hr at darkness), cultivation (7 days), and packaging/storage. We evaluated the growth of mung bean sprout and inspected for necrotic spots.

Statistical analysis. The data were subjected to analysis of variance and expressed as mean ± standard deviation. The experimental design included random group assignment. Statistical analysis of data was performed through an analysis of variance using ANOVA ($P<0.05$) and Duncan's multiple range tests using SAS 8.1 statistical data analytical software.

Results

Pathogen and epiphytic bacterial strains. Of the bacterial and fungal strains identified from mung bean sprout on market samples (Table 1), only *C. acutatum* was responsible for sprout rot incidence. None of the bacteria was associated with mung bean sprout rot; *Corynebacterium aquaticum, Pseudomonas fluorescence* and *Microbacterium liquefaciens* were not virulent on mung bean sprout, suggesting that bacterial strains are associated as epiphytics.

Effect of propolis and ethanol treatment. Propolis treatment prior to sprouting was effective in reducing number of necrotic spots on mung bean sprout with the control value of 85.5% (Fig. 1). The treatment with 40% ethanol for 30 min showed the highest control value among all the treatments. Treatment with 20% ethanol still resulted in about 20 spots on hypocotyls and the control value was calculated to be 80.8% equivalent to the efficiencies of propolis treatment. Apparently retarded growth of mung bean sprout, shown as CK in Fig. 2, was attributed to the anthracnose infection on hypocotyls: their elongation was most profoundly affected by artificial inoculation. Hypocotyl growth was equivalently affected by application of propolis and 20% ethanol: propolis reduced root growth more than ethanol did at given level of infection.

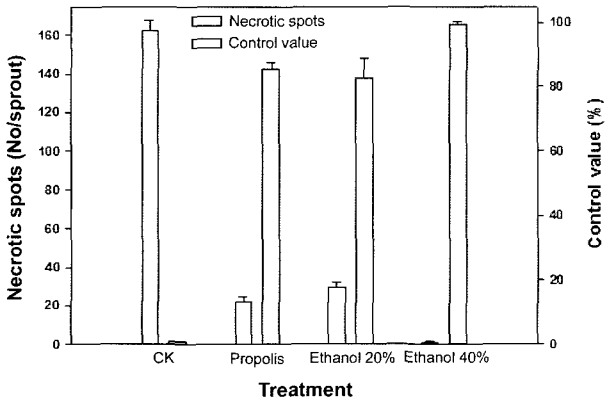

Fig. 1. Effect of propolis and ethanol on the control of mung bean sprout rot incidence. Mung bean seedlots were submerged in conidial suspension (10^{3-4} spores/ml) for 5 min and dried at ambient temperature in clean bench for 1 h. The artificially infested seeds were subjected to propolis (100 X solution for 30 min) and Ethanol (20%, 40%, and 30 min) prior to small scale production procedure for 6 days to determine the efficacies. Values are means of three replicates and bars represent one standard deviation.

Effect of heat treatment on anthracnose fungi *in vitro*. HWT or DHT at 45°C for as long as 30 min resulted in no lethal effect on conidia of *C. acutatum*. HWT at 50°C for 10 min or longer provided significant lethality *in vitro*. However, DHT was not effective at all at 50°C up to 30 min (data not shown). Excellent level of lethality was achieved by HWT for 10 min or longer at 55-65°C. An intermediate level of lethality was apparent up to 55°C for 20-25 min by DHT. No viable counts were retrieved when the temperature was elevated to 60 or 65°C for 10 min (Fig. 3). Therefore, it is suggested that HWT was more effective in eliminating pathogen than DHT. Tolerance of mung bean seedlot, as estimated by hypocotyl elongation and growth, was lower for HWT than for DHT (Table 2). Seedlot germination and growth of sprouts were excellent over the range of the heat treatment, except for those treatments of wet heat at 65°C for 5 min, which resulted in approximately 2% of seeds that failed to sprout to normal germling and less than 5% being retarded sprouts with coarse wrinkled hypocotyls. This

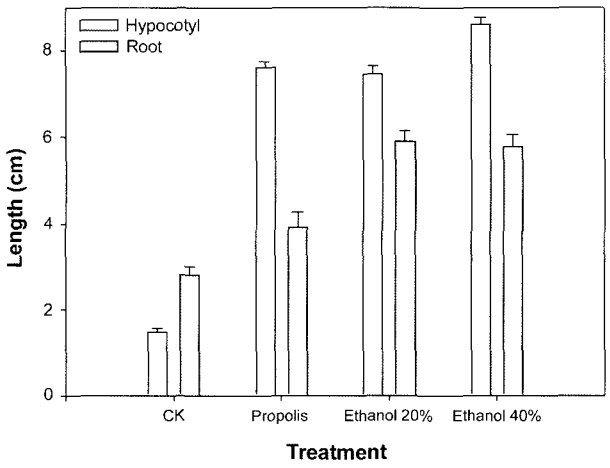

Fig. 2. Effect of propolis and ethanol on the growth of mung bean sprout. Refer to the footnote of Fig. 1 for details. Values are means of three replicates and bars represent one standard deviation.

Table 2. Influence of DHT and HWT on mungbean seedlot on germinability and sprout growth during cultivation

Temperature (°C)	Poor germination[b] (%)		Abnormal sprout[c] (%)	
	DHT (30 min)	HWT (5 min)	DHT (30 min)	HWT (5 min)
CK[a]	0	0	0	0
55	0	0	0	0
60	0	0	0	0
65	0	1 ± 1.2	0	3 ± 1.2

Whole experiment was repeated for three times. Each value represents the mean obtained from five samples per treatment.
[a]Untreated control.
[b]Rate of seed sprouted to abnormal germlings.
[c]Rate of retarded abnormal sprouts with coarse-wrinkled hypocotyl.

result suggested that DHT was more feasible to disinfect mung bean seedlots for commercial sprout production.

Effect of heat treatment HWT and DHT on epiphytic bacteria. Heat treatment scheme was highly effective in eliminating the epiphytic bacterial strains associated with marketed sprout rot samples. None of the bacterial cells were retrieved from any of the heat-treated samples (Table 3).

Effect of HWT and DHW in small-scale sprouting system. HWT of seedlot at 55°C, 60°C and 65°C for 5 min resulted in outstanding lethal effect *in vivo* on mung bean sprout rot incidence with marketable sprout quality. DHT at corresponding temperature for 30 min also gave excellent results through the small-scale sprouting system (Table 4). Both HWT and DTH revealed no apparent necrotic spot and significant effect on hypocotyls and root growth. This DHT method was further proved to be a feasible and reliable method against anthracnose incidence and those bacterial strains associated with marketed sprout samples as well through factory scale mung bean sprout production system (Table 5).

Discussion

The fact that anthracnose fungi and other diverse epiphytic bacterial strains were associated with marketed mung bean sprout attracted our attention to develop disinfection techniques for healthy mung bean sprout production. Pathogen infection provided ecological niches for associated saprobic bacteria (Conway et al., 2000). Fett and Cook (2003) observed that yeast and cocci were abundant but not the filamentous fungi in the native biofilms on the adaxial surface of cotyledons of mung bean sprout. Accordingly, it is urgent to develop a better seedlot disinfection technique

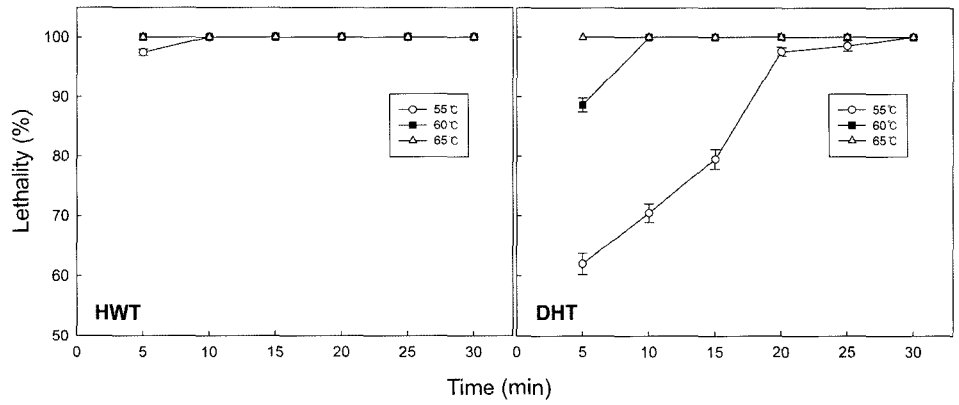

Fig. 3. Lethal effect *in vitro* of HWT and DHT on *C. acutatum in vitro*. Values are means of three replicates and bars represent one standard deviation.

Table 3. Heat susceptibility of bacterial isolates associated with mungbean sprout rot

Isolate	55°C		60°C		65°C		CK[c] (CFU/ml)
	DHT[a]	HWT[b]	DHT	HWT	DHT	HWT	
Corynebacterium aquaticum	0	0	0	0	0	0	740 ± 46
Pseudomonas fluorescence	0	0	0	0	0	0	480 ± 58
Microbacterium liquefaciens	0	0	0	0	0	0	460 ± 32

Whole experiment was repeated for three times. Each value represents the mean obtained from five samples per treatment.
[a]DHT; treated for 30 min at temperature settings in Drying oven.
[b]HWT; treated for 5 min in hot water adjusted to corresponding temperature.
[c]Untreated control: No. of colonies recovered from untreated control sample.

Table 4. Effect of HWT and DHT on mungbean seedlot against sprout rot incidence under small-scale production system

Temperature (°C)	No. of necrotic spots		Hypocotyl length (cm)		Root length (cm)	
	HWT[b]	DHT[c]	HWT	DHT	HWT	DHT
CK[a]	43.2	36	4.58a	4.46a	3.24a	3.80a
55	0	0	7.74b	7.62b	7.00b	7.12b
60	0	0	7.70b	7.82b	7.14b	7.08b
65	0	0	7.64b	7.20b	7.04b	6.74b

Means followed by different letters are significantly different according to Duncan's multiple test at $P < 0.05$. Necrotic spots appeared only untreated control.
[a]Untreated control.
[b]HWT was done for 5 min.
[c]DHT was done for 30 min at corresponding temperatures.

that should eliminate the above seed borne bacterial strains, and to prevent potential carry-over to sprouts economically.

Propolis, a natural product derived from plant resins collected by honeybees, is known to have antimicrobial activities against the 13 different species of plant pathogenic bacteria (Basim et al., 2006) and also against two gram positive human pathogen and/or two gram negative bacteria and yeast (Choi et al., 2006). Soaking seedlots in propolis (100 X) and ethanol (20% for 30 min) appeared promising with control values of 85.5 and 80.8 respectively against sprout anthracnose, but still resulted in up to 20% incidence (Fig. 1) under our *in vivo* screening system. These two agents are cost-ineffective. However, for large-scale production by using non-artificially inoculated commercial seedlots, 15% ethanol for one hour decontaminated seedlot practically in terms of mung bean sprout quality (data not shown).

Recently, heat treatment technology emerged as a feasible and cost-effective method for controlling postharvest decay of horticultural fruit crops (Fallik, 2004). We have found that HWT was more effective to eliminate *C. gloeosporioides* than DHT was (Fig. 3). Surprisingly, mung bean seedlot was fairly tolerant to heat treatment without significant effect on seed germination and growth of sprout at the temperature ranges for satisfactory seedlot disinfestations. Therefore, we optimized the heat treatment scheme at 55-60°C, for 5 min of HWT and 60-65°C for 30 min of DHT by compromising the experimental results obtained through this study. This was supported by Weiss and Hammes (2003, 2005) who reported that mung bean seed, due to its thicker seed coat, tolerated to higher temperature/time regimes from 55°C/20 min, 60°C/10 min, 70°C/5 min to 80°C/2 min.

Table 5. Effect of DHT on mungbean seedlot against sprout rot incidence under standardized production system

Temperature (°C)	No. of necrotic spots	Hypocotyl length (cm)	Root length (cm)
CK[a]	45.5	6.62	2.28
55	0	6.56	2.48
60	0	6.62	2.48
65	0	6.28	2.36

Whole experiment was repeated for three times
[a]Untreated control.

Our HWT and DHT schemes proved to be feasible and reliable methods to effectively eliminate the epiphytic bacterial strains that might potentially contaminate seedlots prior to sprouting. Even though we have not isolated bacterial strains of public concern, i.e., food borne human pathogens as suggested earlier by Fett and Cook (2003), our technique should be effective to decontaminate potential heat-labile microbes on mung bean sprout. One may have noticed the relatively poor growth of mung bean sprout for untreated control plot (UCP), obtained with small scale production system (Table 4), compared to those with standardized system for factory scale production (Table 5). This was attributed to the differences in sprouting process, i.e., for small-scale sprouting system, compared to the standard factory scale production; after disinfection with HWT or DHT, we left out imbibtion and aeration processes prior to sprout, which might have a negative effect on seed germination and growth as shown in Table 4, so that the hypocotyl growth of UCP was significantly shorter. Our heat treatment technique, when incorporated to the factory scale production system, provided marketable quality of mung bean sprout, free of any necrotic spot or decays. The reason why the root length was significantly shorter for factory-scale production system (Table 5) could be attributed to the Benzyladenopurine treatment during imbibtion, which also renders shorter, stouter hypocotyl (Kang et al., 2004b).

Considering the practical value of seedlot disinfection procedures with high precision, it is concluded that DHT would be more feasible to disinfect mung bean seedlots for commercial sprout production. Therefore, our method should be advantageous over others so far available. It provided excellent control against not only the major target

pathogen, but also other bacterial contaminants on mung bean sprout.

Acknowledgements

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