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Ethyl Acetate Extract of *Bacillus pumilus* SH122 Induces Resistance Against Phytophthora Blight in Pepper Plant

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In order to obtain bacterial metabolites inducing disease resistance in pepper plant, two hundred bacterial isolates were isolated from the rhizosphere soil of tobacco, cucumber, and pepper plant. Ethyl acetate extract of each bacterial culture was used to screening for induction of resistance against phytophthora blight of pepper plant. Application of ethyl acetate extract of an isolate SH122 culture to pepper plant conferred resistance against phytophthora blight consistently and significantly. According to cellular fatty acid analysis and other characteristics, the SH122 was identified as *Bacillus pumilus*. Disease severity and lesion length of phytophthora blight on pepper plants treated with ethyl acetate extract of *B. pumilus* SH122 culture were significantly lower than those on control plants treated with ethyl acetate extract of nutrient broth. The *B. pumilus* SH122 itself or ethyl acetate extract of its culture did not show antifungal activity against *Phytophthora capsici* on potato dextrose agar. These results suggest that *B. pumilus* SH122 produces compound(s) induce resistance against phytophthora blight in pepper plants.

Keywords **:** induced resistance, bacterial metabolites, *Bacillus pumilus*, phytophthora blight, ethyl acetate extract.

Induced disease resistance is an enhancement of the plant's defense capacity against a broad spectrum of pathogens after appropriate stimulation. The induced resistance can be triggered by various biotic and abiotic agents, such as the necrogenic pathogens, certain plant growth-promoting rhizobacteria (PGPR), and chemicals (Sticher et al., 1997; van Loon et al., 1998). Nonpathogenic, root-colonizing bacteria have shown to induce resistance in different plants. Colonization of the rhizosphere by *Pseudomonas fluorescens* WCS417 induced systemic resistance against *Ps syringae* pv. *tomato* and *Fusarium oxysporum* f. sp. *raphani* (Pieterse et al., 1996). Seed treatment of three PGPR strains, *Bacillus pumilus* INR7, *B. subtilis* GB03, and *Curtobacterium flaccumfaciens* ME1 resulted in induced systemic resistance mediated disease suppression of angular leaf spot and anthracnose in cucumber in field (Raupach and Klopper, 1998). *Ps. putida* 89B-27 and *Serratia marcesens* 90-166 induced resistance in cucumber against Fusarium wilt and *S. marcesens* 90-166 induced resistance in tobacco against *Ps syringae* pv. *tabaci* (Liu et al., 1995, Press et al., 1997).

Several natural compounds produced by PGPR strains were shown to induce resistance in plants. Lipopolysaccharides (LPS) extracted from *Ps. fluorescens* WCS417 induced systemic resistance against Fusarium wilt in carnation (Van Peer and Schippers, 1992) and radish (Leeman et al., 1995). The purified Pseudobactin, a kind of siderophore, from *Ps. fluorescens* WCS374 also induced resistance in radish against Fusarium wilt caused by *F. oxysporum* f. sp. *raphani* (Leeman et al., 1996). SA produced by *Ps. aeruginosa* 7NSK2 induced resistance against *Botrytis cinerea* in bean, *Phaseolus vulgaris* (De Meyer and Hofte, 1997). Exogenous application of SA were proven to induce resistance in many different plants (Uknes et al., 1992; Delaney et al., 1995; Lawton et al., 1996).

Synthetic chemical compounds, such as 2,6-dichloroisonicotinic acid (INA), DL-β-amino-n-butylic acid (BABA), and benzo(1,2,3)thiadiazole-7-carbothioic acid-s-methylester (BTH) are well known to induce resistance in plants. INA and its methyl ester induced resistance efficiently in dicotyledonous as well as monocotyledonous plant species against a wide spectrum of pathogens, viruses, bacteria, and fungi (Sticher et al., 1997). BABA were shown to induce resistance in tomato, potato, tobacco, and pepper plants against *Phytophthora infestans*, *P. capsici*, and *Peronospora tabacina* (Cohen, 1993; 1994; Sunwoo et al., 1996). BTH induced resistance in a number of plants including wheat, rice, and tobacco (Gorlach et al., 1996). It provided long-lasting protection wheat against powdery mildew, and commercialized in Germany as a plant activator for disease protection.

PGPR and native or synthetic chemical compounds inducing resistance in plants have great potential to provide novel benefits over biocide type fungicide oriented disease control since it could provide more environmentally friendly disease control strategy. The objective of this study

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was to isolate bacterial metabolite(s) inducing resistance against phytophthora blight in pepper plant.

Materials and Methods

Isolation of bacteria Ten grams of rhizosphere soil of tobacco, cucumber, and pepper plant, collected from different locations, was suspended in 100 ml of sterilized water and the suspension was agitated in a rotary shaker for 30 min at room temperature. Bacteria were isolated from the suspension with two ways. First, the suspension was dilution-plated on NA (Bacto beef extract 3 g, Bacto peptone 5 g, Bacto yeast extract 2 g, agar 15 g per liter of distilled water) and various types of colonies grown on the plate were obtained. Second, in order to isolate the *Bacillus* spp., the suspension was kept in 80°C water bath for 50 min before dilution plating. All of the bacterial isolates were cultured pure state and stored in deep freezer at -70°C for further studying.

Ethyl acetate extraction A loopful of bacterial cells on NA was transferred into 50 ml or 250 ml of nutrient broth (NB; Bacto beef extract 3 g, Bacto peptone 5 g, Bacto yeast extract 2 g per liter of distilled water) and cultured in a rotary shaker with 200 rpm at 28°C for 3days. The bacterial culture was extracted with the same volume of ethyl acetate (Merck, Germany) in funnel, and the ethyl acetate layer was collected and air-dried by an evaporator (Oganomation Associates Inc. USA) in safety hood at room temperature. The residue of ethyl acetate remaining in the extract was removed *in vacuo* for 30 min and the extract was kept at -20°C until use. Ethyl acetate extract of NB was prepared with the same method.

Pepper plant and inducer treatment Pepper plant (*Capsicum annuum* L.) cv. Nockwang was cultured in plastic pot (14 cm in diameter) with commercial nursery soil in greenhouse, and 8-9 leaf stage of pepper plants were used in bioassay (Lee et al., 1998). Ethyl acetate extract was resuspended in distilled deionized water with $1/5^{th}$ of the original volume. The suspension was sprayed on leaves and stems of pepper plant until the plant was wet completely. Control plants were sprayed with ethyl acetate extract of NB or 1 mg/ml of DL-β-amino butyric acid (BABA).

Pathogen inoculation and disease assessment *P. capsici* was inoculated on 4 days after the inducr treatment. Zoospore suspension of *P capsici* pc1 was prepared previously (Lee et al., 1998). Two ml of zoospore suspension (10^1 spores per ml) was applied into cotton pad wrapped with parafilm on stem of pepper plant at 2-3 cm above soil line. Disease severity and lesion length of phytophthora blight were determined every other day after inoculation. Disease severity was rated based on 0-5 scale (Sunwoo et al., 1996); 0 = no visible disease symptom, 1 = leaves slightly wilted with brownish lesions beginning to appear on stem, 2 = 30-50% of entire plant diseased, 3 = 50-70% of entire plant diseased, 4 = 70-90% of entire plant diseased, 5 = plant dead. Lesion length was determined by measuring the length of lesion appeared on stem of pepper plant. Plant heights and fresh weights of above ground of pepper plants were determined after a final examination of disease severity and lesion length. Nine plants were used for each treatment with 3 replications.

Identification of isolate SH122 Gram reaction and morphology of SH122 were determined by the methods as described by Leary and Chun (1988). Analysis of the cellular fatty acids of isolate SH122 was carried out by the Microbial Identification System (MIDI Sherock system with Hewlett-Packard 6890 series GC, USA) Preparation and analysis of the cellular fatty acids were followed by manufacturers instruction

Determination of antifungal activity Antifungal activity of *B. pumilus* SH122 and its ethyl acetate was determined by growth inhibition test on potato dextrose agar (PDA, Difco Co. USA). *P. capsici* pc1 was cultured on center of PDA plate for 2days at 28°C, and then 10 μl of filter sterilized ethyl acetate extract of *B. pumilus* SH122 was put into agar plug wells around of the fungal colony. For determination of antifungal activity of *B pumilus* SH122, the bacterium was cultured around the *P. capsici* pc1 colony as the same manner with the ethyl acetate extract of *B pumilus* SH122. The plate was incubated further, and the formation of growth inhibition zones around the well or bacterial colony was observed.

Results

Total two hundred bacterial isolates were obtained from tobacco, cucumber, and pepper plant's rhizosphere soil. For preliminary screening, ethyl acetate extract of 50 ml culture of each bacterial isolate was used to bioassay for induction of resistance against phytophthora blight in pepper plant. Any bacterial isolates of which ethyl acetate extract of 50 ml culture reduced the disease severity and lesion length of phytophthtora blight compared to control plants sprayed with ethyl acetate extract of NB were used to 2^{nd} round screening. After three preliminary screening, the isolate SH122 was selected to further study because it showed the strongest activity of induction of resistance against phytophthora blight in all three bioassays.

The SH122 isolated from tobacco rhizosphere soil after heat treatment of the suspension. It was gram positive by gram staining and 3% KOH test and rod shape on light microscope observation. The result of the Sherock Microbial Identification System suggests that the isolate SH122 is *B. pumilus*. The similarity index of isolate SH122 was 0.744 to the type strain of *B. pumilus*. There were not any other bacteria showed the similarity index more than 0.5 to SH122. The closest one was *Staphylococcus sciuri* of which similality index was 0.232.

Ability of *B. pumilus* SH122 to induce resistance against phytophthora blight in pepper plants was checked further in the larger scale bioassay. Phytophthora blight was significantly lower in the pepper plants sprayed with ethyl acetate extract of *B. pumilus* SH122 than the control pepper plants sprayed with ethyl acetate of NB (Table 1 and Fig. 1). Disease severity of the SH122 extract-treated plants was 0.6 that means almost no disease on the pepper plants while it of control plants was 3.4 indicating more than 50-70% of

Table 1. Reduction of phytophthora blight on pepper plants by pre-treatmrnt of ethyl acetate extract of *B. pumilus* SH122 culture

Treament[Y]	Disease[Z] severity	Lesion length (cm)	Plant height (cm)	Fresh wt. (g)
Control	3.4 ± 2.4 a	6.6 ± 4.8 a	28.3 ± 2.6 b	6.5 ± 4.8 b
SH122	0.6 ± 0.9 b	0.5 ± 0.9 b	32.8 ± 2.4 a	13.7 ± 3.9 a
BABA	1.0 ± 1.6 ab	1.5 ± 2.8 ab	30.4 ± 2.8 ab	11.2 ± 4.3 ab

[Y] Ethyl acetate extract of nutrient broth (control) and *B pumilus* SH122 culture (SH122), and 1 mg/ml of DL-β-amino-n-butyric acid (BABA) were sprayed on 9 pepper plants with three replications. *P. capsici* was inocurated on 4 days after the treatment.

[Z] Disease severity, lesion length, plant height and fresh wt. of above ground part of pepper plant were determined at 14 days after *P. capsici* inoculation. Values represent means ± standard deviations. Values followed by the same letter in each column are not significantly different (p = 0.05) by Duncan's multiple range test

entire plant diseased. Lesion length was significantly lower in the SH122 extract-treated plants than the control plants as well. Both disease severity and lesion length of the SH122 extract-treated plants was even lower than those of the BABA-treated plants. Plant height and fresh weight of above ground part of pepper plant were the highest in the SH122 extract-treated plants.

B. pumilus SH122 did not make growth inhibition of *P. capsici* on PDA (Fig. 2). The ethyl acetate extract of *B. pumilus* SH122 did not show any growth inhibition activity of *P. capsici* on PDA either (data not shown).

Disscussion

Gram positive, rod shape, heat resistance, and the similality index of isolate SH122 by cellular fatty acid analysis with the Sherock Microbial Identification System suggest that the SH122 is *B. pumilus*. It was interesting that the SH122 was identified to *B. pumilus*, because *B. pumilus* was reported as the plant growth promoting rhizobacteria (PGPR) inducing the induced systemic resistance (ISR) on cucumber (Wei et al., 1996; Raupach and Kloepper, 1998). We do not know that *B. pumilus* SH122 has ability for PGPR in tobacco, cucumber, or pepper plant yet.

The result that *B. pumilus* SH122 and its ethyl acetate extract did not show antifungal activity on *P. capsici* suggests the reduction of phytophthora blight on the pepper plants sprayed with ethyl acetate extract of *B. pumilus* SH122 due to induction of resistance in pepper plants rather than fungicidal activity of the extract. Treatment of ethyl acetate extrxact of *B. pumilus* SH122 reduced more phytophthora blight than 1 mg/ml of BABA which is well known as a strong inducer of disease resistance in tomato (Cohen, 1993), tobacco (Cohen, 1994), and pepper plant

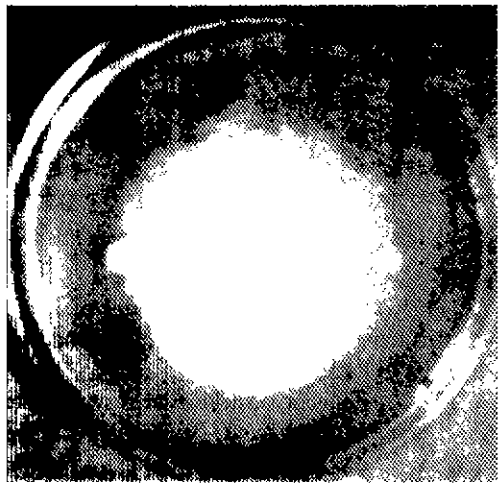

Fig. 2. No growth inhibition zone around *B. pumilus* SH122 against *P. capsici* have been observed on potato dextrose agar.

Fig. 1. Phytophthora blight symptoms on pepper plants treated with ethyl acetate extract of *B. pumilus* SH122 culture (122) and Nutrient broth (control), and 1 mg/ml of BABA. *P. capsici* was inoculated on stem of pepper plants on 4 days after the treatment.

(Sunwoo et al., 1996). These results suggest that *B. pumilus* SH122 produces strong inducer(s) of disease resistance in pepper plants.

Phytophthora blight of pepper plant is one of the widespread and destructive soilborne diseases and it is not readily controlled by fungicide application (Hwang and Kim, 1992). In pepper plants, non-pathogenic strain of *P. capsici*, TMV, and BABA were reported to induce resistance against phytophthora blight previously (Hwang and Kim, 1992; Lee et al., 1998; Sunwoo et al., 1996). The microorganisms or chemicals induce disease resistance in pepper plant, such as non-pathogenic strain of *P. capsici*, BABA, ethyl acetate extract of *B. pumilus* SH122, may provide more effective and environmentally friendly control measure for the disease.

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References

Cohen, Y. 1993. Local and systemic control of *Phytophthora infestans* in tomato plants by DL-3-amino-n-butanoic acids. *Phytopathology* 84.55-59.

Cohen, Y. 1994. 3-aminobutyric acid induces systemic resistance against *Peronospora tabacina*. *Physiol. Mol. Plant Pathol* 44:273-288.

Delaney, T., Friedrich, L. and Ryals, J. 1995. *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. USA* 92: 6602-6606.

De Meyer, G. and Hofte, M. 1997. Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinerea* on bean. *Phytopathology* 87:588-593.

Golach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K., Staub, T., Ward, E. and Kessmann, H. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* 8:629-643.

Hwang, B. K. and Kim, E S 1992 Protection of pepper plants against Phytophthora blight by an avirulent isolate of *Phytophthora capsici*. *Korean J. Plant Pathol.* 8:1-7.

Lawton, K., Friedrich, L., Hunt, M., Weymann, K., Staub, T., Kessmann, H. and Ryals, J. 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10: 71-82.

Leary, J. V. and Chun, W. W. C. 1988. *Bacillus*. In: *Laboratory guide for identification of plant pathogenic bacteria* ed. by N. W. Shaad, pp120-127. APS press, MN., U.S.A.

Lee, S-H., Lee, J.-Y. and Cha, J.-S. 1998. Induction of resistance by TMV infection in *Capsicum annuum* against phytophthora blight. *Korean J Plant Pathol.* 14:319-324.

Leeman, M , Van Pelt, J. A., Den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M. and Schippers, B. 1995. Induction of systemic resistance against Fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. *Phytopathology* 85:1021-1027

Leeman, M., Den Ouden, F. M., Van Pelt, J. A., Dirkx, F. P. M, Steijl, H., Bakker, P. A. H. M. and Schippers, B. 1996. Iron availability affects induction of systemic resistance to Fusarium wilt of radish by *Pseudomonas fluorescens*. *Phytopathology* 86:149-155.

Liu, L., Kloepper, J. W. and Tuzun, S. 1995. Induction of systemic resistance in cucumber against Fusarium wilt by plant growth-promoting rhizobacteria. *Phytopathology* 85:695-698

Pieterse, C. M. J., van Wees, S. C. M., Hoffland, E., van Pelt, J. A. and van Loon, L. C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis related gene expression. *Plant Cell* 8:1225-1237.

Press, C. M., Wilson, M., Tuzun, S. and Kloepper, J. W. 1997. Salicylic acid produced by *Serratia marcesens* 90-166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. *Mol Plant-Microbe Interact.* 10:761-768.

Raupach, G. S. and Klopper, J. W. 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 88:1158-1164.

Sticher, L., Mauch-Mani, B. and Metraux, J. P. 1997. Systemic acquired resistance. *Annu Rev. Phytopathol.* 35:235-270.

Sunwoo, J. Y., Lee, Y. K. and Hang, B. K. 1996. Induced resistance against *Phytophthora capsici* in pepper plants in response to DL-ß-amino-n-butyric acid. *European J. Plant Pathol.* 102:663-670.

Ukncs, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J. 1992. Acquired resistance in *Arabidopsis*. *Plant Cell* 4:645-656.

Van Loon, L. C., Bakker, P. A. H M. and Pieterse, C. M. 1998. Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36:453-483.

Van Peer, R. and Schippers, B. 1992. Lipopolysaccharides of plant-growth promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to Fusarium wilt. *Neth. J. Plant Pathol* 98:129-139.

Wei, G., Kloepper, J. and Tuzun, S. 1996 Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. *Phytopathology* 86:221-224.