Plant Pathol. J. 21(1) : 68-72 (2005)

The Plant Pathology Journal

Enhancement of Biocontrol Efficacy of *Serratia plymuthica* A21-4 Against Phytophthora Blight of Pepper by Improvement of Inoculation Buffer Solution

Shun-Shan Shen, Sin-Hyo Park and Chang-Seuk Park*

Division of Plant Resources and Environment, Gyeongsang National University, Jinju 660-701, Korea

(Received on November 22, 2004; Accepted on January 28, 2005)

The production of antibiotic substances by *Serratia plymuthica* A21-4 was greatly enhanced by modifying components of a growth medium. When the minimal medium containing K_2HPO_4 0.7%, KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4$ 0.01% was used as basal medium, the best carbon source for antibiotic production was glycerol and the most favorable nitrogen source was ammonium sulfate. The modified medium for antibiotic production also increased colonization ability of A21-4 on pepper root and in the rhizosphere soil. When the cells of A21-4 were suspended in modified medium, the population density of A21-4 on pepper root was 10-100 times higher than that suspended in 0.1 M $MgSO_4$. The population density of A21-4 on root did not decrease under 10^6 cfu/groot up to 21 days after treatment although the inoculum of A21-4 was reduced to 10^7 cell/ml. Similar tendency was also observed in the rhizosphere soil. Consequently, Phytophthora blight of pepper was successfully controlled by A21-4 with 10^7 cell/ml suspended in the modified buffer solution instead of 10^9 cfu/ml suspended in 0.1 M $MgSO_4$.

Keywords **:** biological control, Phytophthora blight, pepper, *Serratia plymuthica* A21-4

Since the Phytophthora blight of pepper is frequently destructive, it is considered as the principal limiting factor in pepper production in Korea (Hwang et al., 1995). Fungicide treatment is the main measure to control the disease. However, the chemical treatment was not satisfied and rather brought undesirable effects to environments (Jee et al., 1988). Although numerous attempts have been made to control the disease by biological means, most of them were not practically feasible. Main reason for the failure is that the population of biocontrol agents was not sufficient to suppress the pathogens in soil throughout the growing season. In our previous study a promising biocontrol agent, *Serratia plymuthica* A21-4 showed high efficacy, on control of Phytophthora blight of pepper. The bacterial isolate was also proved to produce strong antifungal substance *in vitro*. For the effective control, however, relatively high density of cell number (10^9 cfu/ml) is required (Shen et al., 2002). In this experiment, we tried to determine favorable carbon, nitrogen sources and mineral by analysis of antibiotic production to improve biocontrol efficacy of the selected isolate *Serratia plymuthica* A21-4 with low concentration of inoculum.

*Corresponding author
Phone) +82-55-751-5442, FAX) +82-55-751-5439
E-mail) changpak@gsnu.ac.kr

Material and methods

Screening of nutritional component for production of antibiotic substance. To select the better carbon source for the antibiotic production, various carbon sources (1.0%) were incorporate to the basal medium (K_2HPO_4 0.7%, KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.01%). A21-4 was cultured in this medium at 28°C for 48 hours, and the antagonistic activity from the culture supernatant of A21-4 was tested.

To select the better nitrogen source for the antibiotic production, various nitrogen sources (0.1%) were added into the basal medium containing glycerol as a carbon source. After the culture at 28°C, the antagonistic activity of A21-4 was evaluated.

To select the salts for the production of antibiotic compound, various salts (5 mM) were added into the basal medium containing glycerol and $(NH_4)_2SO_4$ as carbon and nitrogen sources. After overnight incubation at 28°C, the antagonistic activity of the culture filtrate was evaluated.

***In vitro* evaluation of suppressive effect of A21-4 against *P. capsici*.** For evaluation of inhibition activity of A21-4 against the mycelial growth of *P. capsici*, the mycelial block of *P. capsici* was inoculated on the V8 juice medium that the culture supernatant of A21-4 was added previously, the plates were incubated at 28°C, and the growth rate was examined after 7days incubation.

For evaluation of inhibition activity to cystospore germination of *P. capsici*, a cystospore suspension of *P. capsici* was mixed with an equal volume of culture supernatant of A21-4, and then the mixture was dropped on a glass slide. The slides were kept in a moisten petri plate at 30°C, and the number of germinated cystospores were

examined and recorded under the microscope after 4 hours incubation.

For assay the inhibitory effect of A21-4 to zoosporangia formation, the mycelial block of *P. capsici* which grown on V8 agar for 3 dyas was moved to a new empty petri plate and then culture supernatant was added. The plate was placed under a blue fluorescent light at 25°C. Number of zoosporangia was counted after 16 hours.

Analysis of population density of A21-4 in soil and pepper root. One gram of rhizosphere soil or root were macerated in sterile mortar and pestle containing 9 ml of 0.1M $MgSO_4$ and vortexed for one min. The population density of A21-4 was determined by dilution plate on 1/10 strength of TSA containing 50 μl/ml rifampicin.

Assessment of suppression of Phytophthora blight by A21-4. For assessment of disease suppression of A21-4, cell suspension of A21-4 was prepared with modified buffer (sodium citrate 0.05%, glycerol 0.1%, $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.7%, KH_2PO_4 0.2%, $MgSO_4$ 0.01%) and the generally used 0.1 M $MgSO_4$ buffer and kept in 4 until use.

The roots of 50-day-old pepper seedlings (variety Nok-Kwang) supplied by commercial plug nursery company were soaked in each bacterial suspension of A21-4 (10^7 to 10^9 cfu/ml). Then, the plants were transplanted to plastic pot (9 cm in diameter) containing pot mix soil (Tosilee Sinangro Co, Korea) which was previously infested with zoospore suspension of *P. capsici* (90 g of pot mix soil with 10 ml of zoospore suspension at the concentration of 10^4 spore/ml). The infected plants showing typical Phtophthora blight symptom were examined until 21 days.

Results

Effect of carbon sources on antibiotic production. To select the most effective carbon source for the production of antibiotic compounds by A21-4, various carbon sources were tested using basal medium containing K_2HPO_4 0.7%, KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.01%. Among 11 carbon sources tested, glycerol was the most effective to increase suppressive activity of the bacteria to *P. capsici* and followed by lactose and galactose. When the antagonistic bacterium was cultured in the basal medium containing 0.1% of glycerol at 28°C for 48 hours, inhibition rates of mycelial growth, zoospore formation, and cystospore germination of *P. capsici* were 65.5, 93.3, and 95.9%, respectively, while the inhibitory rates of mycelial growth, zoospore formation, and cystospore germination of the pathogen without carbon source were 10.9, 27.3, and 16.9%, respectively (Table 1).

Effect of nitrogen sources on antibiotic production. To select best nitrogen source for the production of antibiotic compound by A21-4, various nitrogen sources (0.1%) were supplemented into the basal medium containing glycerol as a carbon source. Among 13 nitrogen sources tested, ammonium sulfate was the most effective to increase suppressive activity of the bacterium to *P. capsici* and followed by $(NH_4)_2S_2O_8$. The culture supernatant of A21-4 obtained from the basal medium supplemented with ammonium sulfate inhibited mycelial growth 65.49%, zoosporangial formation 93.3%, and cystospore germination 95.9%, respectively (Table 2).

Table 1. Effect of carbon sources on inhibitory activities of *Serratia plymuthica* A21-4 against *Phytophthora capsici*

Carbon source[a]	Cell number (cell/ml)	Inhibition rate[c] (%)		
		mycelial growth	zoosporangia formation	cystospore germination
None	2.2×10^8 h[b]	10.9 j	27.3 k	16.9 i
Arabinose	6.3×10^8 g	20.5 h	31.4 j	5.2 j
Saccharose	3.1×10^9 ab	31.1 e	86.7 c	18.5 h
Glycerol	1.9×10^9 f	65.5 a	93.3 a	95.9 a
Glucose	2.7×10^9 de	29.5 f	88.2 b	20.3 g
Galactose	2.6×10^9 e	21.5 g	68.4 h	80.8 c
Xylose	2.9×10^9 cd	34.3 d	77.3 e	88.1 b
Lactose	3.0×10^9 bc	54.5 b	80.0 d	73.1 d
Maltose	3.3×10^9 a	35.3 c	75.4 f	24.8 f
Fructose	2.7×10^9 e	15.4 i	65.5 i	73.5 d
TSB	3.0×10^9 bc	20.5 h	73.1 g	51.4 e

[a] Each carbon source (1.0%) was added to the basal medium consisted with K_2HPO_4 0.7%, KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.01% and sodium citrate 0.05% pH 7.0.
[b] Different letter in the column means significantly different at 5% probability level (Turkey's studentized range test).
[c] Inhibition rate = [(Non Treatment – Treatment)/Non Treatment] × 100.

Effect of mineral salts on antibiotic production. To select the best salt for the production of antibiotic compound, various salts at the concentration of 5 mM were added into the basal medium containing glycerol 1.0% as a carbon and $(NH_4)_2SO_4$ 0.1% as a nitrogen sources, the antagonistic activity of A21-4 was evaluated. The most favorable mineral salts for the production of antibiotic by *S. plymuthica* A21-4 was combination of K_2HPO_4 0.7% + KH_2PO_4 0.2% + $MgSO_4 \cdot 7H_2O$ 0.01% (Table 3).

Population change of A21-4 in pepper roots and soil. Effects of buffers on population changes of A21-4 in pepper roots and rhizosphere soils were investigated. Colonization ability of the bacterium suspended in the modified buffer was much higher than that suspended in 0.1 M $MgSO_4$ in both pepper root and rhizosphere soil. The bacterium at 10^9 cfu/ml was treated with the modified buffer, the colonized population density sustained over 10^7 cfu/g in pepper roots until 21 days and at 10^7 cfu/ml was treated with modified buffer, the colonized population density slightly decreased after 21 days (Fig. 1). However,

Table 2. Effect of nitrogen sources on inhibitory activities of *Serratia plymuthica* A21-4 against *Phytophthora capsici*

Nitrogen source[a]	Cell number (cell/ml)	Inhibition rate[c] (%)		
		mycelial growth	zoosporangia formation	cystospore germination
None	2.0×10^8 h[b]	9.6 j	64.0 g	50.2 i
$(NH_4)_2SO_4$	1.9×10^9 c	65.5 a	93.3 a	95.9 a
Urea	1.5×10^9 d	14.9 h	65.2 f	56.2 g
Bacto peptone	4.1×10^8 f	22.1 f	64.0 g	62.8 d
$NH_4H_2PO_4$	2.3×10^9 b	46.9 d	89.0 b	89.8 b
Malt extract	2.8×10^8 g	10.3 j	64.0 g	35.2 l
KNO_3	1.4×10^9 d	19.9 g	64.0 g	32.6 m
Beef extract	1.0×10^9 e	14.8 h	64.0 g	42.9 j
$NaNO_3$	2.3×10^9 b	11.8 i	84.0 d	59.8 e
Yeast extract	1.8×10^9 c	9.6 j	64.0 g	39.6 k
$(NH_4)_2S_2O_8$	3.1×10^9 a	52.2 b	72.7 e	66.6 c
Proteose peptone NO.3	1.7×10^9 cd	39.1 e	64.0 g	23.7 n
$(NH_4)_2HPO_4$	2.4×10^9 b	49.5 c	88.0 c	57.1 f
TSB	3.0×10^9 a	20.5 g	73.1 e	51.4 h

[a] Nitrogen sources (0.1%) were added to the medium [K_2HPO_4 0.7%, KH_2PO_4 0.2%, $MgSO_4\cdot7H_2O$ 0.01%, sodium citrate 0.05% and glycerol 1.0%, pH 7.0] and the *S. plymuthica* A21-4 was cultivated for 48 hours at 28°C.

[b] Different letter in the column means significantly different at 5% probability level (Turkey's studentized range test).

[c] Inhibition rate = [(Non Treatment – Treatment)/Non Treatment] × 100.

Table 3. Effect of salts on inhibitory activities of *Serratia plymuthica* A21-4 against *Phytophthora capsici*

Mineral salts[a]	Cell number (cell/ml)	Inhibition rate[d] (%)		
		mycelial growth	zoosporangia formation	cystospore germination
Salt mix[b]	3.1×10^9 b[c]	65.5 a	93.3 a	95.9 a
$FeCl_3\cdot6H_2O$	2.3×10^7 h	34.3 e	37.3 d	10.5 h
$BaCl_2$	1.1×10^8 e	34.3 e	32.0 d	22.8 d
$NaHPO_4$	3.8×10^7 fg	40.0 c	70.7 b	13.3 g
$CaCO_3$	3.2×10^8 c	31.3 f	53.3 c	47.3 c
$MgCl_2$	9.0×10^7 e	37.1 d	32.0 d	9.1 i
KCl	1.8×10^7 i	34.3 e	64.0 b	17.6 e
K_2HPO_4	1.7×10^8 d	34.3 e	64.0 b	10.3 h
NaCl	1.1×10^8 e	51.4 b	68.0 b	15.3 f
$CaCl_2$	2.0×10^7 hi	37.1 d	53.0 c	0.7 k
$FeSO_4$	4.9×10^7 f	37.1 d	16.0 e	13.3 g
LiCl	3.2×10^7 g	51.4 b	53.0 c	4.3 j
TSB	3.0×10^9 a	20.5 g	73.1 b	51.4 b

[a] Mineral salts (5 mM) were added to the medium [$(NH_4)_2SO_4$ 0.1%, sodium citrate 0.05% and glycerol 1.0%, pH 7.0] and the *S. plymuthica* A21-4 was cultivated for 48 hours at 28°C.

[b] K_2HPO_4 0.7%, KH_2PO_4 0.2%, $MgSO_4\cdot7H_2O$ 0.01%.

[c] Different letter in the column means significantly different at 5% probability level (Turkey's studentized range test).

[d] Inhibition rate = [(Non Treatment – Treatment)/Non Treatment] × 100.

the bacterial population treated with 0.1M $MgSO_4$ was much lower than those of corresponding densities compared to the modified buffer. The bacterial population suspended in 0.1 M $MgSO_4$ at 10^9 cfu/ml was lower than that suspended in the modified buffer at 10^7 cfu/ml after 14 and 21 days after treatment (Fig. 1).

Population densities of A21-4 in the rhizosphere soil of pepper were also different between the two buffers. The bacterial density in pepper rhizosphere soil decreased in all treatments. However, the bacterial population in the modified buffer remained over 10^7 cfu/g soil when treated at 10^7 cfu/ml until 21 days. The bacterium was treated with the modified buffer, population density sustained over 10^7 cfu/g in the soil until 21 days (Fig. 2). However, the bacterial population treated with 0.1 M $MgSO_4$ was much lower than those of corresponding densities compared to the modified buffer. The bacterial population suspended in 0.1 M $MgSO_4$ at 10^9 cfu/ml was lower than that suspended in the modified buffer at 10^7 cfu/ml after 14 and 21 days after treatment (Fig. 2).

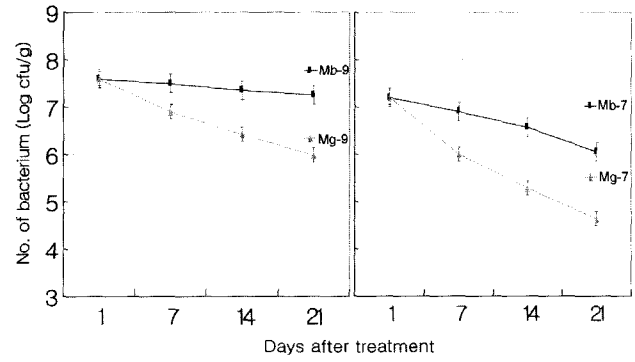

Fig. 1. Population changes of *Serratia plymuthica* A21-4 in pepper root. The bacterium was suspended in either the modified buffer (Mb) or 0.1 M $MgSO_4$ (Mg) with different concentrations (Left: 10^9 cfu/ml, Right: 10^7 cfu/ml).

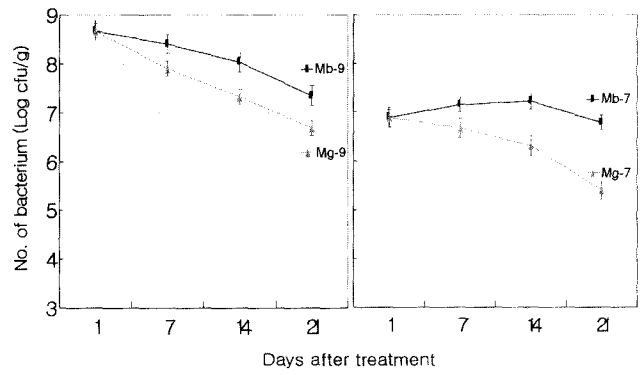

Fig. 2. Population changes of *Serratia plymuthica* A21-4 in the rhizosphere soil of pepper. The bacterium was suspended in either the modified buffer (Mb) or 0.1 M $MgSO_4$ (Mg) with different concentrations (Left: 10^9 cfu/ml, Right: 10^7 cfu/ml).

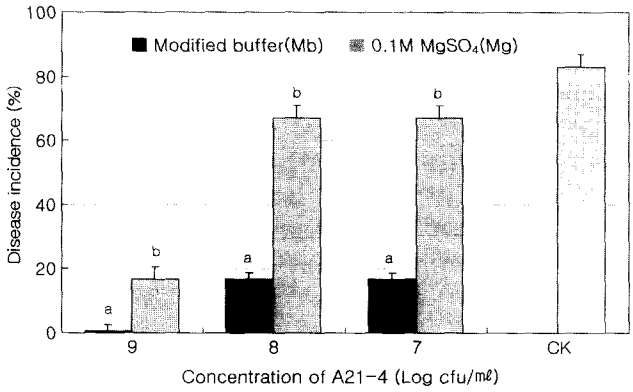

Fig 3. Comparison of the general buffer 0.1 M $MgSO_4$ and the modified buffer on suppression of Phytophthora blight of pepper. Cells of *Serratia plymthica* A21-4 were suspended in each buffer and peppers were soaked and transplanted into artificially infested soils.

Fig. 4. Effect of the modified buffer on increasing bio-activity of *Serratia plymuthica* A21-4 against Phytophthora blight of pepper. Pepper root treated with the bacterium in modified buffer showed health (right), while the pepper roots treated with the bacterial suspension of 0.1 M $MgSO_4$ were diseased (left).

Effect of buffer on activity of A21-4 for suppression of Phytophthora blight of pepper. The modified buffer significantly increased the suppressive activity of A21-4 compared to the general buffer 0.1 M $MgSO_4$. When the bacterial cells were suspended in the buffer 0.1 M $MgSO_4$, only 10^9 cfu/ml effectively suppressed the Phytophthora blight showing lower than 20% disease incidence. In contrast, pepper treated with 10^8 cfu/ml or 10^7 cfu/ml revealed over 60% disease incidence. However, when the bacterial cells were suspended in the modified buffer, pepper treated with 10^8 cfu/ml or 10^7 cfu/ml showed lower than 20% disease incidence and 100% control effect with 10^9 cfu/ml. Consequently, the modified buffer increased the bacterial activity about 100 times on suppression of Phytophthora blight (Fig. 3). Pepper root treated with 10^7 cfu/ml of bacterium in modified buffer showed health, while the pepper roots treated with the bacterial suspension of 0.1 M $MgSO_4$ were diseased (Fig. 4).

Discussion

Antagonistic root-associated bacteria are an important functional group of beneficial bacteria responsible for the control of soilborne pathogens (Weller, 1988). Many promising biocontrol agents, such as, *Pseudomonas cepacia* (Jee et al., 1988; Park et al., 1989), *Pseudomonas fluorescens* (Ryu, 1988), *Trichoderma harzianum* (Jee et al., 1988; Park et al., 1989), *Bacillus* spp. (Jee et al., 1988) and *Enterobacter agllomerans* (Park et al., 1989) were reported for control of Phytophthora blight of pepper. In previous study, the author also selected *Serratia plymuthica* A21-4 that showing strong antibiotic activity *in vitro* and successful suppression of Phytophthora blight of pepper in pots and in a farmer's greenhouses (Shen et al., 2002).

The potential antagonistic microorganisms selected by *in vitro* test often fail to effectively control plant diseases in greenhouse or field trials (Weller et al., 1985). But *S. plymuthica* A21-4 strongly inhibited the germination of cystospore, zoosprangia and mycelial growth of *P. capsici* not only in laboratory experiments, but also protected successfully the infection of pathogenic fungus in pot, small scale vinyl house experiments, and in a commercial scale vinyl houses (Shen et al., 2002).

The most important factor to obtain successful biocontrol of soilborne diseases is how to maintain sufficient population density in the rhizosphere and roots as long as the crop is growing. Many works have been reported how to maintain the population density of biocontrol agent. Some of them developed the formulation of biocontrol agents (Park et al., 1989) and some amended with organic materials into soil (Nam et al., 1988). But it is not easy to maintain the population density of microorganisms which introduced to soil. Excellent root colonization by introduced bacteria is essential for the suppression of root pathogens (Kaiser et al., 1989). Bacteria growing in or near the infection courts on roots are ideally positioned to inhibit root pathogens early in pathogenesis (Kaiser et al., 1989). The strain A21-4 readily colonized on the pepper root system via germinating seed and seedling root inoculation. The colonization of roots with A21-4 may represent for the means preoccupation of possible infection sites of the pathogene. A21-4 readily colonized on pepper roots and remained sufficiently high population density for prolonged period of time. This prerequisite is fulfilled by suspending bacterial cells in modified minimal buffer. The treatment of A21-4 cell suspended in modified minimal buffer greatly enhanced the colonization ability of A21-4 on root and in the rhizosphere soil of pepper plant (Figs. 1 and 2), consequently reducing the inoculum concentration.

For the early parts of this study, cells of *S. plymuthica* A21-4 were suspended in 0.1 M $MgSO_4$ buffer which has

been routinely used in the laboratory. In later parts of this study, we have developed a new formulation to enhancing the biocontrol activity of *S. plymuthica* A21-4. The author found that A21-4 produced much more antifungal substances when grown in the modified minimal medium than other common media used routinely so far. It is presumed that chemical compounds of modified medium might stimulated the bacteria to produce antibiotic substances. Therefore, the modified minimal medium and buffer was substituted for 0.1 M $MgSO_4$ buffer. The newly formulated buffer solution for the suspended bacterial cells provided superior biocontrol efficacy even at 100× lower concentration: treatment of 10^7 cell/ml resulted in disease suppression equivalent to that obtained by 10^9 cell/ml suspended in 0.1 M $MgSO_4$ buffer (Fig. 3). This finding is crucial for commercial large scale production of biocontrol agent formulation.

Acknowledgments

This study was supported by technology Development Program for Agriculture and Forestry, Republic of Korea.

References

Hwang, B. K. and Kim, C. H. 1995. Phytophthora blight of pepper and its control in Korea. *Plant Dis.* 79:221-227.

Jee, H. J., Nam, C. G. and Kim, C. H. 1988. Studies on Biological Control of Phytophthora Blight of Red-pepper I. Isolation of Antagonists and Evaluation of Antagonistic Activity *in vitro* and in Greenhouse. *Kor. J. Plant Pathol.* 4:305-312.

Kaiser, W. J., Hannan, R. M. and Weller, K. M. 1989. Biological control of seed rot and pre-emergence damping-off of chickpea with fluorescent pseudomonas. *Soil. Biol. Biochem.* 21:269-273.

Kurze, S., Dahl, R., Bahl, H. and Berg, G. 2001. Biological control of soil-borne pathogens in strawberry by *Serratia plymuthica* HRO-C48. *Plant Dis.* 85:529-534.

Lee, J. Y., Kim, B. S., Lim, S. W., Lee, B. K., Kim, C. H. and Hwang, B. K. 1999. Field Control of Phytophthora Blight of Pepper Plants with Antagonistic Rhizobacteria and DL-β-Amino-n-Butyric Acid. *Kor. J. Plant Pahtol.* 15:217-222.

Nam, C. G., Jee, H. J. and Kim, C. H. 1988. Studies on Biological Control of Phytophthora Blight of Red-pepper. Enhancement of Antagonistic Activity by soil Amendment with Organic Materials. *Kor. J. Plant Pathol.* 4:313-318.

Park, J. H. and Kim, H. K. 1989. Biological control of Phytophthora crown and root rot of greenhouse pepper with *Trichoderma harzianum* and *Enterobacter agglomerans* by improved method of application. *Kor. J. Plant Pathol.* 5:1-12.

Park, K. S., Jang, S. W., Kim, C. H. and Lee, E. J. 1989. Studies on Biological Control of Phytophthora Blight of Red-pepper . Formulations of *Trichoderma harzianum* and *Pseudomonas cepacia* Antagonistic to *Phytophthora capsici* and Their Preservation. *Kor. J. Plant pathol.* 5:131-138.

Ryu, G. H. 1988. Biological Control of Several Soil-borne Diseases of Vegetables by *Pseudomonas fluorescens*. Ph. D. Thesis. Seoul National Univ., Korea.

Shen, S. S., Kim, J. W. and Park, C. S. 2002. *Serratia plymuthica* strain A21-4: A Potential Biocontrol Agent Against Phytophthora Blight of Pepper. *Plant Pathol. J.* 18:138-141

Shen, S. S., Choi, O. H., Lee, S. M. and Park, C. S. 2002. *In vitro* and *In vivo* Activities of a Biocontrol Agent, *Serratia plymuthica* A21-4, Against *Phytophthora capsici*. *Plant Pathol. J.* 18:221-224.

Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytophthol.* 26:379-407.