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Isolation and Characterization of *Burkholderia cepacia* strain YJK2, Antagonistic Microorganism of Paprika Pathogens

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파프리카 병원균들에 대한 길항미생물, *Burkholderia cepacia* strain YJK2의 분리 및 특성

양수정 · 김형무 · 주호종

Although several adverse effects have been increased in recent years, synthetic agro-chemicals have been widely used to control diseases on paprika. This research was conducted to isolate and to characterize the antagonistic microorganism to control major paprika diseases, gray mold rot, fruit and stem rot, *phytophthora* blight, *sclerotium* rot, and wilt disease. Analysis of the fatty acid and analysis of the 16S rDNA gene sequence revealed that YKJ2 isolated in this research belongs to a group of *Burkholderia cepacia*. Specially, 16S rDNA gene sequence of YKJ2 showed 99% of sequence similarity with *B. cepacia*. Observation through the optical microscope revealed that YKJ2 was effective on suppression of the spore germination and the hyphal growth of pathogens. YKJ2 treatment on pathogens induced marked morphological changes like hyphal swelling and degradation of cell wall. In the case of *phytophthora* blight, the zoosporangium formation was restrained. On the basis of the results of this study, we propose that an antagonistic microorganism, *B. cepacia*, found in this study naming as "*B. cepacia* strain YKJ2" and has great potential as one of biological control agents against major diseases of paprika.

Key words : *antifungal microorganism, biological control, Burkholderia cepacia, paprika*

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Ⅰ. Introduction

Paprika (Capsicum annuum L. var. Grossum) originated from Central America was introduced to Europe (Farooqi et al., 2005; Lim, 2013). Nowadays, it has been successfully produced in many countries including the USA (Farooqi et al., 2005) and Korea (RDA, 2002; Lee, 2012). In Korea, paprika was first grown in the mid-1990s and most of product was exported as a promising export crops (Lee, 2012). In addition, westernization of the Korean diet and the growing interest in health result that domestic demand of paprika is rapidly increasing. Thus, paprika cultivation area in Korea has been increased steadily (Nam, 2003) and there is no doubt that paprika cultivation will be the promising source of income for farmers like hot pepper.

However, like other crops, diseases on paprika are causing economic loss by reducing yield and Paprika is known to have higher susceptibility than hot pepper to more than 15 diseases (Nam, 2003; Jee, 2005, Jee et al., 2005b), which include gray mold by Botrytis cinerea (Yoon et al., 2008), stem and fruit rot by Nectrica haematococca (Jee et al., 2005a), wilt disease by Fusarium spp. (Cha, 2009), blight by Phytophthora capsici (Cho, 2010), sclerotial disease by *Sclerotinia sclerotiorum* (Jeon et al., 2006), viral diseases by Cucumber mosaic virus (CMV) (Kim et al., 2002) and Tomato spotted wilt virus (TSWV) (Mun et al., 2008). Many of disease controls except for viral diseases are mainly dependent on chemical agents to combat the various plant diseases that damage agricultural crops (RDA, 2002).

Recently, the interest in environmentally friendly agricultural products has been grown because negative attention has been increasing to use of agro-chemical causing various adverse effects such as food safety, appearance of tolerant-strain against pesticide and bad influence on ecosystem. In fact, there were two problems on quarantine process in April 2009 and in December 2012, due to pesticide residues exceeding the detection limit values (Lee, 2013). Such problem can occur at any time if pesticides are used in order to control disease. Thus, the importance of the safe use of pesticides for export vegetables is emerging more than ever and the development of alternative technologies that can replace the pesticide is highly required, especially for paprika.

Although there are many alternative to chemical controls, it has been known that biological control, defined as use of one living creature to manage the activity of the plant pathogens, is the best one of the best environmentally friendly methods to replace the chemical control (Noling and Becker, 1994; Lee, 2004; Lee et al., 2008). Biological control has been developed based on the decrease of inoculum and/or of pathogenic activity through the natural (Baker and Cook, 1974). In addition, antagonistic microorganisms improve crop productivity by promoting the activity of the plant root system. They generally colonize the root surfaces and localized at

soil interface acting as plant growth-promoting bacteria (PGPB) (Kloepper et al., 1999). Many agents for biological control include fungi from Trichoderma genus (Hadwan and Khara, 1990; Lin et al. 1994) and bacteria from Streptomyces and *Bacillus* genus have been commercialized (Gasoni et al. 1998) to control plant diseases because of *Pythium*, *Fusarium*, *Phytophthora*, *Rhizoctonia*, powdery mildew, *Colletotrichum*, *Erwinia*, *Pseudononas*, *Xanthomonas*, *Diplocarpon*, *Cercospora*, *Venturia*.

Burkholderia cepacia are abundant in ecosystem including soil, water, and plant surfaces (McArthur et al., 1988). *B. cepacia* was first considered as a pathogenic agent on onions, but it attracted attention as an excellent biocontrol agent to manage the soilborne and the post-harvest diseases (Janisiewicz and Roitman. 1988; Wilson and Chaluz. 1989; Baligh, 1991). Since first recognition as biological agent, *B. cepacia* evaluated to control many plant diseases including Rhizoctonia stem and root rot of poinsettia (Hwang and Benson, 2002), mung bean (Satya et al., 2011). In addition, *B. cepacia* has other potential for soil remediation because of metabolic versatility resulting in degrading chlorinated aromatic substrates which are toxic compounds found in complex pesticides and herbicides (Sangodkar et al., 1988). Thus, use of *B. cepacia* gives many beneficial influences to the organic farming system. This study explores the properties of *B. cepacia*, the antagonistic microorganism, of the major fungal disease in paprika.

Ⅱ. Materials Methods

1. Isolation of pathogens and ability test of antagonistic microorganism

For isolation of pathogens, diseased paprika plants in cultivation area of paprika were collected for isolation of pathogens and 5×5 mm of tissue was excised from border region between lesions and healthy regions of diseased plants. The surfaces of tissues were sterilized with 1% of NaOCl solution for 1 minute and followed by 3 times rinsing with autoclaved distilled water. The treated plant tissues were incubated on potato dextrose agar (PDA, 0.4% potato starch, 2.0% dextrose, 1.5% agar) medium at 28℃ in the dark. Single spore was re-incubated on PDA medium followed by pure isolation of pathogens.

For the isolation and the selection of antagonistic microorganisms, soil samples were collected from paprika cultivation area. One g of the soil samples was added into a test tube containing 9 ml of sterile physiological saline (0.85% NaCl) solution and diluted to 10-6. A hundred μl of diluent was inoculated on nutrient agar (NA, 0.5% peptone, 0.3% beef extract, 1.5% agar)

medium at 28℃ in the dark for 3 days. After that, bacteria were isolated based on the size and the shape of colonies and then used to test activity of antagonistic bacteria against paprika pathogens. Antagonistic activity test was performed using a petri dish filled with PDA medium by dual culture. A 5 mm of mycelia agar block from fresh cultures of pathogens was put on the one side and a loopful of antagonistic bacteria was streaked on the other side of petri dish. Dishes with pathogen and antagonistic bacteria were incubated at 25℃, suitable temperature for the pathogens for 7 days.

After the incubation, percent inhibition of growth (PIG) was calculated based on the formula below

$$PIG(\%) = [(CR - TR)/CR] \times 100$$

where CR indicates the growth of mycelia in plate without antagonistic bacterium and TR represents the growth of mycelia in plate with antagonistic bacterium (Rahman et al., 2007).

2. Identification of the antagonistic microorganism strain

In order to identify bacteria with great antagonistic activity, fatty acid profile by using the Microbial Identification (MIDI, Inc. Newark, DE, USA) and genetic comparisons based on the 16S rDNA gene analysis were used.

Fatty acid analysis using MIDI was conducted as described previously with a little modification (Yang et al., 1993). After antagonistic microorganism was cultured at 28℃ for 24 h on 1.5% tryptone soya agar (TSA, Difco, USA; 0.17% tryptose, 0.3% soytone, 0.25% glucose, 0.5% sodium chloride, 0.25% dipotassium phosphate, 0.15% agar), 40~60 mg of colony was used for purification of fatty acids (FAs). The purified FAs were then separated using gas chromatograph equipped with a fused silica capillary column. Fatty acids were identified and quantified by comparing the FA profiles generated in this study with the MIDI System software (Sherlock database TSBA40, version 4.1) containing various plant bacteria. The relative amount of each fatty acid was represented as a percentage of the total fatty acids and compositions of FA were expressed similarity index at MIDI Library entry (Osterhout et al., 1991; Aritua et al., 2008).

Bacterial cells were cultures overnight in 1 ml of Luria-Bertani broth (LB) (Miller, 1972) at 28℃. Genomic DNA from bacterial cells was purified using the Wizard® Genomic DNA Purification Kit (Promega Korea, Ltd, Seoul) based on the manufacturer's instructions and stored at -20℃ for the further use.

16S rDNA gene of selected isolate was amplified by PCR using primers, 27F (5'-AGAGTT TGATCMTGGCTCAG-3, forward) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3, reverse) (Reysenbach et al., 1992). PCR amplicons were reacted with 27F and 1492R using BigDye® Terminators v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) on ABI 3730XL Genetic Analyzer(Hitachi, Japan). Aligner software was used to generate the consensus sequence. For analysis of the sequence similarity, the 16S rDNA gene sequence was used to conduct BLAST with NCBI (National Center for Biotechnology Information) GenBank database. Phylogenetic trees were constructed using MEGA v4.0 program (Tamura et al., 2007) applying the neighbor-joining method (Saitou and Nei, 1987). Sequence distance was calculated with Kimura 2-parameter method (Kimura, 1980) and bootstrap values were calculated with 1000 replicates (Felsenstein, 1985).

3. The impact of the bacterial culture filtrates (BCF) on conidia germination of *B. cinerea*, *F. oxysporum*, and *F. solani* and the antagonistic microorganism on pathogenic mycelial growth

The influence of BCF on conidia germination of *B. cinerea*, *F. oxysporum*, and *F. solani* was examined by Lee's method with a little modification (Lee, 1994). A hundred μl of spore suspension (1×10^9 cell/ml) was inoculated into test tube containing 2.7 ml of potato dextrose broth (PDB, Difco, USA, 0.4% potato starch, 2.0% dextrose) with 0.3 ml of BCF. After inoculation, stand incubation allowed for 24 hours, 48 hours, and 72 hours to for incubation. The spore germination was examined at 400× using the optical microscope (Leica, ICC50).

The influence of antagonistic microorganism on mycelial growth of *B. cinerea*, *F. oxysporum*, *F. solani*, *P. capsici*, and *S. sclerotiorum* was examined by Lee's method with a little modification (Lee, 1994). Five pathogens and antagonistic microorganism were incubated in each tryptic soybean broth (TSB, Difco, USA, 1.7% tryptone, 0.3% soytone, 0.5% sodium chloride, 0.25% dipotassium phosphate, 0.25% dextrose). A two hundreds μl of antagonistic microorganism cultured for 24 hours was inoculated to 1.8 ml of pathogens in culture and allowed incubation for 48 hours. The morphological change of mycelia was observed at 400× using the optical microscope (Leica, ICC50).

4. Statistical analysis

Statistical analyses were carried out with Statistical Analysis System (SAS version 9.1, SAS

Institute Inc., Cary, NC, USA). Treatment means were compared using Fisher's protected LSD test ($P < 0.05$).

Ⅲ. Results and Discussion

1. Isolation and antagonistic ability test of antagonistic microorganism

The five major pathogens of paprika, *B. cinerea*, *F. oxysporum*, *F. solani*, *P. capsici*, and *S. sclerotiorum* and antagonistic microorganisms were isolated from diseased plants and soil samples collected at field of paprika growers, respectively. Dual cultures of the isolated pathogens with antagonistic microorganisms were conducted. An antagonistic microorganism strain showing 40% or more in pathogen growth inhibition was selected. This particular strain was named as YKJ2 and used for the experiments (Table 1 and Fig. 1).

In dual culture, an inhibition zone was observed between antagonistic microorganism and five fungal pathogens showing that mycelial growth of *B. cinerea*, *F. oxysporum*, *F. solani*, *P. capsici*, and *S. sclerotiorum* was inhibited by this bacterial agent with antagonistic activity (Fig. 1).

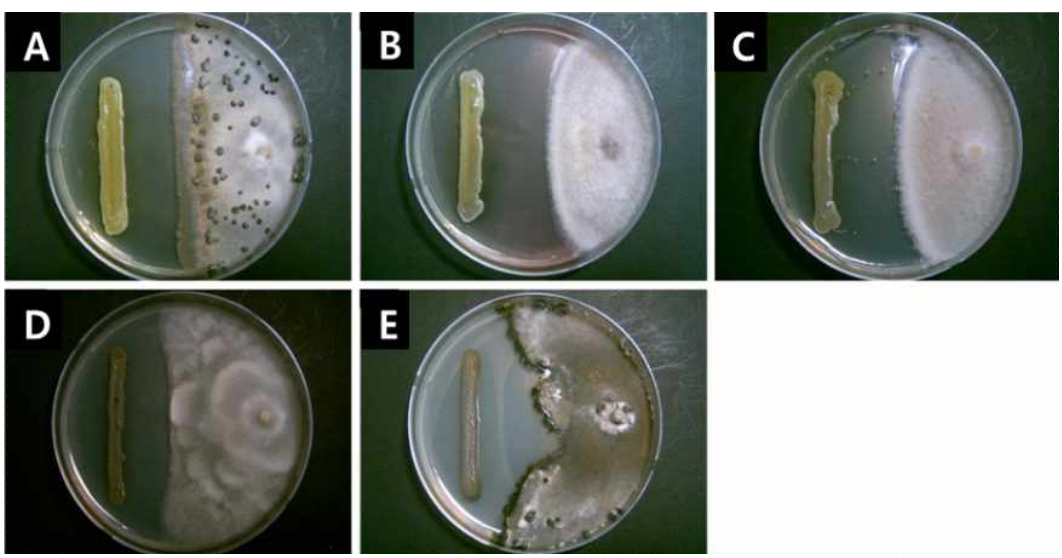

Fig. 1. Inhibitory patterns of the YKJ2 on growth of the 5 major pathogenic mycelial in dual culture for 10 days.

A) *Botrytis cinerea*, B) *Fusarium oxysporum*, C) *Fusarium solani*, D) *Phytopthora capsici*, E) *Sclerotinia sclerotiorum.*

Table 1 showed that inhibition of mycelial growth is 50.77%, 50.00%, 48.72%, 45.64%, or 47.18% for *B. cinerea*, *F. oxysporum*, *F. solani*, *P. capsici*, or *S. sclerotiorum*, respectively. As expected, *B. cinerea* and *S. sclerotiorum* formed sclerotia at dual culture with YKJ2 but *F. oxysporum*, *F. solani*, and *P. capsici* did not form sclerotia (Fig. 1).

Table 1. Antagonistic effect of the YKJ2 on mycelial growth of pathogen in dual culture

Major Pathogens	YKJ2	Control
	----------Inhibition rate (%)----------	
Botrytis cinerea	50.77 [a]	0[b]
Fusarium oxysporum	50.00 [a]	0[b]
Fusarium solani	48.72 [a]	0[b]
Phytopthora capsici	45.64 [a]	0[b]
Sclerotinia sclerotiorum	47.18 [a]	0[b]

* The same letters are not significantly different according to a fisher's protected least significant difference (LSD) at 0.05 probability level

Chung (2011) selected Pseudomonas putida strain P84 by dual culture with Pathogens *B. cinerea*, *F. solani*, *P. capsici* which were causing diseases in paprika and reported possibility of environmentally friendly formulation selection to control paprika major pathogens by antagonistic microorganisms. Kim and Kim (1997) used *Bacillus* sp. showing the mycelial growth inhibition for rice blast about 30%. YKJ2 strains isolated in this study showed a greater than 40% inhibition.

Therefore, taken together, YKJ2 strains are expected to be excellent biological agent to control the major pathogens that occur on the paprika.

2. The fatty acid and the 16S rDNA sequence analysis

Sasser (1990) employed the principle that the fatty acid compositional proportions of membrane among different species differ but is constant at the same microbial species to identify microbes. In general, if the similarity value is greater than 0.5, the significance is recognized in the MIDI (Osterhout et al., 1991).

The MIDI similarity value of the bacterium isolated from this study was 0.592, which was greater than that reported previously (Li et al., 2011). GC (gas chromatography) analysis revealed

that isolate YKJ 2 mainly was consisted of cyclo fatty acids. C16, C17, C18, and C19 fatty acid were 30.24%, 23.24%, 14.38%, and 17.77%, respectively (Table 2). This fatty acid composition was very similar to the main fatty acid component in another *Burkholderia* spp. (Li et al., 2011).

Table 2. Cellular fatty acid composition of YKJ2 analyzed by gas chromatography

Fatty acids	Retention time	Percentage
12:0	4.082	0.14
13:1 AT 12-13	5.000	1.49
14:0	6.287	4.58
15:0	7.692	0.30
16:1 ω5c	9.102	0.66
16:0	9.241	24.54
17:1 ω7c	10.589	0.20
17:0 CYCLO	10.694	22.64
17:0	10.881	0.40
16:1 2OH	10.952	0.35
16:0 2OH	11.269	1.38
16:0 3OH	11.753	3.31
18:1 ω7c	12.266	9.88
18:1 ω5c	12.437	0.10
18:0	12.570	2.21
11methyl 18:1 ω7c	12.695	0.26
19:0 ISO	13.663	0.25
19:0 CYCLO ω8c	14.098	17.52
18:1 2OH	14.409	1.93
20:2 ω6,9c	15.519	0.25
Summed Feature1*	11.701	3.78
Summed Feature2	8.952	3.81

* Summed features represent group of two fatty acids which could not be separated by gas-liquid chromatography with the MIDI system. Summed feature 1 contained one or more of following fatty acids: unknown 14:0 3OH/16:1 ISO I, summed feature 2 contained one or more of following fatty acids: 15:0 ISO 2OH/16:1 ω7c

16S rDNA analysis of YJK2 showed homology of 99% with *B. cepacia* (accession no. KM018333) registered in the NCBI GenBank database and phylogenetic analysis revealed that YKJ2 appeared to belong to the same group as *B. cepacia* (Fig. 2). Therefore, we propose YKJ2 naming as "*Burkholderia cepacia* strain YKJ2".

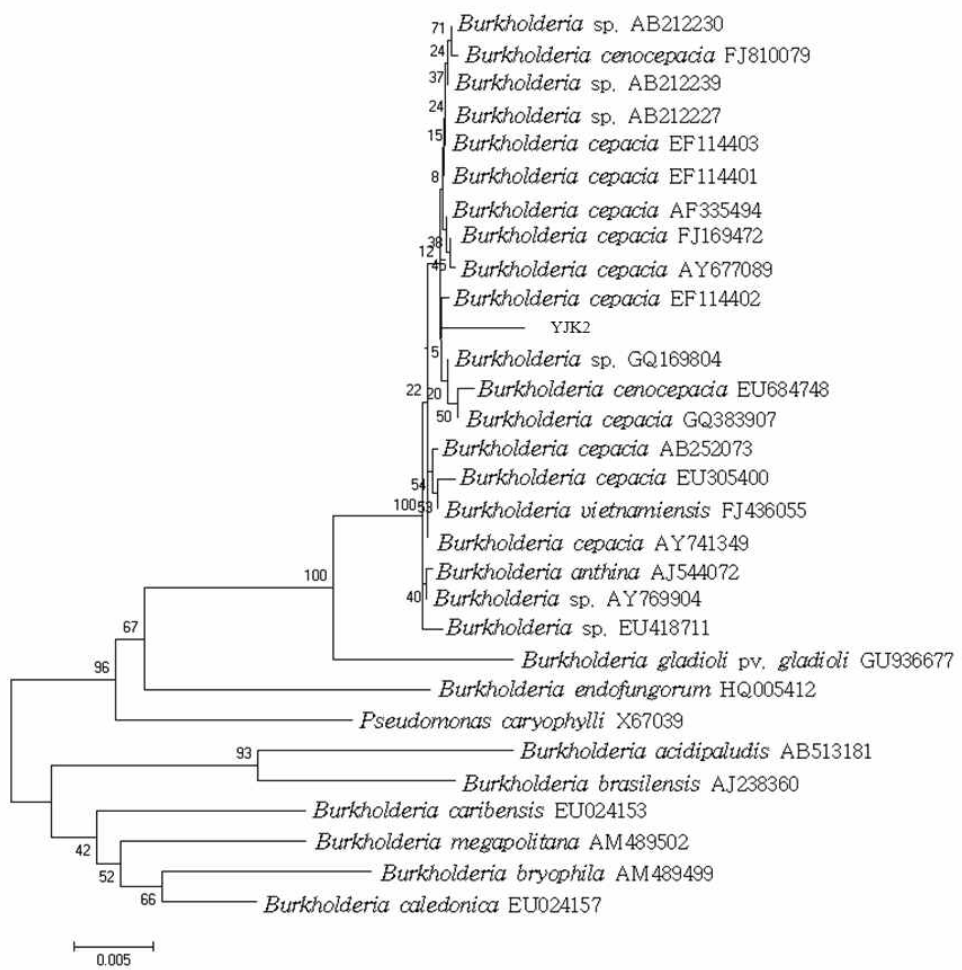

Fig. 2. Phylogenetic tree generated from the partial 16S rDNA sequence of YKJ2 and that of other related Burkholderia species acquired from NCBI GeneBank.

The tree was produces using the neighbor-joining method. The percentage on trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

calculated using the Kimura 2-parameter correction and are in the units of the number of base substitutions per site

3. The impact of the bacterial culture filtrates (BCF) on conidia germination of *B. cinerea*, *F. oxysporum*, and *F. solani*

Since *B. cinerea*, *F. oxysporum*, and *F. solani* formed conidia, the impact of the culture filtrate of YKJ2 on spore germination was tested for *B. cinerea*, *F. oxysporum*, and *F. solani*. Conidia of all pathogens including *B. cinerea* significantly were inhibited germination by YKJ2 cultural filtrates. None of them geminated at 24 hours (Table 3).

Table 3. Effect of cultural filtrates of YKJ2 on spore germination of pathogens causing diseases on paprika

		YKJ2	Control
		----------Spore germination (%)----------	
Botrytis cinerea	24h	0[b]	67[a]
	48h	2[b]	75[a]
	72h	5[b]	93[a]
Fusarium oxysporum	24h	0[b]	47[a]
	48h	4[b]	65[a]
	72h	8[b]	98[a]
Fusarium solani	24h	0[b]	58[a]
	48h	1[b]	69[a]
	72h	3[b]	95[a]

* The same letters are not significantly different according to a fisher's protected least significant difference (LSD) at 0.05 probability level.

There were many reports regarding the effect and use of antagonistic microorganisms to control pear gray mold disease occurring after harvest (Nunes et al., 2001). Especially antagonistic microorganism, *P. putida* strain 84 (Chung, 2011) and *P. antimicrobica* (Walker et al., 2001) showed the suppressing effect on the germination of spores produced by *B. cinerea*. Moussa and Rizk (2002) reported that *Streptomyces aureofaciens* inhibited germination of *F. solani* and suggested that the secretion of spore germination inhibitor during the culture of many microbes. This study showed similar results to previously published studies. During the incubation process, it was thought that *B. cepacia* strain YKJ2 might secret effective conidial germination inhibitor

for *B. cinerea*, *F. oxysporum*, and *F. solani*.

4. The effect of the B. cepacia strain YKJ2 on pathogenic mycelial growth

In general, the mechanism of inhibiting plant pathogens by microorganism is described as antibiotic activity, cell wall degradation, membrane degradation, and nutrient completion (Gunji et al., 1983).

YKJ2 was treated on *B. cinerea*, *F. oxysporum*, *F. solani*, *P. capsici*, *S. sclerotiorum* to evaluate the impact on the mycelial growth. Several phenomena were observed on hyphae such as pathogen's cell wall dissolution at the distal end (Fig. 3A), swollen hyphae (Fig. 3B, C and E), and degradation of zoosporangium (Fig. 3D), indicating that YKJ2 possess antagonistic mechanism of various plant pathogens.

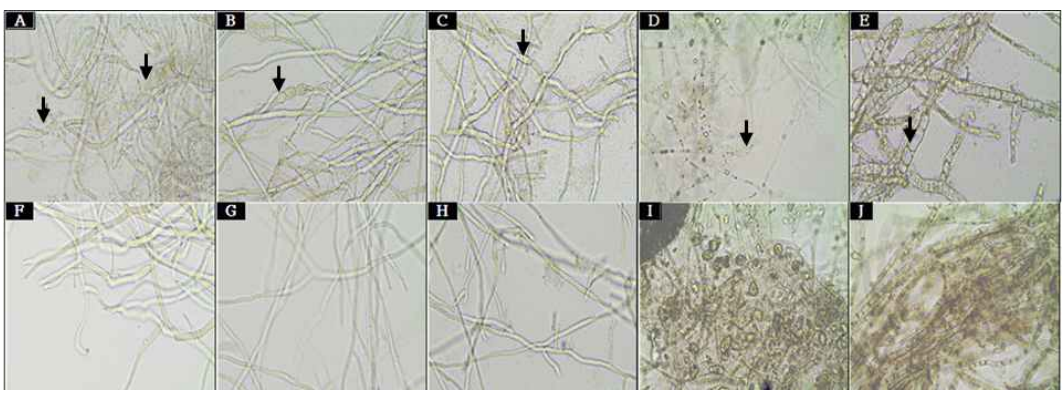

Fig. 3. Hyphal morphological abnormalities induced by culture of YKJ2 on several pathogenic microbes. Co-cultivation of YKJ2.

A) *Botrytis cinerea*, B) *Fusarium oxysporum*, C) *Fusarium solani*, D) *Phytopthora capsici*, E) *Sclerotinia sclerotiorum* Control; F) *B. cinerea*, G) *F. oxysporum*, H) *F. solani*, I) *P. capsici*, J) *S. sclerotiorum*

Lee et al. (1999) and Lim and Kim (1997) was reported Pseudomonas sp. producing a siderophore in the soil inhibited the mycelial growth of F. solani and Seong and Shin (1996) described that *Pseudomonas* sp. suppressed mycelial growth of hyphae for *Phytopthora. capsici*, *Pythim ultimum*, and *Pyricularia oryzae*. Our results were agreed with their study. As shown at Fig. 3, it was hardly formed zoosporangium at YKJ2 treated *P. capsici* (Fig. 3D) compared control which formed a lot of zoosporangia (Fig. 3I) in agreement with previous study that *Bacillus polymixa* AC-1 suppressed the formation zoosporangium of the pathogen (Kim, 1995).

Taken together all, YKJ2 isolated from paprika field is thought to be a strain of *B. cepacia* in terms of molecular biological aspects and has great potential for eco-friendly organic farming as a biological agent.

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