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Chili - Pepper Protection from *Phytophthora capsici* and *Pectobacterium carotovorum* SCC1 by Encapsulated Paromomycin Derived from *Streptomyces* sp. AMG-P1

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The efficacy of an antibiotic paromomycin in encapsulated form with kaolin alginate and non-encapsulated paromomycin was studied for the control of two important pathogens, *Pectobacterium carotovorum* SCC1 (SCC1) and *Phytophthora capsici* in chili-pepper through systemic resistance under greenhouse conditions. Paromomycin, one of the antibiotic depsipetides isolated from *Streptomyces* sp. AMG-P1 (AMG-P1), can be used as one of the biocontrol agents to suppress the soilborne diseases in Korea. The compound was granulated with sodium alginate-kaolin by immobilization technique. The treatment at 0.1-10μg/g soil exhibited strong activity against *P. capsici* and *P. carotovorum* SCC1. After treatment with non-encapsulated paromomycin or encapsulated paromomycin, upon pathogen challenge, there was a greater reduction of soft rot and phytophthora blight infections caused by *Pectobacterium carotovorum* SCC1 and *Phytophthora capsici*, respectively, through systemic resistance. There was an increased reduction of soft rot infection in whole leaf bioassay significantly, when chili-pepper plants were treated with encapsulated paromomycin beads in granular form. While, there was a lower disease reduction in the treatments with BTH (0.1mM) and untreated control. The treatment with encapsulated paromomycin reduced the disease severity of phytophthora blight significantly ($P<0.05$) at lower dosage compared to non encapsulated paromomycin, where the reduced disease severity was observed at 1.0ppm concentration. This is the first report that the immobilized paromomycin with kaolin exhibits protection against plant pathogens. Hence, AMG-P1 can play a role in enhancing plant defense by secretion of ISR elicitors including paromomycin.

Key words: Paromomycin, Encapsulation, Soft rot, Phytophthora blight, Chili pepper, Induced Systemic Resistance.

Encapsulation can improve the properties of biological control agents or their antibiotic substances such as easy handling and application. Protection of the biocontrol agent from biotic and abiotic stress factors, enhanced shelf life, controlled release in environmental conditions, and enhanced efficiency in the soil. Recently, encapsulation techniques based on ionotropic gels well-known have successfully been used to formulate entomopathogenic nematodes (Patel and Vorlop, 1994), bacterial antagonists (Patel *et al.,*2003), while in our study, we demonstrate the efficacy of antibiotic paromomycin in encapsulated granules on disease control in chili-pepper.

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Elicitation of plant resistance has successfully been demonstrated using different groups of biocontrol agents in many crops against major pathogens. Various chemical fungicides have been used to control root diseases. There is a need to reduce the pesticide application to food crops to reduce the environmental pollution.

Phytophthora capsici is a soil-borne pathogen that causes root rot, foliar blight and pod rot in nearly all cultivars of chili-pepper (*Capsicum annum*) (Park *et al.* 1989; Alcantara and Bosland 1994; Goldberg 1995). It is one of the most devastating soil-borne diseases of chili-pepper worldwide (Hausbeck and Lamour 2004). The pathogen is among the most economically destructive to chili-pepper production causing losses annually in chili-pepper growing regions throughout the world. *Pectobacterium carotovorum* subsp *carotovorum*, formerly known as *Erwinia carotovora* subsp. *carotovorum* mainly affects crops in subtropical and temperate regions, and is likely among the phytopathogenic bacteria that have a wide host range (Hibar *et al.,*2007). It is the causal agent of bacterial soft rot, a severe disease of many economically important food crops such as potato, tomato, chili-pepper, eggplant and Chinese cabbage (Catara *et al.,*2001; Fiori and Schiaffino 2004).

In initial screening of antifungal compounds originating from micro-organisms, some aminoglycoside antibiotics from actinomycetes were found to be selectively active against *Phytophthora*, *Pythium* and *Pectobacterium* species (Lee *et al.,*2005). To date, most of the studies of the biological properties of aminoglycoside antibiotics have focused on activities against bacteria, yeast and protozoa (Gracenea *et al.,*1998; Beers and Berkow 2004). So far, only limited information is available on the activity such as paromomycin against oomycetes of *P. capsici*, and antibiosis, but there is lacking of disease suppression through induced systemic resistance (ISR). Thus, in the present investigation, we report for the first time, that the protection exerted by paromomycin active against the two major plant pathogens such as phytophthora blight caused by *P. capsici* and soft rot disease caused by *P. carotovorum* SCC1 in chili-pepper plants.

MATERIALS AND METHODS

Source of paromomycin

The isolated and purified antibiotic paromomycin from *Streptomyces* sp. AMG-P1 was provided by Dr. J. C. Kim, Biological Function Research Team, Korean Research Institute of Chemical Technology, Daejeon, Korea.

Granulation of paromomycin by encapsulation with kaolin and sodium alginate

Sodium alginate (Sigma-Aldrich) solution was prepared by dissolving 20 g of dry sodium alginate in a minimum volume (10 ml) of sterile distilled water (SDW). This mixture was poured into 1 L of swirling, warm, distilled water and allowed to mix on the stirrer for 30 min until a homogeneous suspension was obtained. The alginate solution was sterilized for 15 min at 121°C. The antibiotic agent, paromomycin at 1000ppm was mixed thoroughly with 200g of previously sterilized kaolin (aluminum silicate, Sigma-Aldrich) and the mixture was added in small portions (2g) into 1 L of swirling, SDW supplemented with 4 drops of Tween 20. The kaolin-paromomycin mixture was allowed to swirl in the stirrer until ready for mixing with the sodium alginate solution. A droplet forming devise was constructed by attaching a 1-L reagent bottle with a spout at the bottom to a T-valve outlet system. The entire devise was sterilized for 15 min at 121°C before use. The sodium alginate-kaolin mixture amended with paromomycin were added to the reagent bottle and stirred continuously while the suspension was allowed to drip through an Ependorff pipette tip, attached to the T-valve, into a sterile solution of 0.1 M $CaCl_2$. The resulting alginate-kaolin-paromomycin beads were then allowed to stand in a fresh 0.1 M $CaCl_2$ solution for 30 min, filtered through a sterile cheese cloth and wash at least three times with SDW. The beads were air dried at 40°C and their dry weight was recorded. This granular formulation of paromomycin (hereafter referred to as beads) was stored at room temperature.

Preparation of spore suspensions of *P. capsici* and bacterial pathogen SCC1 inoculums

P. capsici inoculum was prepared as described by Ploetz *et al.,*(2002). A 5 mm diameter mycelial plug of an isolate was transferred to a V8 agar plate. After one week of incubation at 25°C, V8 agar plugs with mycelia were placed into a Petri-

dish containing V8 broth, and allowed to grow for another week at 25°C. The V8 broth was then drained and each plate was washed twice with sterile distilled water (SDW). SDW was added to cover the mycelia on each plate, and then the plates were placed under wide-spectrum light at room temperature for 24–48 h to induce sporangial development. The sporangia were chilled at 4°C for 45 min to induce the release of zoospores. The cyst spores were adjusted to 1×10^5 spores/ml and used for challenge inoculation under greenhouse conditions. For the preparation of SCC1 inoculum, the bacterial cell suspension was prepared from 24 h old culture at 28°C. Ten ml of SDW was poured on TSA culture plate and scraped with sterile plastic loop and adjusted to 1×10^8 cfu/ml (OD_{600} = 0.8) before application.

Disease assessment of *P. carotovorum* SCC1 and *P. capsici* by paromomycin or encapsulated paromomycin in chili-pepper plants under greenhouse conditions

Chili-pepper (*Capsicum annum* L.) cv. Hanbyul seedlings at first-branch stage were used in this study. The seeds were germinated in a plastic tray (55cm×35cm×15cm) containing steam-sterilized soil, sand and compost (1:1:1, v/v/v). Seedlings at the two-leaf stage were transplanted to plastic pots (5cm×15cm×10cm) containing the same soil mix. Complex fertilizer was applied to plants after transplanting once in a week. The plants were raised in a growth room under 16 h/day illuminations at 27±2°C. The plants were treated with the antibiotic paromomycin or encapsulated paromomycin granules with kaolin by soil drench at different concentrations (0.1, 1.0, and 10 ppm) of 30mL or 10 g of granules to each pot under greenhouse conditions. BTH, a common systemic fungicide at 0.1mM and distilled water served as positive and negative control, respectively. One week later, the ISR activity against *P. carotovorum* SCC1 was performed by whole leaf assay in square plates (Bio-Assay dish, nunc™, Apogent Company, Denmark) (24×24 cm). The leaves from treated plants were brought to the laboratory and placed in square plates containing moisture tissue paper. Pathogen inoculated paper disks were placed onto the leaves. The percent of diseased lesion was calculated after incubating for 48 h at 28°C. Twelve replicates were used per treatment. For induced suppression of phytophthora blight, all the seedlings were treated with only encapsulated paromomycin under greenhouse conditions. One week later, the plants were challenge inoculated with zoospore suspensions of *P. capsici* (1×10^5 zoospores/mL) by pipette near the stem region in the pots. The plants were transferred to greenhouse bench for one week. The percent of disease severity of phytophthora blight was recorded.

Statistical analysis

Data were analyzed (mean±SE) with SAS JMP software, SAS Institute, USA (SAS, 1995). All the experiments were repeated at least once with similar results. For each experiment, data were analyzed separately, and the results of one representative experiment are shown. Significant differences in treatment means on each sample data were determined using LSD at P=0.05.

RESULTS AND DISCUSSION

The alginate-kaolin based formulation of an antibiotic paromomycin derived from *Streptomyces* sp. AMG-P1 was generated as pale-gray colored, spherical beads with an average size of 4 mm diameter were used for the biocontrol of soft rot and phytophthora blight diseases in chili-pepper plants (Fig. 1a). The incorporation of air dried paromomycin into the alginate-kaolin beads produced viable dry formulation. This formulation involved simple technology and are time- and cost-effective. Furthermore, encapsulation of biocontrol agent (BCA)s in natural carrier substances such as flour, starch or bran is environmentally friendly (Lewis *et al*, 1995; Lumsden *et al*, 1995) and can also serve as nutrient sources for BCAs and, thereby, help in the establishment of BCA at the application site (Lewis *et al*, 1995). Paromomycin might be better survival rate in encapsulated beads than in any other forms. Alginate-kaolin beads also possess many desirable qualities, such as uniformity in size, light weight and more suitable in greenhouse for application by mechanical seeders (Lumsden *et al*, 1995).

The treatment of chili-pepper plants with paromomycin at 1.0ppm or encapsulated paromomycin in granular form at 0.1ppm suppressed the soft rot infection caused by *Pectobacterium carotovorum* SCC1 completely in soil application (Fig. 2). Either of these two

treatments reduced the disease severity significantly (P<0.05) when compared to chemical treatment BTH (0.1mM) or water treated control upon pathogen inoculation. The encapsulated paromomycin could reduce the disease severity only at lower concentration (0.1ppm), while the non-encapsulated paromomycin was at 1.0ppm concentration. This implies that the systemic resistance was developed in the plants at lower concentration of paromomycin than higher concentrations. The treatment with encapsulated parmomycin beads or non-encapsulated paromomycin at various concentrations against phytophthora blight disease caused by

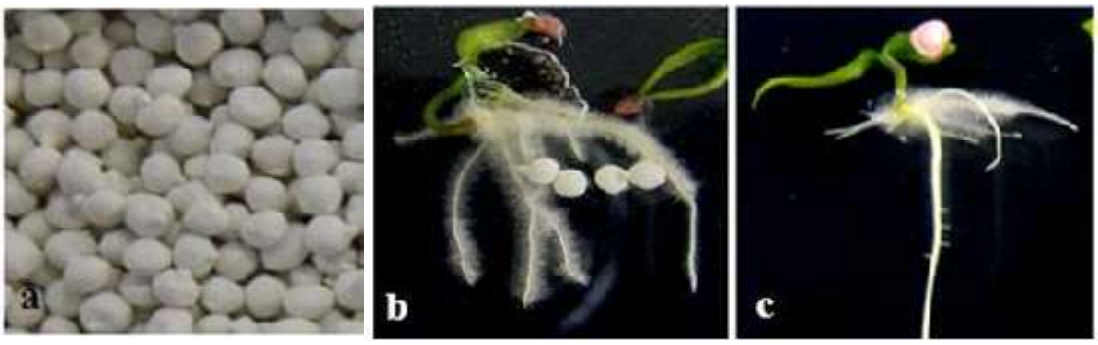

Fig. 1. Encapsulation of paramomycin by alginate-kaolin granular beads (a). The ability of the paromomycin granules in root growth development (b), compared to untreated control without granules (c) in chili-pepper seedlings

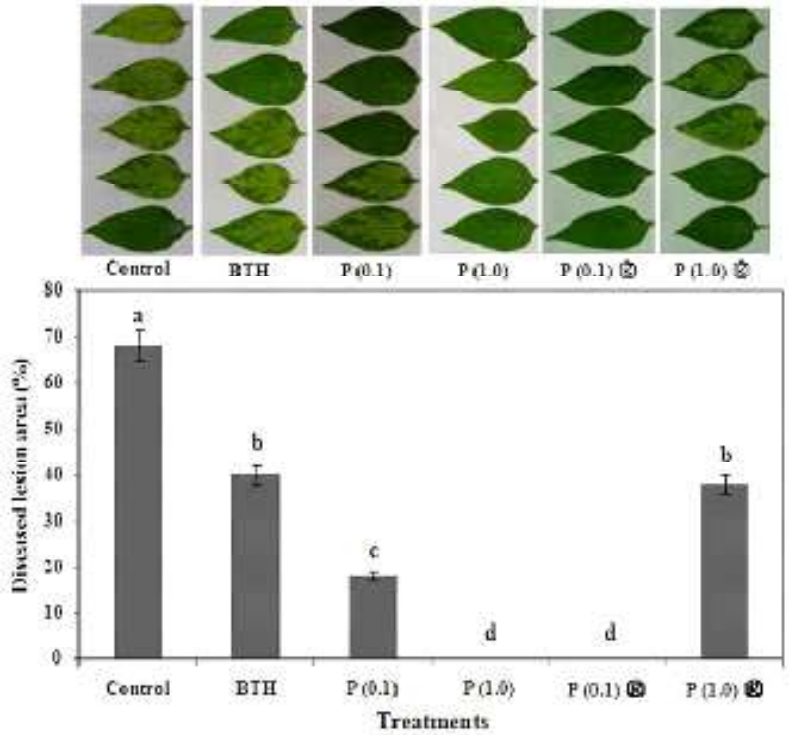

Fig. 2. Effect of encapsulated paromomycin with kaologin on diseases suppression of soft rot infection caused by *P. carotovorum* SCC1 in chili-pepper compared to non encapsulated paromomycin. BTH (0.1mM) and distilled water served as positive and negative controls respectively. P: Paromomycin; P ®: Paromomycin granule.

Phytophthora capsici revealed that, there was significant (P<0.05) reduction of disease severity upon pathogen challenge when compared to BTH and untreated control (Fig. 3). The phytophthora blight disease was completed controlled by encapsulated paromomycin at lower concentration. While, there was 18% disease severity in BTH (0.1mM) treated plants upon pathogen challenge.

Treatment of chili-pepper seedlings with both encapsulated and non-encapsulated paromomycin resulted in a desirable plant response compared to the untreated control plants. The systemic resistance of the plants, treated with encapsulated paromomycin showed significant (P<0.05) increase compared to the plants treated with non-capsulated paromomycin after seven days of growth under greenhouse conditions. Smith, (1992) stated that, the kind of carrier utilized defines the physical form of the biofertilizer. The carrier is the major portion of the inoculants that helps to deliver a suitable amount of BCA in a good physiological condition. Dry inoculants can be produced using different kinds of soil materials or inert materials such as vermiculite, perlite, bentonite, silicates, including kaolin. The materials constituting the carrier can be of various origins which include organic, inorganic, or synthesized from specific molecules. Availability and cost are the main factors affecting the choice of a carrier. The carrier should be designed to provide a suitable microenvironment for the BCA and should assure a sufficient shelf life of the product. A good carrier normally possesses the properties that include good moisture absorption capacity, easy to process and free of lump-forming materials, near-sterile or easy to sterilize by autoclaving (Keyser et. 1993). The survival of paromomycin in encapsulated beads is an important both during the storage period of the bioproduct after being introduced into the soil (Trzcinski *et al.,*2011). Carrier materials that make available nutrients and habitable micropore to the BCA. The results of this study confirm that the treatment of the seedlings with an improved encapsulated paromomycin beads could be a simple, effective and applicable system for delivering antibiotic agent to control soft rot and phytophthora blight pathogens in chili pepper.

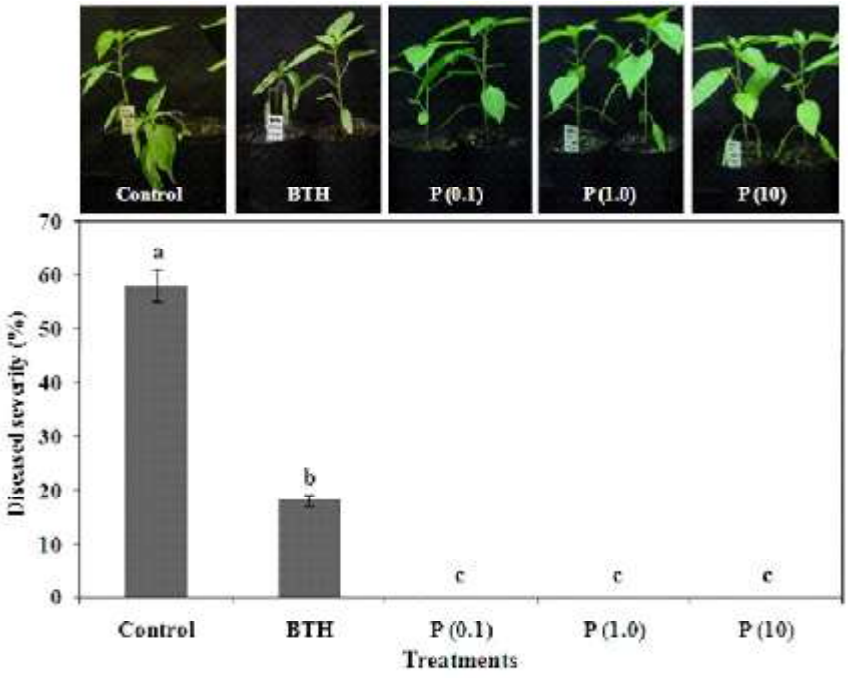

Fig. 3. Chili-pepper protection against *Phytophthora capsici* by encapsulated paromomycin with alginate-kaolin granules. BTH and distilled water served as positive and negative controls, respectively

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