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BIOLOGICAL CONTROL OF VEGETABLE AND FRUIT FUNGAL DISEASES BY BACTERIAL ANTAGONISTS

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Executive summary

In recent years, cereals, vegetables, and fruits have almost replaced cotton as a leading crop in Uzbekistan. The protection of these newly introduced crops against plant diseases has become an important agricultural problem. An additional concern is the severe environmental pollution throughout Uzbekistan and other CAR that has resulted from massive and immoderate applications of pesticides to cotton in the past. In spite of these facts the farmers in Uzbekistan still have to rely on chemical pesticides since at present there are no efficient alternatives to chemical usage. Therefore, the problem of protection of these newly introduced crops against plant diseases has become significantly important in this region stimulating the search for new approaches to plant disease control. One of the most promising is biological control, the use of microorganisms capable of attacking or suppressing plant pathogens in order to reduce disease incidence. Biological control of plant pathogens based on natural interaction between organisms (e.g. antagonisms, competition etc.), offers potential means to overcome ecological problems induced by chemical pesticides. The main aim of the present project was to develop new products for biological control of diseases caused by phytopathogenic fungi in vegetables and fruits. The bacterial strains *Pantoea (Enterobacter) agglomerans* IC1270, *Pseudomonas fluorescens* IC111, *P. putida* IC27, *P. chlororaphis (aureofaciens)* 449 and *Serratia plymuthica* IC14 isolated in Uzbekistan and Israel and characterized by broad range of antagonistic activity and multiple mechanisms of action against, mainly, of plant pathogenic fungi, were used for the biocontrol products elaboration. The results of experiments in vitro, in greenhouses and micro-plot field conditions performed collaboreatively in Israel and Uzbekistan have shown that the strains could be considered as new, potential biological control agents of sail-borne, aerial and post-harvest fungal pathogens. Thus, strain IC1270 was effective against soilborne diseases caused by *R. solani* on cucumber and beans, *P. aphanidermatum* on cucumber, *S. rolfsii* on beans; against aerial diseases caused by *B. cinerea* and *S. sclerotiorum* on cucumber, and in addition was suppressive of the post-harvest fungal pathogens *Monilinia* and *Rhizopus* on peaches. Foliar application of strain IC14 protected cucumber against *Botrytis cinerea* gray mold and *S. sclerotiorum* white mold diseases under greenhouse conditions. Strains IC27 and IC111 were found efficient against *F. oxysporum* and *S. rolfsii* while strain 449 provides moderate control of *R. solani* and *S. sclerotiorum* on cucumber, while. Several protocols for the antagonistic bacterias' formulation and application were developed. Under greenhouse and field conditions, the bacterial isolates revealed proficient biocontrol activity, dependent upon the crop and disease specificity. Some of the bacterial antagonists were found to be resistant to a chemical fungicide commonly employed in Uzbekistan for the control of fungal diseases, showing that the bacteria can be used in combination with substantially lower doses of these fungicides as part of an integrated pest management approach. Immobilization of the chitinolytic strain IC1270 bacterium in freeze-dried alginate-based beads sharply improved its survival in non-sterile irrigated and dry soils. The project enhanced the ability of Uzbekistan Institutions to exploit biological control of plant diseases. The training of scientific staff from Uzbekistan in Israel contributed to the establishment of a modern level of basic and applied research as well as teaching of biotechnology in the Samarkand State University. The data obtained in the framework of this project is already being implemented in several agrofirms in Uzbekistan.

Research Objectives.

The specific research objectives include: i) testing of bacterial selected antagonists for ability to control soil-borne, aerial and post-harvest fungi under laboratory, greenhouse and small-plot field conditions; ii) testing of selected antagonists for properties requisite for acceptable biocontrol agents (persistence in soil, plant-growth promoting, survival on leaves and fruits); iii) fermentation and formulation of the selected strains; iv) testing of compatibly of the selected strains with pesticides commonly used against plat pathogenic fungi in Uzbekistan; v) study of mechanisms of antagonistic activity and approaches for further improving of the selected strains biocontrol capacity.

Methods.

Bacteria. *Pantoea* (*Enterobacter*) *agglomerans* IC1270, *Pseudomonas putida* IC27 and *Pseudomonas fluorescens* IC111 were isolated in the Samarkand region (Republic of Uzbekistan) from the rhizosphere of grape bushes in vineyards, melon and tomato, growing in the fields, respectively. Strains *Serratia plymuthica* IC14 and *P. chlororaphis* (*aureofaciens*) were isolated in Israel and Uzbekistan from rhizosphere of melon and maize, respectively.

Plant pathogenic fungi. The fungal plant pathogens used in this work were obtained from the collections of corresponding laboratories and institutes. The fungi include *Rhizoctonia solani, Sclerotium rolfsii, Pythium apahanidermatum, Fusarium oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *licopercici*; *Botrytis cinerea* Pers; Fr., *Sclerotinia sclerotiorum* (Lib.) de Bary, *Monilinia fructicola* (Wint.) Honey, *Penicillium expansum* Link, *Rhizopus stolonifer* (Ehrenb.: Fr.) Lind.

Growth media. Bacteria grown in the rich media, e.g. nutrient broth, or meat-peptone broth, with aeration at 28°C to a final concentration ca 5 x 10^8 to 1 x 10^9 cells/ml were washed in tap water and applied to the plants as described below. For spraying on leaves 0.1% Twin-20 was added to the tap water.

Plants. The test plants were bean (*Phaseolus vulgaris* L.), cucumber (*Cucumber sativus* L.), melon (*Cucumis melon* L. cv. "Ein Dor"), tomato (Lycopersicum), rape (*Brassica napus* var. *oleifera*), white cabbage (*Brassica oleracea capitata spherica alba*), and sunflower (*Helianthus*).

Fruits. The fruits tested for control of post-harveesr diseases were apple (Pirus Malus L. cv. "Golden Delicious") and peach (*Prunus persica* (L.) Batsch. cv. "Swelling"). **Strain delivery.** The bacteria in tap water were applied at a concentration of 3-5 x 10^8 cfu/g soil by: 1) coating the seeds; 2) drenching the soil on the day of sowing and 1 week later; 3) mixing with soil in pots (for protection of seedlings against *Fusarium*). For seed coating methylcellulose or metamucil (49% refined mucilloid of psyllium seed, Searle & Co. Ltd, High Wycombe, England) powder was added as a sticker to aliquots of bacterial cells (~ 5 x 10^8 cells/ml) to a final concentration of 0.4 to 0.5%. Within each experiment, test seed treatments, varying in their formulation, were prepared along with a control in which seeds were encapsulated in tap water containing the same sticker.

Biocontrol assay. Plants were grown under greenhouse conditions in sandy loam soil (pH 7.2) or a peat-vermiculite (1:1 v/v) mixture. For protection of plants against fusariosis *Fusarium oxysporum* f. sp. *meloni* – melon fungal inoculum was obtained from a one day old liquid culture: 200 µl of it was spread on plates with Yeast Extract agar, the plates were grown for one week at 25°C. Then tap water (ca. 10 ml) was added to each plate, and the mycelium was scratched with a microscope slide. The liquid was collected, filtered with gaze and centrifuged (7000 rpm; 4° C; 15 min). The pellet was resuspended in ca. 100 ml.

of tap water and the spore's concentration was measured using the hemocytometer. ($2.3x10^7$ spores/ml). One week old seedlings of melon (var. "Ein Dor" and "Kukalapush") were prepared in pots with sandy loam soil (pH 7.2) and then infected by immersion for 3-5 minutes in two fungal suspensions in the titles indicated. obtained by serial dilutions from the original suspension. Seedlings treated with each dilution were placed in groups of 7 in pots containing soil previously (24 h. before) mixed with bacteria (ca.10^7 cells/gr.of soil). A second treatment was performed by drench on the 8^{th} day, using the same amount of cells. Each variant, including the disease control, was performed in 4 replicates. In control of germination (3 replicates), the seedlings have been treated with water instead of fungal suspension. For protection of plants against root rot disease caused by *Rhizoctonia solani* or *Sclerotium rolfsii* polypropylene boxes (7 x 19 x 14 cm) were filled up to 2/3 with sandy loam soil (pH 7.2) and each of them contained 10 seeds of cotton (var. "Pima 177"). A seed cover layer (1/3 of the pot's content) was infested by the fungal suspension. For this aim *R. solani* was grown for 5 days in PDB (10 ml with 3 discs of fungi). The mycelium (2.3-2.7 gr/flask) was collected (enough to obtain 0.3gr/pot) and blended in 15 ml of water (ca. $3x10^5$ spores/ml). Then an additional amount of tap water was added in order to use 6 ml per pot of fungal suspension, which was mixed with the top soil. In case of infection with *S. rolfsii* the soil was infested with the fungus sclerotia (100 mg/kg^{-1} of soil). Bacteria in tap water were applied by seed coating and then by drench on the 7^{th} day from sowing (30 ml suspension of each isolate per pot to give an initial concentration of 10^6-10^7 cells g-1 soil. This soil was used only as a cover layer, on top of the seeds (15 mm depth). In the disease control of tap water was used instead of the bacterial suspension. In the seeds germination control the soil was not infected by fungal pathogen and the pots were irrigated just by water without treatment by bacteria. Disease incidence was determined after 10-14 days as the percentage of the seedlings with root rot, to the total amount of the plants appeared. Each strain was tested in six replicates and the experiments were carried out at 28°C. For protection of plants against *Pythium aphanidermatum* the fungus was grown on Corn Meal Agar (Difco) for 3 days at 25°C, then 4 small squares of the mycelium were placed in empty Petri dishes, and covered by one or two pieces of sterilized grass (boiled in sterile water for 30 min and cooled before application). All the plates were closed with parafilm and put back in the incubator. The day after, 30 ml of sterile water were added to each plate. Two days after, the content of all the plates was transferred into an Erlenmeyer flask, closed with parafilm, and put in the shaker at 30°C for 10 min. Then the liquid was filtrated with sterile gaze and the amount of oospores was measured at the microscope using an hemocytometer (~10^5 spores/ml): the fungal suspension was then ready to be mixed to the soil which was a mixture of peat-vermiculite (1:1). About 10 ml/pot was used to get a strong pre-emergency damping-off in the disease control (non-treated by antagonistic bacteria). In order to see a post-emergency disease the amount of suspension was reduced to about 3 ml./pot. In this case the level of germination was very close to that observed in non-infected control but the post-emergency damping-off was observed in about 50% of germinated seedlings. Each pot contained ten seeds of cucumber (var. "Dalila"). The bacteria in tap water were added by drench, but in the non-infected control of germination and in the disease control, tap water was used instead of bacterial suspension. Each strain was tested in six replicates and experiments carried out at 28°C. The pots were treated with tap water just after the preparation of the pots and daily. For protection of cucumber against *B. cinerea*, the fungus was grown for 17 to 25 d on PDA at 28 °C in the dark. Spores or cells were washed from the agar with a mixture of PDB and tap water (1:1, v v^{-1}) containing 0.1% agar, to prepare inoculum suspensions of 1 x 10^6 spores ml^{-1}. Bacteria grown in NB for 24 h at 28 °C were washed in tap water and used for application to cucumber leaves. Before infection with the fungi, cucumber

(*Cucumis sativus* L. cv. "Cfir 413") seedlings with 2 to 3 true leaves were sprayed with the bacterial (10^6 cells ml^{-1}) suspension (about 50 ml in tap water supplemented with 0.01% Tween-20 for 15 to 20 seedlings). A *B. cinerea* spore suspension was applied to the each true leaf in triple drops (10 µl per drop). To assay for protection against *S. sclerotiorum*, the cucumber seedlings were first treated with the bacterial suspension as described above, then disks (6 mm diameter) covered with 5 to 7-d-old mycelium of S. *sclerotiorum* grown at 28 °C in the dark, were taken from PDA plates and applied to the leaves (one disk per each true leaf). In both cases, 15 to 20 seedlings were used in each treatment variant and experiments were repeated four times. The infected plants were incubated in a controlled-climate glass chamber at 25°C under 90% humidity until necrosis appeared on the control plants. The effect of the bacterial application was monitored 72 h after the fungal application, when obvious spots of necrotic tissue were observed on infected leaves of plants used as controls.

To determine bacterial survival on the leaf surface, the amount of bacteria was counted under the same conditions of application but without fungal infection. A piece (0.5 cm^2) of tissue was cut from the same leaf just after the bacteria had been sprayed and then every 24 h for 3 d. Four samples of leaf tissue were taken from two seedlings per treatment variant and analyzed for bacterial contamination and the experiment was repeated twice. The cut tissues were soaked in sterile water, vortexed and diluted. Then samples from the dilutions were seeded onto LBA plates with appropriate antibiotics (rifampicin in the case of strain IC14, rifampicin plus kanamycin in the case of the miniTn5 mutants). Cell number was counted after 24-h incubation at 28°C.

Suppression of fungal pathogens on peaches and apples in postharvest. Peaches (*Prunus persica* (L.) Batsch.) cv. "Swelling" and apples (*Pirus Malus* L.) cv. "Golden Delicious" were surface disinfected and uniformly wounded using a sterile needle. A drop (20 µl) of the bacteria suspension in tap water (10^7 up to 10^9 ml^{-1}) or purified Prn preparations (1 µg in 10 µl of methanol per wound) was pipetted onto the wound side. In control tap water was used instead of bacteria cells and 10 µl of methanol was applied to the wound instead of the antibiotic. The pathogen inoculums in tap water were prepared from 4- to 10-day-old cultures grown on PDA plates to yield a final concentration of 10^3 up to 10^5 CFU ml^{-1} for each pathogen. 20 µl aliquots of the pathogen suspensions were applied to the wound 2 h after bacteria. Fruits were air-dried, then put into firmly closed plastic trays at room temperature and about 95% relative humidity. The infection incidence and lesion diameter were observed after 3 days and 7 days in case of *Monilina* or *Rhizopus* infection and *Penicillium* infections, respectively.

Formulation of strain *E. agglomerans* IC1270 in the alginate beads. Alginate with a molecular mass of 60 to 70 kDa, containing 61% mannuronic acid and 39% guluronic acid (Sigma, LV, St. Louis, MO), was dissolved in distilled water (2% w/w). Glycerol (1-70% w/w, final concentration) was added to the alginate solution (2% w/w, final concentration). For the alginate-chitin bead preparation, a colloidal chitin suspension was added to an alginate or alginate-glycerol solution to a final concentration of 1% (w/w). All mixtures were sterilized by autoclaving. Strain IC1270 (~10^9 or ~10^{11} cells/ml) was then added at a 1:9 volumetric ratio to alginate, alginate-glycerol, alginate-chitin or alginate-glycerol-chitin solutions. This final mixture was dripped into a 1% (w/w) sterile solution of calcium chloride and stirred for 30 min (the volumetric ratio between the alginate mixture and the calcium chloride solution was 1:100). A spontaneous cross-linking reaction produced spherical beads with an average diameter of about 4 mm, containing either ca. 10^7 or ca. 10^9 cells/bead. The beads were frozen at -80°C for 1 h before freeze-drying, which was carried out at -50°C at a pressure of 1.1 Pa (Martin Christ model ALFA I-5; Osterode am Harz, W. Germany).

Preparation of extracellular proteins and detection of chitinolytic enzymes. The procedure was generally performed as described previously (Chernin et al., 1995). For analysis by gel electrophoresis, the filtrate proteins were first concentrated and dialyzed in an ultrafiltration system with a cut-off of 10 kDa (Vivascience, Linkoln, UK). Secreted proteins denaturated with sodium dodecyl sulfate (SDS) were separated by 10% polyacrylamide gel electrophoresis (PAGE) and then reactivated by removing SDS using the casein-EDTA procedure. Enzyme activity was detected on gels by using fluorescent substrates 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, 4-methylumbelliferyl-β-D-N, N'-diacetylchitobioside, and 4-methylumbelliferyl-β-D-N, N', N''-triacetylchitotriose (all from Sigma).

Antibiotic compound purification and assay. The crude extracts of the antibiotic from cells grown on PDA plates were prepared as described previously (Chernin et al., 1996). The extracts were fractionated by thin-layer chromatography (TLC) on SIL G-25 (Macherey-Nagel, Duren, Germany) plates using a chloroform/methanol (19:1) mixture as the solvent. After the run, the silica gel was divided into equal segments, which were scratched, dissolved in MeOH and used in a PDA plate bioassay against R. solani. The active fraction was re-applied on two TLC plates, one of which was used for visualization of the antibiotic with Erlich reagent (2% para-dimethylaminobenzaldehyde in 5 N HCl). The corresponding fraction scratched from the second TLC plate was analyzed by high-pressure liquid chromatography (HPLC) (Spectrum Chromatography, Houston, TX) with a "Luna" (Phenomenex, Tottance, CA) reverse-phase C18 column (5 μm, 250 x 4.6 mm), eluted isocratically (45% H_2O, 30% acetonitrile, 25% MeOH) at a flow rate of 1 ml min^{-1}. Antibiotic absorption was monitored with a diode array detector (UV6000) at 225 nm, 310 nm, and 278 nm, the wavelengths commonly used to monitor Prn, pyoluteorin, and Phl, respectively (Corbell and Loper, 1995). Purified Prn was kindly supplied by Karl-Heinz van Pee (Institute for Biochemie, TU Dresden, Dresden, Germany) and used as a standard. Samples (1 ml) were collected over 1 min, concentrated by SpeedVac and tested in a bioassay against R. solani.

Assays for glucanase, protease, HCN, IAA and hemolytic activities. β-1,3-glucanase activity was assayed using laminarin (Sigma) as a substrate and measuring the liberation of glucose from the incubation mixture by the glucose oxidase method described by Fridlender et al. (1993). Proteolytic activity was assayed on Bacto Litmus milk (Difco) agar plates by, monitoring the appearance of haloes of casein lysis around bacteria the colonies. HCN assay was perform using the "Aquaquant 14417- Testsystem" (Merck, Darmstadt, Germany) with a culture broth of the bacteria. The method described by Sarwar and Kremer (1995) was used to determination IAA, with some modification: bacteria were grown in RM containing mannitol (10 g l^{-1}) and L-tryptophan (0.2 g l^{-1}) for 3 d at 28 °C. A cell-free supernatant of the tested cultures was dispensed into microplate wells (Nunclone, Denmark) followed by the addition of Salkowski reagent. After 30 min of incubation at room temperature, the pink color's intensity was measured at 530 nm by Micro-Plate Reader EL311SX (Bio-Tek Instrument Inc., Winooski, VT). Various concentrations of IAA (Sigma) solution were prepared and measured to obtain the standard curve. The hemolytic activity was assayed by the appearance of clearing zones of blood hemolyses on blood agar (Difco) supplemented with 5% rabbit blood after incubation at 30 °C and 37 °C for 48 h.

Detection of siderophores. The plate assay (Alexander and Zuberer, 1991) was performed using the ternary complex chrome azurol S/iron (III)/hexacyltrimethyl-ammonium bromide as an indicator. Siderophore production was quantified using Cu-CAS reagent according to Shenker et al. (1995). The absorption was measured at 582 nm and the exact concentration

of siderophore(s) present in the culture medium, forming ligands with the Cu from the Cu-CAS reagent, was determined.

Isolation of mutants deficient in biocontrol activity by Tn5 mutagenesis. Spontaneous mutants of strains IC1270 and IC27, resistant to rifampicin (40 μg/ml) or streptomycin (100 μg/ml), respectively, were used as a recipient in matings with *Escherichia coli* strains carrying the plasmids pSUP2021::Tn5, pUT-miniTn5 or ColIb-drd::Tn5, used as donors of the Tn5 transposons.

DNA manipulations. Extraction of plasmid pUT-miniTn5-Km2 DNA from E. coli by the alkaline lysis method, isolation of genomic DNA from strain IC14 and its miniTn5 mutants digestion of the DNA by restriction endonucleases, electrophoretic separation of the restriction fragments, isolation of the fragments from the agarose gel and Southern hybridization were performed using standard procedures (Ausubel et al., 1994). The 1.7-kb HindIII fragment of plasmid pUT-miniTn5-Km2 was extracted from the agarose with a QIAquick Gel Extraction Kit (Qiagen, Germany) and used as a Km-DNA probe. Labeling of the fragment with α-^{32}P-labeled CTP (3,000 Ci mmol^{-1}, Amersham England) was carried out with a random-prime DNA-labeling kit (RediprimeII, Amersham). Restriction enzymes were purchased from Fermentas MBI (Vilnius, Lithuania) and used under the conditions recommended by the manufacturer. Heat-lysed bacterial suspensions used in polymerase chain reaction (PCR) analysis were prepared as described (Raaijmakers et al., 1997). PCR amplification was carried out in a 25-μl reaction mixture containing 5 μl of diluted heat-lysed cell suspension, 2.5 μl 10X buffer Taq-polymerase (Promega Co., Madison, WI), 200 μM each dATP, dTTP, dGTP, and dCTP, 10 pmol of each primer and 1 U of Taq DNA polymerase. The primers for determination of genes chiA, prnC, phl and pca, encoding the synthesis of a 58-kDa endochitinase, the antibiotics pyrrolnitrin, 2,4-diacetylphloroglucinol, and phenazine-1-carboxyl acid, respectively, were designed to the conserved region of these genes. The primers were: chiA (Chernin et al., 1997) 5'-TATCCTCTCGGAATAAAGGAAT (forward) and 5'-GAATTCACTCAAACAACTCT (reverse); prnC (Mavrodi et al., 2001) 5'- CCACAAGCCCGGCCAGGAGC (forward) and 5'- GAGAAGAGCGGGTCGATGAAGCC (reverse); phl2 (Raaijmakers et al., 1997) 5'-GAGGACGTCGAAGACCACCA forward and 5'-ACCGCAGCATCGTGTATGAG (reverse); pca (Raaijmakers et al., 1997) 5'-TTGCCAAGCCTCGCTCCAAC (forward) and 5'-CCGCGTTGTTCCTCGTTCAT (reverse); nptII (de Lorenzo and Timmis, 1994) 5'-GAGGCTATTCGGCTATGACT (forward) and 5'-AATCTCGTGATGGCAGGTTG (reverse). Amplifications were performed in the PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) using PCR programs described for chiA (Chernin et al., 1997), prnC (Mavrodi et al., 2001), and for phl2 and pca (Raaijmakers et al., 1997). The program for nptII was the following: 94°C, 2 min; 60°C, 30 s; 72°C, 1.5 min; then 94°C, 45 s; 60°C, 45 s; 72°C 1.5 min for 30 cycles, followed by a final extension at 72°C for 10 min. PCR products were separated on a 1% agarose gel in 0.5 x Tris-acetate-EDTA buffer at 100 V.

Statistical analyses. Data were statistically analyzed at a significance level of P= 0.05 by Student't-test and the Excel's Descriptive Statistical Tool. Disease protection rate was calculated as relative disease reduction (DR, %) = 100 x [(test treatment disease rating) – (control disease rating)]/(control disease rating).

RESULTS

I. Biocontrol of soil-borne diseases

I.1. Biocontrol of *Fusarium* wilt

I.1.1. Protection of melon seedlings against *F. oxysporum* f. sp. *melonis* under laboratory greenhouse conditions. A soilborne chitinolytic *E. agglomerans* strain IC1270 isolated in Uzbekistan from rhizosphere of grape was found to be a strong antagonist of about 30 species of plant-pathogenic bacteria and fungi in vitro and an efficient biocontrol agent of several diseases caused by soilborne fungal pathogens (Chernin et al., 1995). The strain produced and excreted a set of chitinolytic enzymes consisting of two N-acetyl-β-D-glucosaminidases with apparent molecular masses of 89 and 67 kDa, and a 58-kDa endochitinase. The chitinolytic activity was induced when the strains were grown in the presence of colloidal chitin as the sole C source; the observed chitinolytic enzymes seemed to be the most abundant proteins secreted by the bacteria under this condition. In addition to its chitinolytic activity, the strain IC1270 produces an antibiotic pyrrolnitrin {3-chloro-4-(2'-nitro-3'-chlorophenyl)pyrrole} with a wide range of activity against many phytopathogenic bacteria and fungi in vitro (Chernin et al., 1996). The ability of *E. agglomerans* IC1270 to produce pyrrolnitrin in combination with chitinases would be advantageous in attacking fungal phytopathogens. The *chiA* gene of the 58-kDa endochitinase (ChiA_Entag) was cloned from strain IC1270 in *E. coli* and the ChiA_Entag-producing *E. coli* strain decreased the disease incidence of root rot caused by *R. solani* on cotton under greenhouse conditions (Chernin et al., 1997). Strain *P. putida* IC27 produces still non-identified siderophore, while strain *P. fluorescens* IC111 was shown produce exo-protease and still non-identified antibiotic activity. The experiments were performed in 1998-1999 in the in Israel during first training visit of the Uzbek partners. Fig. 1 shows the results of four independent experiments.

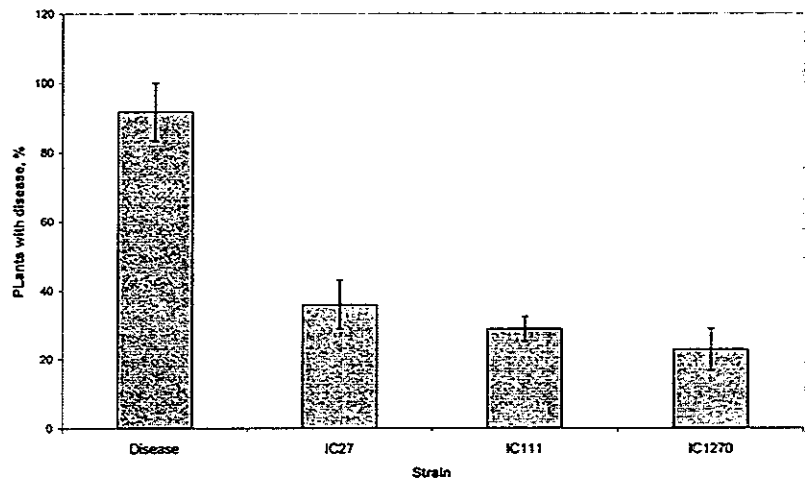

Fig. 1. Protection of melon speedlings against *Fusarium oxysporum* f. sp. *melonis* by application of bacterial antagonists to the potting soil

Rather rapid decline of the strains *Pantoea* (*Enterobacter*) *agglomerans* IC1270, *Pseudomonas putida* IC27 and *Ps. fluorescens* IC111 survival in soil was observed. The amount of cells of all these strains dropped from ca. 10^8 per g^{-1} of soil at the day application, to ca. 10^7, 10^5 and 10^2 after 1, 3, and 6 weeks after application, respectively.

Therefore seed coating and repeated treatments with bacteria at least at sowing and 7 days after was found substantive requirement to get efficient plant disease protection. Significant decrease of the wilt incidents was observed in case when the 7-10-days old seedlings of melon were infected by *F. oxysporum sp. f. meloni* and then transferred to the pots containing soil mixed with the bacterial antagonists of the fungus. The results were cooperatively published (Chernin et al., 1999).

I.1.2. The experiments on biocontrol of *Fusarium* spp. were continued with tomato and melon in Uzbekistan under greenhouse and small-plot field conditions (trials were performed in years 1999-2001). At the first stage the bacteria were tested for resistance to fungicide Vitavax 2000 commonly used in Uzbekistan for protection of crops against fungal disease. A clear mutagenic effect of the fungicide Vitavax 2000 was observed using a *Saccharomyces cerevisiae* test system: treatment of yeast cells with Vitavax 2000 at 0.1 to 10% led to a four- to nine-fold increase in mutant formation. The data indicate the importance of further decreasing fungicide doses for practical use; this can be achieved by combining its application with biocontrol agents (Linder et al., 1999). An in-vitro assay on petri plates revealed that all three bacterial antagonists are highly resistant to Vitavax 2000, at least up to 80% of the original preparation (which is equivalent to the 75% solution recommended for use with 2.5-3.5 kg per ton of seeds). Resistance was tested from 0.1% to 100% of the 75% fungicide solution (Linder et al., 2000). Since the bacterial antagonists were found resistant to Vitavax, the fungicide was tested in combination application oof the bacterial antagonists to show whether the bacteria can be used as a part of integrated pest management approach.

The results on protection of melon against *F. oxysporum* f. sp. *melonis* by the bacterial antagonists under greenhouse conditions in experiments performed in Uzbekistan are presented in Table 1. The trials were performed in commercial greenhouse of the Bagizagan scientific experimental Center of the vegetable-gourds crops (Samarkand region) on square of 100 m^2. The results are showing that treatment of seeds before sowing by the bacteria or by mixture of the strains with fungicide Vitivax increased the amount of germinated seeds. A small decrease of germination in the disease control towards the non-treated control was observed. All the three stains stimulated the germination at the same level as the chemical fungicide Vitavax, while only strain IC1270 and the fungicide slightly decreased the amount of diseased plants. The most remarkable decrease of the diseased plants was observed when strain IC1270 was used in combination with Vitavax. Moreover, percentage of plants without visible symptoms of the disease in the disease control was 37%, in comparison to 75% in case of combination of IC1270 with Vitavax. However, if the calculation will take into the consideration the amount of seeds sowed in that case in the disease control only 16% of plant looked healthy vs. 80% in variant of IC1270+Vitavax (Table 1).

Table 1. Protection of melon (cv. Kukalapush) against *F. oxysporum f. sp. melonis* under greenhouse conditions.

Variant	Total number of plants	Non-germinated*	Germinated*		Plants with disease*		
			Totally	%	Number	% to germinated	% to sowed
Control.	60	27	33	55 (100)	0	-	-

Disease control.	60	33	27	45 (81.8)	17	**63**	28.3
IC1270	60	16	44	73.3** (162.9)	16	**36.4**	26.7
IC27	60	27	43	71.7** (159.3)	22	51.2	36.7
IC111	60	15	45	75** (167.7)	23	51.1	38.3
Vitovax (Vt)	60	14	46	76.7** (170.4)	20	**43.5**	33.3
IC1270+Vt	60	12	48	80** (177.8)	12	**25**	20
IC27+Vt	60	10	50	83.3** (185.1)	27	54	45
IC111+Vt	60	26	34	56.7** (126)	12	35.3	20

Note: Variants of the experiment: Control (tap water), Control of disease, Strains of the bacterial antagonists tested: *P. agglomerans* IC1270, *P. putida* IC27, *P. fluorescens* IC111, fungicide Vitovax (Vt), combination of the bacteria with Vitavax. Adding of the fungus (10^6 spores/ml) formed infection background. The spores' concentration was determined by direct counting in hemocytometer. The fungus was grown on plates with PDA for ca. 5 days at 28°C, then the mycelium was scratched from the agar surface and suspended in 0.89% NaCl. The pots were filled on ½ with local river' yellow sand, covered by a layer (0.5-0.7 cm) of the soil infected by the fungus. Bacteria were grown for ca. 36 h at 28°C in minimal medium 925 and the obtained bacterial suspension (2-4 x 10^8 cells/ml) was directly used for treatment of the melon seeds. The seeds were soaked for 4h in this suspension or in the Vitavax solution (0.2%) or in a mixture of bacterial suspension and Vitavax solution in 1:1 ratio. The treated seeds were places the infected soil layer then covered by the same layer of the infected soil. Additional layer of the non-infected sand was placed on the top of each pot. In control only uninfected soil was used. * Each variant of the experiment was repeated 3 times and the averages from three repetitions are presented. ** % to the disease control. Seeds of melon local variety "Kukalapush" were used. 20 seeds were seeded in each pot. Results showing biocontrol effect of strain *P.a.* IC1270 alone or in combination with fungicide Vitavax are bolded.

The ability of the bacterial antagonists to protect tomato against fusaryosis in open ground (micro-plot field conditions) was studied in the Agrofirm "Uzbekistan" on square of 140 м^2 and 120 m^2 (20 м^2 per variant) in years 1999 and 2001. During the 5 last year this field was exclusively used to tomato growing. As the result high natural infection background of *Fusarium* spp. was formed: usually the level of the *Fusarium* wilt disease in the middle of the vegetation time reached up to 85-100%. Table 2 summarized the results obtained in year 1999 trials. The data showing the level of the disease at the day of the experiment finishing. Measuring of the disease symptoms on the various parts of the plants revealed that treatment by strain IC1270 lead to decrease of the symptoms of the fusariosis on the plants leaves, while the effect of strains IC27 and IC111 was more obvious on fruits. In all cases the effect of the bacteria was quite similar to that observed while fungicide Vitovax was used (Table 2). Very similar results were obtained in repeated trials obtained

in year 2001 (data not shown). However, it is necessary to improve the strain(s) consistency to achieve more stable results in the field conditions.

Table 2. Protection of tomato against *Fusarium* under open ground conditions

Variant*	Leaves	t_d	Fruits	t_d	Whole plant	t_d
Disease control	3,7+0,2	-	0,6+0,1	-	2,6+0,6	-
Vitivax	2,0+0,2	4,5	0,35+0,07	1,9	2+0,2	1
E.agglomerans IC1270	2,1+0,2	0,15	0,55+0,2	0,5	2,2+0,2	0,1
P. putida IC27	2,65+0,1	0,1	0,4+0,3	2,1	2,1+0,2	1,5
P. fluorescens IC111	2,4+0,2	1,1	0,4+0,2	1,9	2.3+0,2	0,7
Mixture of strains	2,2+0,1	1,1	0,4+0,1	0,1	2 +0,2	0,4
Local control	3,5+0,1	1,1	1,1+0,25	1,6	3,0+0,17	0,6

Note: The seedlings of tomato var. "Volgograd" 10-12 cm high with cut off upper part were soaked in the suspension of the tested strain (ca 10^8 cells/ml) for 2 h before sowing in soil. In the control the seedlings were treated with tap water or tap water containing 0.2% of the fungicide Vitavax (which corresponds to 2.5 g per kg of seeds according to the manufacturers recommendation). The mixture of bacteria contained the same strains in ratio 1:1:1. The "Local control" means the incidence of the disease in plants sowed into the soil by farmer's 3 day before we started our experiment in the nearly located plot The experiment was started on 19.05 and finished at 27.09.1999. *, 20 plants per each variant. Rank: 0 – no visible symptoms of the disease; 1 – less than 10% of the square of the indicated part or the hole plant was affected; 2 – between 10 and 25% was affected, 3 – between 25 and 50% was affected; 4 – more than 50% was affected.

In vitro assay was designed to study whether the bacterial antagonists are able to stimulate growth of tomato seedlings. The obtained data revealed that 7th day after the seeds germination on the surface of water soaked Wathman paper in petri dishes the strain IC1270 significantly stimulated the seedling growth. The length of seedlings was found 6,7±0,5 cm vs. 2,8±0,3 in the control (soaking of the seedlings in the minimal medium 925 for 4 h). The effect of this and three other strains was further tested under another experimental system, which included growing of seedlings in soil (Table 3). In this case besides of the increase of the total length of the seedlings, the increase of the amount and the square of leaves, and specially the content of the chlorophyll in leaves were observed.

Table 3. Influence of the bacteria on various characteristics of the tomato seedlings

Variant	Root	Total weight of seedlings (g)		Leaf		Chlorophyll (mg/g)*
	Length	Dry	Wet	Amount	Square (cm^2)	
Control	1,6	0,13	0,07	3,3	1,6	1,11
P. putida 27	1,4	0,26	0,11	3,2	1,5	0,95
P. fluorescens 111	1,5	0,26	0,09	3	1,2	0,85
P. agglomerans 1270	1,6	0,26	0,13	3,7	2	1,53

The seedlings after germination in the presence of bacteria were washed in water, dried on filter paper, then transferred into the soil (the river' yellow sand). In each variant 20 seedlings were tested. *Concentration of chlorophyll was determined by acetone' extraction method. The leaf tissues have been grind with 80% acetone, drew for 2-3 min, then filtrated through Whatman N3 filter. The procedure was repeated several times until the extract was become clear. The amount of pigment was determined by using spectrophotometer and the correspondent calibrating curve. The content of the pigment (A) in the tissue (in mg/g) = C x V/P x 1000, where C - concentration of the pigment (mg/l), determined according the formula ($C = 6.4 \times D_{663} + 18.8 \times D_{644}$), where D - optical density at wave length indicated; V - volume of the pigment-containing extract in ml; P - the material in gr.

I. 2. Biocontrol of damping-off diseases

In addition to biocontrol effect observed with *Fusarium oxysporum* under greenhouse and micro-plot field conditions, the bacterial antagonists efficiently protected cucumber against dumping-off (*Pythium aphanidermatum*) and beans against root rot disease (*Rhizoctonia solani).* The experiments were performed in 1998-2002 under laboratory greenhouse conditions in Israel during visits of the Uzbek partners. When the bacteria were applied by seeds coating and additionally to the soil seed cover layer, the number of seedlings with root rot symptoms caused by the fungus drastically decreased and disease reduction rate between 50 and 95% was observed, depending on the strain tested. (Table 4). It worth note that in *Pythium* – cucumber model on severe infection background when the emergency of the seeds in variant "Disease control" was very low, the strains were shown able to restore it to almost normal level (Table 4, Exp. N1). In case when the lower doses of fungal infection were used, the strains were found able efficiently control seedlings against post-emergency damping-off disease (Table 4, Exp. N2 and 3). The significant biocontrol potential of the bacterial antagonists was also demonstrate in *R. solani* – beans model (Table 4.). Usage of the strains mixture revealed only moderate additional increase in the fungus biocontrol in comparison with the same strains applied separately.

To go more inside mechanisms of the bacteria ability to suppress fungal pathogens, the transposon Tn5-induced mutagenesis was used to obtain the mutants of the strains IC1270 and IC27 deficient in antagonistic activity. Totally about 5,000 clones were screened for deficiency in suppression of *R. solani* growth in vitro (in case of both strains), deficiency in chitinolytic activity (in case of strain IC1270) and/or for loss of pigment-forming ability (in case of strain IC27). Two selected mutants of strain IC1270, designated IC1270-E1 and

IC1270-2h, were found deficient in chitinolytic activity. Two mutants of strain IC27, designated as IC27-23 and IC27-56, were considered unable to produce a siderophore. All four mutants failed in biocontrol activity as was shown in experiments on protection of beans against *R. solani* (Table 4). The data prove suggestion that the ability to produce chitinolytic enzymes or siderophores may significantly contribute to the antagonistic activity of the IC1270 and IC27 strains, respectively. The results of this part of the project were published (Chernin et al., 1999).

Table 4. Biocontrol effect of bacterial antagonists under greenhouse conditions

Variant	Exp.N1 (pre-EC) Germination (%)	*P. aphaniderma tum* Exp.N2 (post-EC) Plants with disease,%	Exp.N3 (post-EC) Plants with disease,%	*R. solani* Plants with disease,%
Control non-infected	100	0	0	0
Disease control	15±3.7*	49±15.7	68±10.4	73±10.9**
E.a. IC1270	88±9.4	4.4±0.9 (91)	3.5±0.7 (95)	28.9±6.1 (60.4)
P. p. IC27	82±9.9	5±1.6 (89.8)	15.8±4.8 (76.8)	11±2.8 (85)
P. fl. IC111	96±11.6	0	8.8±2.1 (79.1)	15±2.3 (79.5)
E.a. IC1270-E1				64±12.9 (12.3)
E.a. IC1270-2h				78.1±11.4 (NR)
P.p. IC27-23				69.3±17.3 (5)
P.p. IC27-56				75.4±17.7 (NR)
Mixture ***				23.1±4.1 (68.4)

* Each of the experiments was performed in 6 pots per variant with 10 seeds sowed per pot. Mean±S.E. is indicated. Disease reduction rates (%) are shown in parentheses), NR, no disease reduction. **, the data of 4 independent experiments are averaged; ***, three strains (IC1270, IC111, and IC27) were mixed in equal proportion; EC-emergency control.

II. Biocontrol of airborne diseases.

S. plymuthica strain IC14 was isolated in course of this project execution was studied as biocontrol agent of aerial diseases in more details. background in an experimental field in Rehovot, Israel. The objectives of the study were: (i) to study the strain IC14 antagonistic activity against economically important plant pathogenic fungi in vitro and under greenhouse conditions; (ii) to determine the mechanisms of this bacterium antifungal activity; (iii) to study the role of chitinases in the biocontrol activity of strain IC14 against *B. cinerea* and *S. sclerotiorum* foliage diseases. These necrotrophic fungi are among the world's most dangerous fungal plant pathogens due to their effects on flowers, leaves, fruits or stems under high humidity or when free moisture is present on the plant surface. Biocontrol is an environmentally friendly and efficient alternative to chemical fungicide management of these pathogens. However, of the approx 80 commercial biocontrol agents (BCAs) available today for use against crop diseases, only a few are specifically

recommended for use against these pathogens. The strain IC14 efficiently protected cucumber against *B. cinerea* and *S. sclerotiorum* by foliar application reducing the number of affected leaves by 76% and 84%, respectively, while neither mutant differed appreciably from the parental strain in protection of plants against *B. cinerea* and *S. sclerotiorum*, suggesting that chitinolytic activity is less essential for biocontrol of these pathogens by strain IC14 (Table 5).

Table 5. Protection of cucumber seedlings against leaf mold caused by *Bc* and *Ss*.

Fungus	Disease control, %	Treatment by strain		
		IC14	IC14::miniTn5#9	IC14::miniTn5#18
		Disease, %		
Bc	62.7±6.4[b]	13.7±3.3[a] (75.9±7.2)	15.4±5.5[a]	10.8±3.1[a]
Ss	69.4±7.8[b]	12±5.9[a] (84±5.3)	31.4±12.3[a]	24.3±4.9[a]

The data of four independent experiments for each of the fungal treatment are summarized. Different letters in the same row indicate significant differences between means using the All Pairs Tukey-Kramer test ($\alpha = 0.05$, $p < 0.001$). Homogeneity of the variances between repetitions in all experiments was proven with Bartlett test (>0.05).

A large number of microorganisms, including fungi, bacteria, and actinomycetes, as well as plant species, possess the ability to excrete cell-wall hydrolases such as chitinases, β-1,3-glucanases, and proteases. These hydrolases play an important role in the reactions between BCAs and pathogens (rev. by Chernin and Chet, 2002). The ability to produce chitinases is considered crucial for antifungal activity of strains of *E. agglomerans* (Chernin et al., 1995). Similar to strain *P. agglomerans* IC1270, the strain *S. plymuthica* IC14 has chitinolytic activity, produces antibiotic pyrrolnitrin and some other antifungal compounds. The ability to produce the plant-growth-promoting hormone IAA and its lack of hemolytic activity at human body temperature could be considered additional advantages for this bacterium's further development as a BCA of a wide range of crop diseases (Kamesky et al., in press). However, in the case of strain IC14, the same level of antifungal activity *in vitro* and *in vivo* was observed with the parental chitinolytic strain and its two miniTn5 mutants, one of which (IC14::miniTn5#9) is a super-producer of chitinase, while the other (IC14::miniTn5#18) is deficient in chitinolytic activity. These data indicate that other antifungal compounds produced by strain IC14, which are not chitinases, play a key role in the strain's activity, at least against *B. cinerea* and *S. sclerotiorum*. Actually, strain IC14 and both its mutants did not differ in their ability to produce pyrrolnitrin, siderophores or proteolytic enzymes, suggesting the predominant role of either one or all of these compounds in the strain IC14's biocontrol activity towards foliage fungal pathogens, while the role of chitinolytic enzymes appears to be less essential. The ability to produce the plant-growth-promoting IAA and its lack of hemolytic activity at human body temperature could be considered additional advantages for this bacterium's further development as a BCA of a wide range of crop diseases.

Strains IC1270 and IC111 also were tested for ability to protect cucumber plants against airborne diseases. Bacterial application via leaf spray in sunflower and rape seedlings reduced the incidence on cucumber seedlings up to ca. 90% (Table 6) and the disease severity (size of the lesion area) up to ca. 80% (data not shown). The similar effect

was obtained in cabbage and cucumber seedlings infected by *S. sclerotiorum* or *B. cinerea* (Chernin et al., 1999).

Table 6. Control of *S. sclerotiorum* in rape and sunflower by the bacterial antagonists

	Sunflower – S. s.		Rape –*S. s.*		Cucumber-*B. cinerea*
Treatment*	Length of lesion, mm, %	Incidence (%)	Length of lesion, mm, (%)	Incidence (%)	Incidence (%)
Control	40.2 (100)	100	38.8 (100)	100	100
IC1270	8.4 (21.6)	12.1	5.5 (24.1)	14.3	25
IC111	13.9 (34.6)	15	10.4 (45.6)	18.3	32.5

*, Between 10 and 20 leaves were inoculated and measured in each treatment. Average data are presented. Percentage of incidence was calculated as amount of diseased leaves per total amount of treated leaves. Data of five independent experiments are summarized. In each of the experiments two to three plants were used and in total, 15 to 20 leaves were infected.

The results of this work were presented on the Seventh IOBC/WPRS Working Group Meeting "Influence of A-Biotic and Biotic Factors on Biocontrol Agents" (Pine Bay, Kusadasi, Turkey 22-25 May 2002) and will be published in Soil Biol. and Biochemistry (Kamensky et al., in press). Strain IC14 was transferred to the Uzbek partners for further testing under commercial greenhouse conditions.

Pseudomonas aureofaciens 449 isolated from rhizosphere of maize suppresses numerous plant pathogens *in vitro*. The strain was found to produce three phenazine antibiotics (PCA, 2-OH-PCA, 2-OH-PHZ), hydrogen cyanide, sidrophore(s) and exoprotease(s).

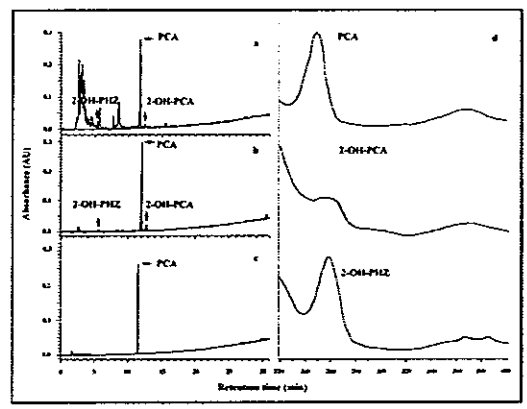

Fig. 2. HPLC analysis of phenazine compounds produced by strains *P. aureofaciens* 449(**a**), *P. aureofaciens* 30-84(**b**), *P. fluorescens* 2-79(**c**), Peak identity of PCA, 2-OH-PCA and 2-OH-PHZ, confirmed by spectral analysis (**d**).

Like some other phenazine producing strains ofr fluorescent pseudomonads strain 449 was found to produce N-acyl-homoserine lactone (AHL) quorum sensing signal molecules HSL known to regulate production of secondary metabolites in a wide range of bacteria,

including biocontrol strains. AHL production by *P.a.* was studied using thin-layer chromatography (TLC) on reverse-phase (RP) C_{18} TLC plates using three different bioreporter strains able to detect acyl-HSL based on the appearance of violacein purple pigment (*Chromobacterium violaceum* CV026), on the cleavage of X-gal by β-galactosidase (*Agrobacterium tumefaciens* NTL4/pZLR4) and on bioluminescence (*E.coli* S17-1/pSB401) in response to the presence of various acyl-HSLs in the growth media. The bacterium was found to produce at least three types of HSLs: N-butanoyl-HSL (BHL), N-hexanoyl-HSL (HHL) and N-(3-oxo-hexanoyl)-HSL (OHHL). Detection of BHL and HHL was carried out on an RP18-TLC plate overlaid with short-chain biosensor CV026 (Fig. 3A) Detection of OHHL was carried on an RP18-TLC plate overlaid with *E.coli* S17-1/pSB401 biosensor (Fig. 3B) and biosensor NTL4/pZLR4 (Fig. 3C).

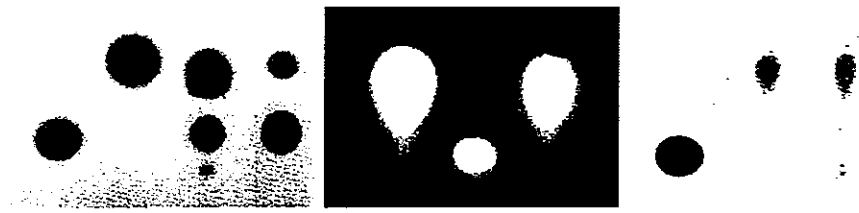

Fig. 3 Thin-layer chromatogram of AHLs extracted from cell-free culture supernatants of strain 449. In A, B and C, TLC plates were overlaid after chromatography with strains CV026, S17-1/pSB401 and NTL4/pZLR4, respectively. A) Lanes: 1, HHL standard ; 2, BHL standard ; 3, strain 449 extract ; *P.a.* strain 30-84 extract. B) Lanes: 1, OHHL standard; 2, HHL standard; 3, strain 449 extract. C) Lanes: 1, HHL standard ; 2, OHHL standard ; 3, strain 449 extract.

Tn5-mutants of strain 449 deficient in PCA production were isolated. Two of them were compared with the parental strain for ability to protect beans and cucumbers against *Rhizoctonia solani* and *Sclerotinia sclerotiorum in* greenhouse conditions. Treatment with the parental strain decreased the incidence of the diseases caused by these fungi by 70% and 50%, respectively. Contrary to that, both mutants were unable to control the pathogens. Thus, indicating the critical role of phenazines in the inhibition of these fungi (Table 7).

Table 7. Biocontrol activity of strain P. aureofaciens 449 and its mutants

Strain/Fungus	*R. solani*	*S. sclerotiorum*
P. a. 449	31.8±8.9^{a}	31.8±8.9^{a}
P. a. 449::miniTn5#3	99.3±0.7^{b}	96±4^{b}
P. a. 449::miniTn5#14	85.8±4^{b}	92.3±7.6^{b}

*Disease incidence percentage estimated by four independent experiments for each of the fungal treatments. Disease incidence refers to 100% disease control (the average actual disease control in the experiments was 86.7 ± 12.6 for *R. solani* and 66 ± 10.7 for *S. sclerotiorum*). *R. solani* disease incidence was determined after 12 to 14 days as the percentage of seedlings with root rot and *S. sclerotiorum* disease incidence was determined as percentage of necrotic zones from agar discs covered with mycelium applied on the surface of the leaves. ** Different letters in the same row indicate significant differences between means using the All Pairs Tukey-Kramer test ($\alpha = 0.05$, $p < 0.001$) using the JMPIN 3.2.2. (SAS Institute Inc., Cary, NC) Program.

Homogeneity of the variances between repetitions in all experiments was proven with a Bartlett test ($p>0.05$).

III. Biocontrol of post-harvest diseases

Rots caused by *Rhizopus stolonifer* (Ehrenb.: Fr.) Lind. and *Monilinia fructicola* (Wint.) Honey and blue mold caused by *Penicillium expansum* Link are among the most destructive postharvest diseases of stone and pome fruits through the world. Chemical treatment is generally an efficient way for controlling plant pathogenes, including those causing postharvest diseases, however development of fungicide-resistant pathogens and public concerns over the presence of chemical residues in food has resulted in cancellation of some of the most effective fungicides. Therefore, research has been focused on the development of management alternatives that are both effective and economically feasible. The biocontrol of postharvest diseases has been widely reported and microbial biocontrol agents (BCA) have shown promise as potential alternatives to fungicides. Several yeast and bacteria were found promising for biocontrol of postarvest diseases caused by *Rhizopus*, *Penicillium* and *Monilinia* spp.

Suppression of post-harvest fungi by strain IC1270 and Prn produced by this bacterium in vitro. Strain IC1270 suppresses the growth of several fungal pathogens which cause diseases in fruits post-harvest. The inhibition zones between pathogenic fungi and the strain tested were up to 14 mm for *P. expansum* and *M. fructicola* and up to 7 to 9 mm in the case of *R. stolonifer* and *B. cinerea*. Prn (0.5 μg ml^{-1}) purified from strain IC1270 was able to suppress on plates the same set of fungal post-harvest pathogens as the bacterium itself. The antibiotic drastically inhibited growth of all four tested fungi. Percentage inhibition of fungal radial growth on a plate with antibiotic as compared to a control plate was up to 70% for *P. expansum*, up to 100% for *M. fructicola* and *R. stolonifer* and up to 90% in the case of *B. cinerea*

Control of postharvest diseases on peaches and apples. Application of strain IC1270 (10^9 CFU ml^{-1}) on peaches infected by *M. fructicola* (10^3, 10^4 or 10^5 conidia ml^{-1}) reduced the incidence of the disease to 4, 46 and 80% from 60, 94 and 100% in the diseased control, respectively. In addition the size of lesions were decreased respectively from 11, 24 and 34 mm in the disease control to 5, 11 and 22 mm in peaches treated by the bacterium. Strain IC1270 (10^9 CFU ml^{-1}) also reduced the incidence of rot caused by *R. stolonifer* (10^3, 10^4 or 10^5 spores ml^{-1}) on peaches to 8, 17 and 58% from 53, 92% and 100% in inoculated control, respectively. In addition strain IC1270 was shown to be highly efficient in control of blue mold disease caused by *P. expansum* (10^5 spores ml^{-1}) on apples: at 10^8-10^9 CFU ml^{-1} the antagonist reduced blue mold incidence by approx 95%.

We have tested if the ability to produce chitinases is considered crucial for biocontrol activity of strain IC1270 against post-harvest fungal pathogens. For this a new set of Tn5-mutants of IC1270 deficient in chitinolytic activity but producing Prn were obtained by insertion mutagenesis using mini-Tn5 transposon- carrying plasmid pUT/mini-Tn5Km2. The mutants of IC1270 were obtained by the Uzbek partners (Drs. Z. Ismailov and F. Kabulova) during their training in Israel in year 2000. Southern hybridization of EcoRI-, HindIII- or SmaI-digested DNA from strain IC14 and these two mutants demonstrated that in both of them miniTn5 was inserted at only one site, since only one EcoRI fragment hybridized with the miniTn5 probe and only two bands of hybridization appeared after digestion of the mutant genomic DNA with HindIII or SmaI (Fig. 4).

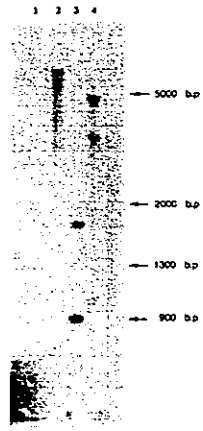

Fig. 4. Southern-blot analysis of Tn5 mutants #3 and #14 of strain IC1270. Lines: 1 – DNA from strain IC11170 digested by EcoRI; 2, 3, and 4 - DNA from mutants IC1270::miniTn5#3 digested by EcoRI*, SmaI and HindIII, respectively.

Despite the deficiency in chitinolytic activity the mutants showed a similar efficiency towards both these pathogens as the parental strain. Moreover, Prn purified from strain IC1270 and co-inoculated with the fungi into the fruit wound completely suppressed *Rhizopus* rot and reduced the *Monilinia* rot by 83%. (Fig. 5).

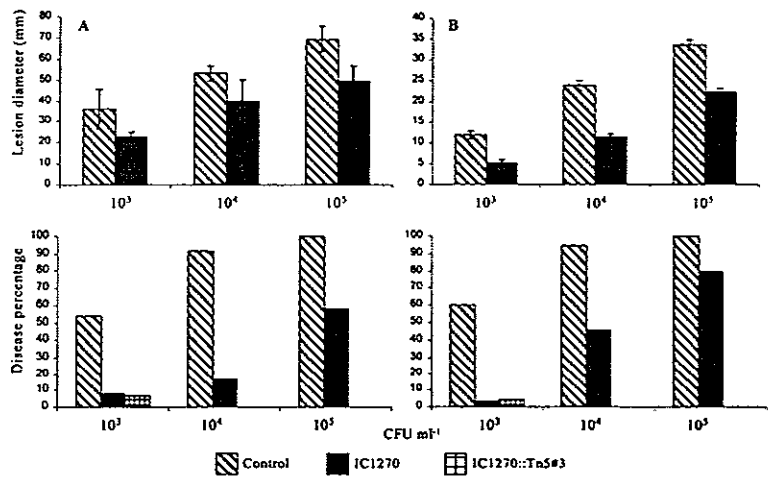

Fig. 5. Dependence of the strain IC1270 biocontrol activity on concentration of *R. stolonifer* (A) and *M. fructicola* (B) used for the fruits inoculation.

The low mammalian toxicity of Prn makes the antibiotic-producing strains reliable for some agricultural uses. The obtained results extend the range of strain IC1270 antagonistic activity indicating that the bacterium is also a perspective candidate for biological control of postharvest diseases of stone and pome fruits. The results of this collaborative work were presented on the Seventh IOBC/WPRS Working Group Meeting "Influence of A-Biotic and Biotic Factors on Biocontrol Agents" (Pine Bay, Kusadasi, Turkey 22-25 May 2002) (Ritte et al., 2002).

IV. Formulation of biocontrol agents.

Two strategies of formulation were tested using strains *P. agglomerans* IC1270 as model organism. Uniform freeze-dried alginate-based beads were developed as possible carriers for the immobilization of the strain. Bacterial survival was examined immediately after lyophilization and under three different storage conditions (at 4°C, –18°C and ambient temperature). The beads differed in their bacterial counts, glycerin and colloidal chitin contents. The higher the initial bacterial content immobilized within the beads, the better the survival detected after lyophilization. Beads including 30% glycerin were more effective in preserving bacterial potency. When 10^9 cells were immobilized in 30% glycerin-1% chitin alginate freeze-dried beads no bacterial death during freeze-drying occurred. Long-term survival of strain IC1270 depended on storage temperature and chitin inclusion. The highest death rate was detected at ambient temperature, in contrast to more stable preparations stored at 4°C and –18°C. Changes in carrier composition and storage had a major influence on *P. agglomerans* efficacy as a biocontrol agent. It was shown that the alginate beads-mediated chitinolytic strain IC1270 may serve as a promising biocontrol agent of soil-borne phytopathogenic fungi, especially in case when the beads contained also colloidal chitin as compound necessary to induce the bacterium chtinolytic activity. Beads with chitin showed highest biocontrol activity against *R. solani*. Uniform freeze-dried alginate-based beads were developed as possible carriers for the immobilization of the strain. Bacterial survival was examined immediately after lyophilization and under three different storage conditions (at 4°C, –18°C and ambient temperature). The beads differed in their bacterial counts, glycerin and colloidal chitin contents. The higher the initial bacterial content immobilized within the beads, the better the survival detected after lyophilization. Beads including 30% glycerin were more effective in preserving bacterial potency. When 10^9 cells were immobilized in 30% glycerin-1% chitin alginate freeze-dried beads no bacterial death during freeze-drying occurred. Long-term survival of strain IC1270 depended on storage temperature and chitin inclusion. The highest death rate was detected at ambient temperature, in contrast to more stable preparations stored at 4°C and –18°C. Changes in carrier composition and storage had a major influence on *E. agglomerans* efficacy as a biocontrol agent. It was shown that the alginate beads-mediated chitinolytic strain IC1270 may serve as a promising biocontrol agent of soil-borne phytopathogenic fungi, especially in case when the beads contained also colloidal chitin as compound necessary to induce the bacterium chtinolytic activity. Beads with chitin showed highest biocontrol activity against *R. solani* (Fig. 4).

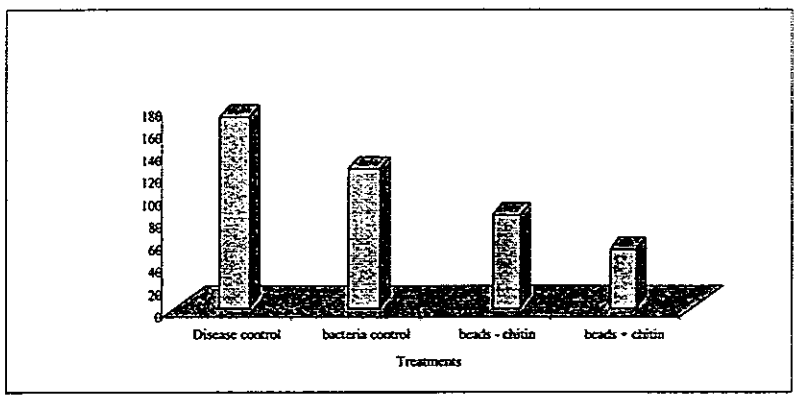

Fig. 4. Effect of soil inoculation by non-formulated and formulated into alginate beads strain IC1270 on protection of beads against *R. solani.*

The data of two independent experiments with 4-6 repetitions in each variant are averaged. The results of this experiments were published (Zohar-Perez et al., 2002).

V. Study of approaches for enhancement of the strains biocontrol activity.

Production of the exoenzymes and secondary metabolites in some strains of *Pseudomonas* is known to be controlled by a response regulator protein (GacA), an environmental sensor protein (ApdA), and by the sigma factors of transcription σ^{38} (RpoS) and σ^{70} (RpoD). Manipulation of the regulatory systems responsible for the production of various antifungal substances could significantly improve the bacteria's antagonism toward plant pathogens. This novel strategy is further extended in our work. We found that the antagonistic activity of strain IC1270 can be further enhanced by transferring heterologous regulatory genes of *P. fluorescens*. The three-parental mating method was used for the construction of four IC1270 derivatives, carrying hybrid plasmids pME3066, pME3424, pJEL5649 and pJEL5771, containing *gacA, rpoD, rpoS* and *apdA* genes of *P. fluorescens*, respectively. The chitinolytic activity of the proteins excreted by the IC1270 strain and the obtained derivatives was determined. The patterns of chitinolytic enzymes secreted by strains grown with colloidal chitin were determined using SDS-PAGE and the 4-methylumbelliferyl fluorescent analogues of disaccharide, trisaccharide and tetrasaccharide chitin derivatives as described earlier. The obtained results showed that transfer of each of *P. fluorescens* regulatory genes into strain IC1270 the led to enhancement of total chitinolytic activity and an increase in the intensity of both N-acetylglucosaminidase bands, of 89- and 67 kDa. However, a new 35 kDa-band representing otherwise latent chitinolytic enzyme was found only in proteins secreted by IC1270 derivatives carrying *P. fluorescens gacA* or *rpoD* genes (Fig.5). This enzyme can be classified as a chitinase with an endo-mode of chitin splitting since it hydrolyze 4-MU from 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$ but not from 4-MU-GlcNAc.

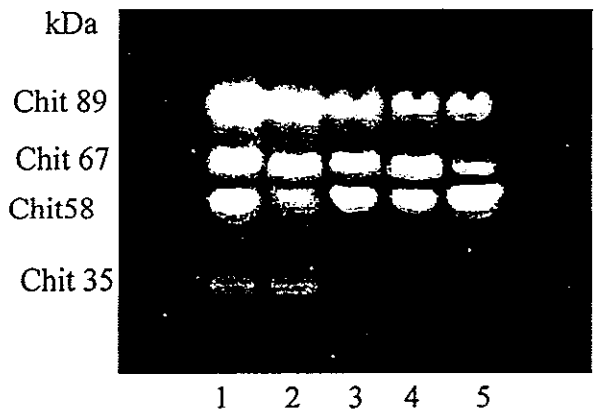

Fig. 5. Detection of chitinolytic enzymes in extracellular proteins after separation by SDS-PAGE using 4-MU-$(GlcNAc)_2$ as the substrate.
Lanes 1-4, extracellular proteins from derivatives of strain IC1270, carrying regulatory genes *gacA, rpoD, apdA,* and *rpoS* of *Pseudomonas*, respectively. Lane 5 extracellular proteins from strain IC1270. Strains were grown on a medium supplemented with chitin.

Strains IC1270/pME3066 (*gacA*) and IC1270/pME3424 (*rpoD*) suppressed the growth of *S. sclerotiorum* and *R. solani* in vitro more markedly than the parental strain (Table 8).

Table 8. *In vitro* antifungal activity of *E. agglomerans* IC1270 and its derivatives carrying different heterologous regulatory genes

Treatments	*S. s.*	*R. s.*	*P. a.*
IC1270	4.7* de	9.3 de	6.3 bc
IC1270/pME3066 (*gacA*)	14.3 ab	11.3 c	6.0 bc
IC1270/pME3424 (*rpoD*)	13.0 b	12.7 bc	2.7 cd

*, Distances (mm) between edges of the fungus and bacterium zone of growth. The data were treated statistically using Statistics Version 3.1 NH Analytic Software. Values in each column were average of three replications. Means within each column followed with the same letter are not differed from each other significantly at P=0.05 level. *S.s., Sclerotinia sclerotiorum, R. s., Rhizoctonia solani, P. a., Pythium aphanidermatum.*

Additionally, we compared the efficiency of strain IC1270 and its derivatives carrying the regulatory genes of *Pseudomonas* to control *S. sclerotiorum* in cruciferous crop *Brassica napus* L (rapeseed) under greenhouse conditions. Application of strain IC1270 via leaf spray was found efficient for reduction of the disease severity (length of the lesion area) (Table 9) and for decrease of the incidence of the disease as well (data not shown). The two IC1270 derivatives carrying *rpoD* or *gacA* were found more proficient in control of the disease than the parental strain.

Table 9. Suppression of rapeseed sclerotinia stem rot

Strain	Lesion length (mm)	
	Average	Inhibition rate (%)
IC1270	22.5±4.4 b	18.5
IC1270/pME3066(*gacA*)	16.2±2.9 c	41.3
IC1270/pME3424(*rpoD*)	17.2±5.5bc	37.7
Disease control	27.6±3.3 a	0

The data were obtained in two independent experiments and treated statistically as six replication at random complete block design using the Statistics Version 3.1, NH Analytic Software.

The results of this part of the project were presented on the 3rd International Symposium on Chitin Enzymology, Senigallia (Ancona), Italy, 6 - 10 May 2001 and the 1st Eurasian Congress on Molecular Biotechnology (ECOMB-2001), October, 2001, Trabzon, Turkey (Zhou et al., 2001a, b) and summarized in a review (Chernin and Chet, 2002).

Impact, relevance and Technology Transfer.

During the project execution the Co-PI from Uzbekistan and his collaborators visited the Israeli partners lab totally for about one year for training and collaboration. During these visits Uzbek partners learned the protocols of laboratory and greenhouse experiments previously developed in HUJ. In addition Dr. Ismailov successfully used the case of his work in our lab in Israel to learn the Internet facilities to get most recent information about current progress in biological control practice and teaching. He also got a free access to all journals and books in the Faculty library. Besides of that the Uzbek partners purchased in Israel several small dispensable equipment, chemicals and other materials (including strains of microorganisms and seeds of the tested plants) and also several most recently edited scientific books and laboratory manuals which will help very much for training his colleagues in Samarkand and for his teaching activity in Samarkand State University as well. The materials, equipment and scientific books purchased from the project funds and delivered to Samarkand, are now in use in the laboratory "Biomethods" and the Department of Genetic and Biotechnology headed by Prof. Ismailov. This further contributes to teaching, research and practical application of biocontrol methods in Uzbekistan. Profs. I. Chet and L. Chernin consulted Drs. Ismailov and Kabulova about technical details of the trials performing and discussed protocols of the experiments. Additionally the Senior Researcher Dr. F. Kabulova visited HUJI for 10 days in December 2001 to continue laboratory and greenhouse trials. During this period Dr. Ismailov and his team acquired all necessary technical facilities and help for performing described molecular biology and genetic engineering experiments. Profs. I. Chet and L. Chernin consulted the Uzbek partners about technical details of the performing trials and discussed protocols of the experiments. The results of both groups obtained during this period started to be implemented in Uzbek partners laboratory in the Samarkand State Univ. with goal to study the available biocontrol agents mechanisms of antifungal activity. The materials, equipment (laboratory pipette-aid, plastic tubes, software, etc.), scientific books and manuals purchased by funds provided by the project and delivered to Samarkand, are available for use to all members of FAN and the Samarkand State University' laboratory headed by the Co-PI.

In September 1999 the PI from Israel Profs. I. Chet and L. Chernin visited Prof. Z. Ismailov, group in Samarkand for one week. The partners discussed the results obtained during the first 1.5 years of the project execution and details of the protocols of several laboratory, greenhouse and field experiments that were planning to be performed during this year. They also discussed details of the project management. This further contributed to teaching, research and practical application of biocontrol methods in Uzbekistan. At the second time Prof. Chernin visited Samarkand in 2001 being engaged in organization of the first Israel-Uzbekistan conference on agriculture biotechnology "Achievements of Biotechnology for the future of mankind" (Samarkand, 11-18 June, 2001, see below).

The results of both groups obtained during the project execution already started to be implemented in Uzbekistan and Israel. In particularity crop growers from vegetable-gourds firms ("Mehrobod" and "Dustlik"), manufacture of a grain of the wheat ("Kukbulak"), and also "Elita-Pharm" firm (Samarkand) which specialize on manufacture of the broad of cultural and herbs have been interested in attained results. Furthermore plant-breeders from Galla-aral Station of the Andijan Scientific Research Institut of the Grain and Bagizagan Scientific Experimental Center of the Vegetable-gourds Crops expressed great interest in collaborative testing of the proposed biocontrol agents. The micro-plot field trials will be performed in 2003-2005 seasons.

Project Activities/Outputs. In frame of the project execution the collaborators engaged in organization of the first Israel-Uzbekistan conference "Achievements of Biotechnology for the future of mankind" which was held in Samarkand, 11-18 June 2001. The main goals of the conference were: exchange of scientific information with Israeli colleagues in the field of biotechnology; development of joint studies between Uzbekistan and Israel in the fields of agriculture, food production and environmental quality sciences; help in introducing developed Western biotechnology into an agricultural complex into an Central Asia; enhancement of the level of education in current biotechnology (see APPENDIX).

Project productivity. The current project was usefully finished according to the originally proposed goals, objectives and plans.

Future work. As a result of the collaboration in frame of the present project, several new strains of bacteria with a broad spectrum of antagonistic activity were isolated. The potential of these four strains as biocontrol agents for the control of plant pathogenic fungi has been demonstrated in greenhouse and micro-plot field experiments performed in Israel and Uzbekistan. However, as with most other biocontrol agents, they sometimes function inconsistently, which is why we consider them logical candidates for genetic improvement via the use of recombinant DNA techniques that offer a technologically sound and efficient means of enhancing their biocontrol activity. Biocontrol PGPB may be improved by genetically engineering them to overexpress one or more of these traits so that the strains with several different anti-phytopathogen traits, which act synergistically, are created. Genetic improvement is of vital importance for the enhancement of the biological control capability of BCAs, and even for upgrading their adaptability to different stresses. Until now, genetically modified (GM) inoculants have been tested under laboratory conditions, but their commercial exploitation has lagged.

This year we have applied to USAID-CDR a new proposal entitled "Genetic improvement of bacterial biocontrol agents of plant pathogenic fungi" (Proposal Identification Number: CA23-036; collaborators – L. Chernin (HUJI), I. Chet (the Weizmann Institute of Sciences), Z. Ismailov (SamSU) and L. Thomashow (USDA-ARS Root Disease and Biological Control Research Unit, Agricultural Research Service, WSU, Pullman, Washington). The potential scientific benefits of the proposed research lie in the significant advancements to the exploitation of already well-characterized biocontrol genes to enhance the performance of microbial inoculants with known potential to increase agricultural productivity while reducing agrochemical usage. The proposed research will result in the construction of new, more effective inoculants that can be evaluated for consistent performance as well as potential long-term risks under contained conditions and in the field, providing a means to address important concerns about the impact of GM microorganisms on the environment. The goals outlined in the statement of research objectives will be achieved by employing combinations of strategies and biological resources already available in the laboratories of the co-PIs, thereby assuring that the objectives will be accomplished successfully and in a timely manner. The proposed development of products for the biological control of crop diseases that are ubiquitous in Uzbekistan and other CAR has important scientific and practical value. The program will include the construction of several PGPB that have been genetically modified to enhance their activity primarily towards economically important fungal pathogens of vegetables and cereals in Uzbekistan and worldwide. It worth mention that Prof. Z. Ismailov during his last (in years 2001-2002) about 6 month training in the Israeli partners laboratory in Rehovot learned and practically used modern

techniques of DNA manipulations and performed several molecular cloning and gene-directed mutagenesis experiments. This training will be used as a very good basis for successful execution of the new proposed USAID-CDR project (in case it will be approved). Further studies are needed also in order to rationally integrate the original and genetically improved bacterial antagonists to a sustainable plant pathogens management program in Uzbekistan and Israel.

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Appendix

Selected pages from the publications supported under grant No TA-MOU—96-CA16-02

Samarkand State University

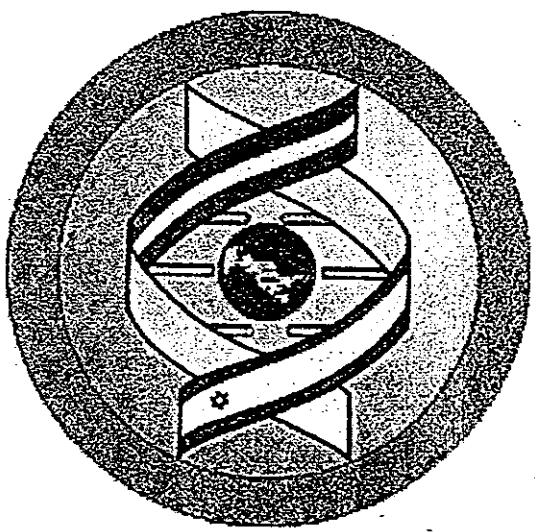
Uzbekistan

Hebrew University of Jerusalem

Israel

PROCEEDINGS OF THE INTERNATIONAL CONFERENCE «ACHIEVEMENTS OF BIOTECHNOLOGY FOR THE FUTURE OF MANKIND»

June 11 - 14, 2001



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1.1. МИКРОБИОТЕХНОЛОГИЯ

GENETIC MANIPULATIONS OF BACTERIAL ENZYMES AND ANTIBIOTICS TO ENHANCE BIOCONTROL OF PHYTOPATHOGENS

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A beneficial organism used to protect plants against pathogens is referred to as a biological control agent (BCA) or, often, as an antagonist, because it interferes with, the target organisms which damage the plant. Some BCAs have been genetically modified to enhance their biocontrol capabilities or other desirable characteristics. Many agrochemical and biotechnological companies throughout the world are increasing their interest and investment in the biological control of plant diseases and pests. For plant pathogens alone, the current list of microbial antagonists available for use in commercial disease biocontrol includes around 40 preparations.

Plantgrowth promoting bacteria (PGPB) control the damage to plants from bacterial and fungal pathogens by a number of different mechanisms including production and secretion of iron-chelators siderophores antibiotics, and cell-lytic enzymes (chitinases, glucanases, cellulases and proreases), the production of substances which promote plant growth and stimulation of the systemic resistance of the plant. Additionally, successful colonization of the root surface is considered a key property of prospective antagonists. The most effective BCAs use two or three different mechanisms. Antagonists also can be combined to provide multiple mechanisms of action against one or more pathogens. An understanding of this me-chanism of action is important because it provi-des a wealth of information that can be useful in determining how to maintain, enhance, and implement this form of biological control.

Chitinase activity has been found in a wide variety of bacteria. Bacteria produce chitinase to digest chitin, primarily to utilize it as a C and energy source. The ability to produce lytic enzymes is a widely-distributed property of soil, marine and rhizosphere bacteria. Many of these are potential biocontrol agents of chitin-containing plant pathogens. Considerable interest has been focused on the role and production of cell-wall-degrading enzymes in bacteria and the ability of chitinolytic bacteria to protect plants against diseases and pests. Antifungal properties of chitinolytic soil bacteria may enable them to compete successfully with fungi for chitin.

A soilborne chitinolytic *E. agglomerans* strain IC1270 isolated in Uzbekistan from rhizosphere of grape was found to be a strong antagonist of about 30 species of plant-pathogenic bacteria and fungi in vitro and an efficient biocontrol agent of several diseases caused by soilborne fungal pathogens (Chernin et al., 1995). The strain produced and excreted a set of chitinolytic enzymes consisting of two N-acetyl-...-D-glucosaminidases with apparent molecular masses of 89 and 67 kDa, and a 58-kDa endochitinase. The chitinolytic activity was induced when the strains were grown in the presence of colloidal chitin as the sole C source; the observed chitinolytic enzymes seemed to be the most abundant proteins secreted by the bacteria under this condition. In addition to its chitinolytic activity, the strain IC1270 produces an antibiotic pyrrolnitrin {3-chloro-4-(2'-nitro-3'-chlorophenyl)pyrrole} with a wide range of activity against many phytopathogenic bacteria and fungi in vitro (Chernin et al., 1996). The ability of *E. agglomerans* IC1270 to produce pyrrolnitrin in combination with chitinases would be advantageous in attacking fungal phytopathogens. The *chiA* gene of the 58-kDa endochitinase (Chi A_Entag) was cloned from strain IC1270 in *E. coli* and the ChiA_Entag-producing *E. coli* strain decreased the disease incidence of root rot caused by *R. solani* on cotton under greenhouse conditions (Chernin et al., 1997).

Biocontrol PGPB may be improved by genetically engineering them to over express one or more of these traits so that the strains with several different anti-phytopathogen traits which act synergistically are

created. Genetic improvement is of vital importance for the enhancement of the biological control capability of BCAs, and even for upgrading their adaptability to different stresses. At present, a widely-used approach is to genetically improve useful antagonists by introducing foreign target genes, e.g. genes with biocontrol potential such as chitinase gene, b-1,3-glucanase gene and even both genes together.

In many Gram-negative bacteria, including plant-growth-promoting pseudo-monads, three types of control elements are involved in the production of some secondary metabolites and enzymes which are synthesized at the end of exponential growth or during the stationary phase and are involved in biological control. These are: i) two-component global regulatory systems which mediate transduction of environmental signals into the cells; ii) sigma-factor-mediated transcription by RNA-polymerase; and iii) a diffusible N-acyl-homoserine lactone (N-acyl-HSL) quorum sensing-signals. The cur-rent knowledge of regulatory mechanisms of various antifungal substances expression may help in the construction of strains with enhanced biocontrol activity. Manipulation of regulatory systems responsible for the production of lytic enzyme and antibiotics resulted in significantly improvement of the bacteria biocontrol potential. The advantages of this approach were demonstrated by increasing of the doses of genes encoding the GacA-GacS system of global regulation or sigma factors of transcription in some biocontrol strains of *P. fluorescens* and other Gram-negative bacteria (Ligon et al., 2000).

In our work the strain IC1270 was chosen as the model organism in aim to exploit heterologous regulatory genes and exogenous quorumsensing signals for further enhancement of the bacteria biocontrol capacity. We have shown that transferring genes of global regulation system or transcription sigma factors from P. fluorescens or treating with exogenous AHL signal molecules, can increase strain IC1270"s biocontrol potency against some fungi diseases under greenhouse conditions (Zhou et al., 2001).

Future research should focus on the development of strains expressing "multigene" combinations while preserving the intrinsic vigor and ecological competence of microbial antagonists. A definition of environmental signals which interact with the microbial recognition and regulatory complexes, such as two-component global regulation and quorum-sensing systems, will help elucidate the competitive mechanisms in pathogen suppression to fully benefit from soil and rhizosphere biocontrol microorganisms. Further greenhouse and field experiments should be conducted to investigate these microorganisms' biological control potential. Once transgenic strains capable of producing highly efficient synergistic combinations of enzymes are obtained, a higher level of plant-pathogen and pest control should become possible. Transgenic BCAs therefore offer the potential of substantially reducing the amount of chemical fungicides required to produce crops protected from diseases and pests. A combination of transgenic BCAs and transgenic plants resistant to pathogens and pests would appear to yield a very environmental friendly and efficient strategy of plant protection as we approach the third millenium – where chemistry will meet ecology.

ACKNOWLEDGMENTS

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Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases

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Abstract

Plant-associated strain IC14 of the Gram-negative bacterium *Serratia plymuthica* isolated from soil around melon roots was shown to suppress a wide range of phytopathogenic fungi in vitro. Foliar application of strain IC14 protected cucumber against *Botrytis cinerea* gray mold and *Sclerotinia sclerotiorum* white mold diseases of leaves under greenhouse conditions, reducing disease incidence by 76 and 84%, respectively. The strain possessed chitinolytic and proteolytic activities, produced the antibiotic pyrrolnitrin [3-chloro-4-(2′-nitro-3′-chlorophenyl)pyrrole] and siderophores, and secreted the plant growth hormone indole-3-acetic acid. An endochitinase with an apparent molecular mass of 58 kDa, was estimated to be the main secreted chitinolytic enzyme. Two mutants, one with increased chitinolytic activity and the second deficient in chitinolytic activity, were obtained by miniTn5-insertion mutagenesis. Neither mutant differed appreciably from the parental strain in the production of other antifungal compounds or in suppression of *B. cinerea* and *S. sclerotiorum* on plates or in the greenhouse, suggesting that chitinolytic activity is less essential for biocontrol of these pathogens by strain IC14. The obtained results present novel information concerning the potential of the soil-borne *S. plymuthica* strains as biocontrol agents of foliar diseases caused by plant pathogenic fungi.

Keywords: Biocontrol; Bacterial antagonists; Chitinases; Pyrrolnitrin; Siderophores; Indole-3-acetic acid

1. Introduction

The necrotrophic fungi *Botrytis cinerea* Pers; Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary are among the world's most dangerous fungal plant pathogens due to their effects on flowers, leaves, fruits or stems under high humidity or when free moisture is present on the plant surface (Zhou and Boland, 1998). Biocontrol is an environmentally friendly and efficient alternative to chemical fungicide management of these pathogens (Köhl and Fokkema, 1998). However, of the approximately 80 commercial biocontrol agents (BCAs) available today for use against crop diseases (Paulitz and Belanger, 2001), only a few, based on the fungal antagonists *Trichoderma harzianum* (Elad, 2000) and *Coniothyrium minitans* (Whipps and Davies, 2000), are specifically recommended for use against *S. sclerotiorum* and/or *B. cinerea*.

Bacteria able to reduce plant diseases caused by these fungi have also been described. Thus, *Pseudomonas fluorescens* and *P. putida* strains show good control of sunflower sclerotial disease caused by *S. sclerotiorum* (Expert and Digat, 1995). Two *P. cepacia* strains applied as a seed treatment caused increased sunflower emergence in the field in the presence of *S. sclerotiorum* (McLoughlin et al., 1992). *Burkholderia* (previously *Pseudomonas*) *cepacia* and *Bacillus subtilis* formulations have been registered in Taiwan for biocontrol of *Sclerotinia* stem rot of rapeseed (Gu, 1996). Strains of *Erwinia herbicola* and *B. polymixa* have been evaluated for the suppression of *S. sclerotiorum* white mold disease in the phyllosphere (Yuen et al., 1994). Bacterial strains with the potential to serve as BCAs against *B. cinerea* have also been described (Dik et al., 1999; Walker et al., 2001).

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suggesting the predominant role of either one or all of these compounds in the strain IC14's biocontrol activity.

5. Conclusion

Overall, the soil-borne strain IC14 of *S. plymuthica* was found to be a proficient BCA of the foliar pathogens *B. cinerea* and *S. sclerotiorum*. The antibiotic pyrrolnitrin, siderophores and/or proteases produced by this bacterium are suggested to be key factors in the strain's ability to suppress these fungal pathogens, while the role of chitinolytic enzymes appears to be less essential. However, the chitinolytic activity could be more important for the biocontrol of soil-borne diseases. The ability to produce the plant-growth-promoting IAA and lack of its hemolytic activity at human body temperature could be considered additional advantages for this bacterium's further development as a BCA of a wide range of crop diseases.

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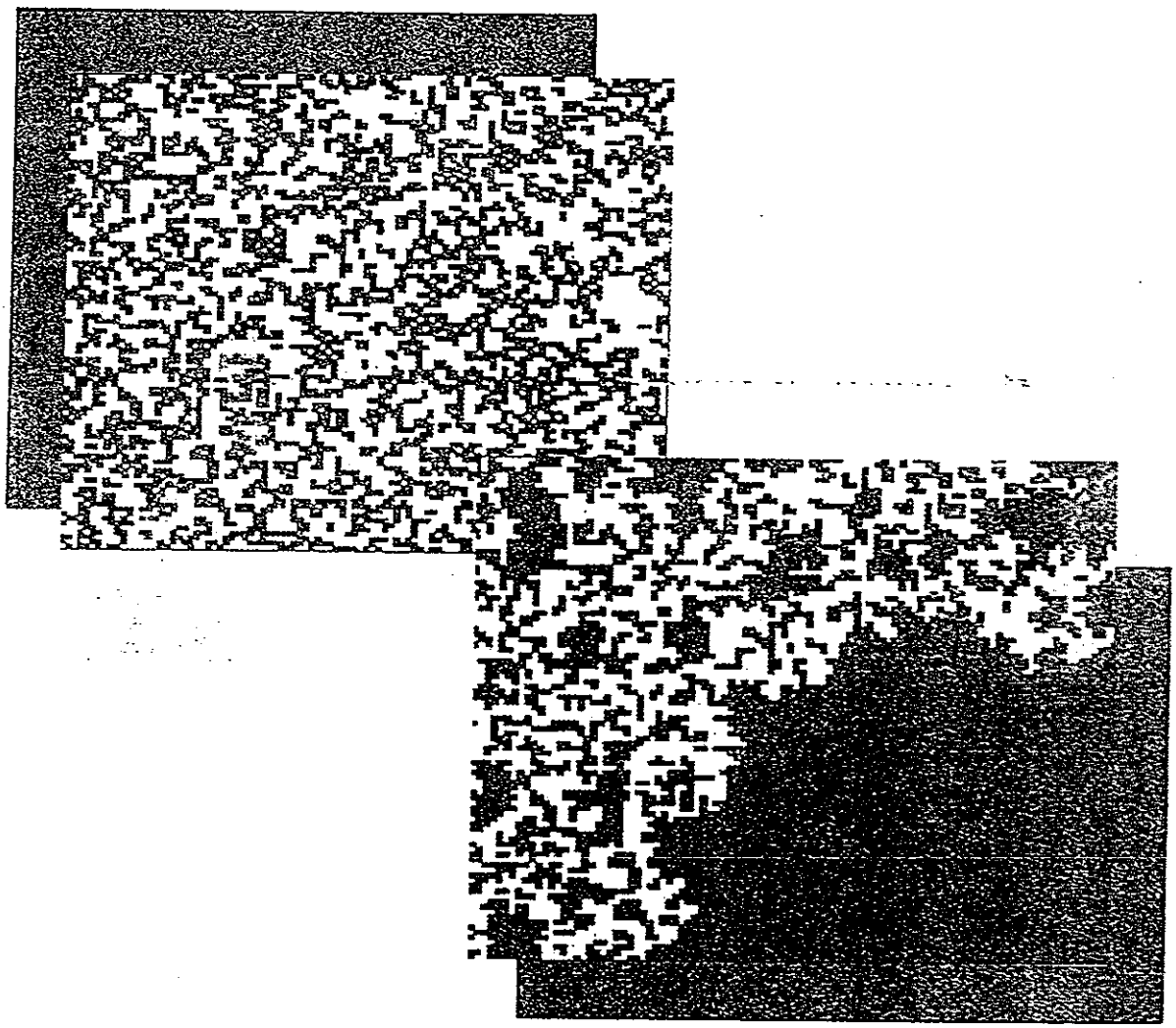

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DEVELOPMENT OF NEW APPROACHES FOR THE BIOLOGICAL CONTROL OF CROP DISEASES: A JOINT ISRAEL – CIS VENTURE

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Biological control of plant pathogens based on natural interactions between organisms offers a potential means of overcoming the ecological problems induced by chemical pesticides. Bacterial strains of *Enterobacter* and *Pseudomonas* spp. isolated in various regions of the former USSR and characterized by a broad range of antagonistic activity against microbial plant pathogens were used as the basis for biocontrol product elaboration. Several protocols for the antagonistic bacterias' formulation and application were developed. Under greenhouse and field conditions, the bacterial isolates revealed proficient biocontrol activity, dependent upon the crop and disease specificity. Application of two strains of *Pseudomonas* to a nonflowing hydroponics system significantly decreased the incidence of fusariosis in mother carnation plants. Some of the bacterial antagonists were found to be resistant to chemical fungicides commonly employed for the control of fungal diseases, showing that the bacteria can be used in combination with substantially lower doses of these fungicides as part of an integrated pest management approach. The advantage of the bacteria used in this work lies in their multiple antagonistic activities, enabling them to protect plants against a number of pathogens simultaneously.

Key words: Biological Control, Plant Disease, Fungal Phytopathogen, Antagonistic Bacteria.

INTRODUCTION

Plant diseases affect agricultural production with losses amounting to 13 to 20% annually, resulting in significant economic losses worldwide. Intensive usage of chemical pesticides for the protection of agronomically important plants against pests and diseases caused by phytopathogenic bacteria and fungi has led to significant pollution and instances of ecological disaster. In addition, excessive application of pesticides has led to an increased proportion of pathogens, which are resistant to the chemicals. Thus, each year, fungal diseases cause millions of dollars worth of crop damage, despite the extensive use of pesticides. Agricultural biotechnology offers a new approach to the problem: the development of alternative, efficient and ecologically safer biological methods of plant disease control [1-4]. One of the most promising options in this field is the use of microorganisms capable of suppressing pathogens in order to reduce disease incidence. There is much evidence of the biocontrol activity of different microorganisms in the laboratory, in greenhouses and under limited field conditions [5-10]. Research on microorganisms as antagonists in the biological control of plant pathogens has markedly increased in the last few years as an alternative to pesticides. About 20 different genera of bacteria and fungi have been shown to be potential biocontrol agents for numerous plant diseases [10-12]. The mechanisms of action of these organisms as biocontrol agents include: a plant response to challenge by pathogens, known as induced resistance; competition for active sites; production of antibiotics, siderophores, cell-wall lytic enzymes (e.g. chitinase and β-glucanase); and mycoparasitism, among others [2, 6, 8, 13-15]. Successful colonization of the root surface is considered to be a key property of the prospective antagonist [16]. Although many agrochemical and biotechnological companies throughout the world are now investing time and money in the biological control of plant diseases, there are still a very limited number of products based on bacterial or fungal strains with biocontrol activity against microbial phytopathogens, and the existing products have a low market share [10, 11, 17, 18]. Therefore, the creation of products focused on the biocontrol of plant diseases – being of both scientific and practical value – remains a vital research focus in agricultural biotechnology.

In our previous research, several strains of bacteria belonging to the genera *Bacillus*,

3. Protection of plants growing in a hydroponics system

The growth of mother carnation plants in a hydroponics system (a water-aeration method of carnation production in nonflowing cultivation chambers) for the production of rooted carnation grafts as elite planting material very often leads to mass-scale infection of the cultivated mother (M1) plants (the grafts rooted in perlite and then transferred to the hydroponics system) by F. oxysporum. Plant loss to these diseases starts from the first month of growth, reaches 30 to 40% after 3 to 4 months and increases by 5 to 10% each subsequent year. In the tested plants, latent infection with Fusarium reached 70%, making these mother plants unfit for the production of healthy plant material. In the case of plants obtained from meristem (Mo), loss was ~ 25 to 30% at the beginning of the third month of cultivation in the hydroponics system, with an additional 30% every month thereafter. Application of B-4117 or CR330D strains in the hydroponics system drastically decreased plant damage (Table 4). Optimal prevention of plant loss and latent infection was obtained when 1 l of the bacterial suspension (10^8 – 10^9 cells/ml) was added to 1 m^3 of hydroponics solution once every 1 to 2 months for 3 months of growth. By the end of the first month the applied antagonist made up ~18 to 48% of the total bacterial population in the hydroponics solution. However, after 2 to 3 months, the amount of antagonists significantly decreased. In the plant root area, the applied bacteria could be found for at least 3 months, making up ~ 50 to 60% of the total microflora in the rhizosphere. The antagonists were thus able to colonize the carnation roots and this probably was one of the most important reasons for the bacteria's ability to protect plants against fungal infection in this system. This suggestion correlates with results presented in Table 4 showing that the effect of the strains was higher when the meristemic (Mo) plants has been grown under sterile conditions (in tubes). In this case the monthly loss of plants reached only 0.1 to 0.3% during 12 to 14 months of cultivation. In contrast, M1 plants grown in perlite before their placement in the hydroponics system were probably already infected by the pathogen at the grafting/ rooting stage, and the effect of the bacteria was therefore not so pronounced. In both cases, these data show that applying the bacteria to a nonflowing hydroponics system leads to an at least fivefold decrease of mother carnation plants afflicted with fusariosis and offers the possibility of their prolonged cultivation under non-sterile hydroponics conditions.

Table 4. Effect of bacteria on loss of carnation plants due to fusariosis in a hydroponics system

Variant	Type of plants	Number of plants in the system	Number of plants lost during a 2-month period (%)
Control I	Mo	7,500	2,250 (30)
B-4117	Mo	11,160	22 (0.2)
CR3300D	Mo	8,280	8 (0.1)
Control II	M1	10,600	2,332 (22)
B-4117	M1	12,600	517 (4.1)
CR330D	M1	14,440	462 (3.2)

Bacteria were applied to the hydroponics system once every 2 months (1 l of suspension, $5x10^8$ cells/ml per 1 m^3 of hydroponics solution); Mo, plants obtained from meristem; M1, mother plants – the graft rooted in perlite and then transferred to the hydroponics system.

CONCLUSIONS

Results from several groups in Israel and the CIS, some obtained within the framework of this collaborative research, demonstrate the high potential of several bacterial antagonists to serve as wide-range biocontrol agents in a number of economically important crops and ornamentals. Further studies are needed to integrate these bacterial antagonists into a sustainable plant-pathogen management program. The refined products should be feasible to produce and suitable for low-input and sustainable farming, benefiting farmers in the partner countries and worldwide.

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Chitinolytic activity of *Enterobacter agglomerans* enhanced by *Pseudomonas*' regulatory genes or exogenous quorum-sensing signals

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Introduction

In *Pseudomonas fluorescens* and some other gram-negative bacteria production of the exoenzymes and secondary metabolites was shown to be controlled by a two-component system of global regulation consisting of a cytoplasmic response regulatory protein GacA and a cognate transmembrane sensor kinase (ApdA or GacS); by relative changes in the concentrations of the transcription sigma factors RpoS (σ^{38}) and RpoD (σ^{70}), and/or by AHL molecules, regulating a cell density-dependent phenomenon known as quorum sensing (1, 2). In *Chromobacterium violaceum* AHLs have been found to control the production of chitinolytic enzymes (3). In general, the presence of complex global cell-regulation mechanisms is considered to be one of the reasons for the exceptional environmental and evolutionary success of microbes. Manipulation of the regulatory systems responsible for the production of various antifungal substances could significantly improve the bacteria's antagonism toward plant pathogens (4). This novel strategy is further extended in the present work. We show here that the chitinolytic activity of *Enterobacter agglomerans* strain IC1270, a broad-spectrum antagonist of plant pathogenic fungi and bacteria producing two N-acetyl-β-D-

despite strain IC1270's inability to produce AHL molecules, its regulatory systems may interact with exogenous quorum-sensing signaling to modulate the expression of genes responsible for chitinolytic enzymes production.

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ARTICLES

Preservation of Chitinolytic *Pantoae agglomerans* in a Viable Form by Cellular Dried Alginate-Based Carriers

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Improved viability of Gram-negative bacteria during freeze-dehydration, storage, and soil inoculation is of crucial importance to their efficient application. The chitinolytic *Pantoae* (*Enterobacter*) *agglomerans* strain IC1270, a potential biocontrol agent of soil-borne plant-pathogenic fungi, was used as a model organism to study the efficacy of freeze-dried alginate-based beads (macrocapsules) as possible carriers for immobilized Gram-negative bacterial cells. These macrocapsules were produced by freeze-dehydration of alginate gel spherical beads, in which different amounts of bacteria, glycerol, and colloidal chitin were entrapped. Subsequent drying produced different unexpected structures, pore-size distributions, and changes in the outer and inner appearance of the resultant dried cellular solid. With increasing glycerol content, the proportion of larger pores increased. These structures can be related to changes in the slow-release properties of the dried beads. The amount of glycerol in the beads differed from that in the alginate solution as a result of leakage during the beads' preparation and dehydration. Entrapping 10^9 cells per bead produced from alginate solution containing 30% glycerol and 1% chitin resulted in improved (in comparison to other studies) survival prospects (95%) during freeze-drying. Moreover, immobilization of the bacterium sharply improved its survival in nonsterile irrigated and dry soils compared to bacteria in a water suspension. The results suggest that optimized conservation of Gram-negative bacteria in dry glycerol-containing alginate-based cellular solids is not only possible but applicable for a variety of uses.

Introduction

Recent advances in biotechnology have led to the isolation of many microorganisms (many Gram-negative bacteria among them) with a wide range of potential applications, e.g., to improve crop growth and protect plants in agriculture. However, success of a microorganism in vitro does not guarantee success in field applications (*1*, *2*). Cell immobilization technology provides a number of advantages over free cell inoculation. The proper formulation should provide desirable characteristics for the inoculant, such as long shelf life and appropriate survival at its destination, as well as sufficient cell density and performance (*1*, *3*). For such preparations, water-soluble polymeric materials (gums) such as agar, λ- and κ-carrageenan, alginate, low-methoxy pectin (LMP), gellan, chitosan, and blends of xanthan and locust bean gum (LBG), among many others, are commonly used (*4*). All of these materials have been used to produce natural-based carriers for the encapsulation of microbial and fungal cells in the food, biotechnology, and agriculture industries (*3*–*5*).

By entrapping the living cells, the carriers protect the microorganism against various stresses. It is desirable that, for their conservation, such inoculated carriers can be dried. Less information can be found on using dried (instead of "wet") beads for adding encapsulated cells to soils or any other purpose.

Dehydration is performed by spray-, freeze-, and fluidized bed-drying. The main problem with these techniques, however, lies in the survival prospects of microorganisms during the dehydration process and storage, particularly with the Gram-negative bacteria that are non-spore-formers (*6*). After matrix dehydration, water availability within the polymer-entrapped-cell preparation decreases until the cells reach a dormant state during which metabolism slows, sometimes to a complete standstill. Final cell survival depends on many parameters, such as the organism being immobilized, the composition of the suspension medium, and the method and conditions of drying. For example, freeze-drying of a Gram-positive lactic acid bacteria starter suspension reduces the population by 90% (*7*).

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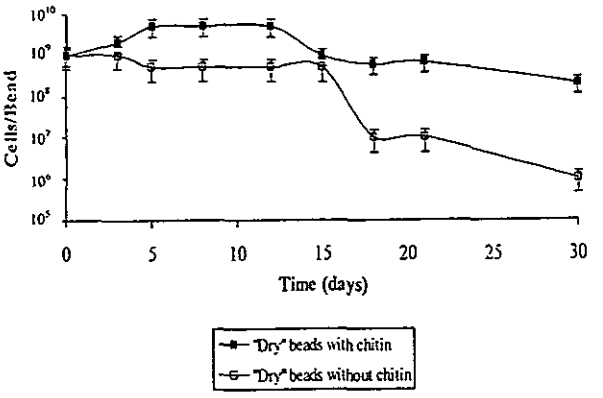

Figure 7. Survival of strain IC1270 immobilized in freeze-dried alginate beads in irrigated soil. Data represent the mean of three independent experiments.

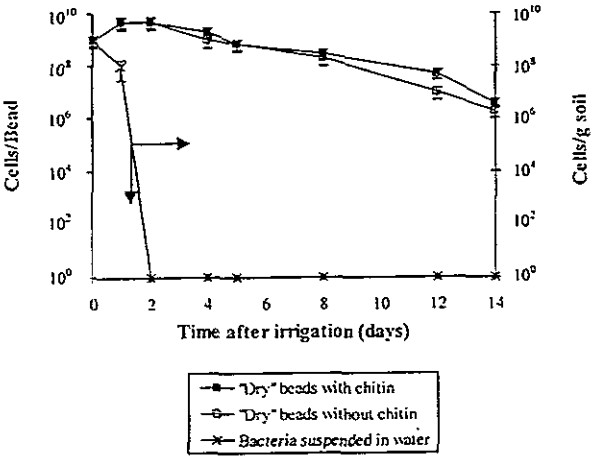

Figure 8. Survival of strain IC1270 immobilized in freeze-dried alginate beads or in a water suspension in dry soil. Data represent the mean of three independent experiments.

formulation. Therefore, bacteria immobilized in dry alginate carriers can be used efficiently when inoculating plants irrigated daily, such as greenhouse vegetation, or irrigated rarely, such as industrial and field crops; the bacteria will remain viable in the dry soil during the intervals between irrigations and will proliferate rapidly when water is available, as previously discussed.

Conclusions

The advantages of the described immobilized bacteria over free cells are almost complete survival during freeze-drying, a long shelf life at low and freezing temperatures, and improved survival in nonsterile, dry, or moist soil. Moreover, immobilization of bacteria in a dry alginate carrier enables the slow release of cells, ensuring a constant supply of the bacteria over a relatively long period. The formulation composition of the gum solution and the procedure's stages and conditions can be optimized to achieve a tailor-made dry cellular carrier with the requested structure and porosity for immobilization purposes.

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ENZYMES IN THE ENVIRONMENT

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Microbial Enzymes in the Biocontrol of Plant Pathogens and Pests

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I. INTRODUCTION

Despite many achievements in modern agriculture, food crop production continues to be plagued by disease-causing pathogens and pests. In many cases, chemical pesticides effectively protect plants from these pathogens. However, public concerns about harmful effects of chemical pesticides on the environment and human health have prompted a search for safer, environmentally friendly control alternatives (1–3). One promising approach is biological control that uses microorganisms capable of attacking or suppressing pathogens and pests in order to reduce disease injury. Biological control of plant pathogens offers a potential means of overcoming ecological problems induced by pesticides. It is an ecological approach based on the natural interactions of organisms with the use of one or more biological organisms to control the pathogen. Generally, biological control uses specific microorganisms that attack or interfere with specific pathogens and pests. Because of their specificity, different microbial biocontrol agents typically are needed to control different pathogens and pests, or the same ones in different environments.

Agriculture benefits, and is dependent on, the resident communities of microorganisms for naturally occurring biological control, but additional benefits can be achieved by introducing specific ones when and where they are needed (4–9). Many agrochemical and biotechnological companies throughout the world are increasing their interest and investment in the biological control of plant diseases and pests. For plant pathogens alone, the current list of microbial antagonists available for use in commercial disease biocontrol includes around 40 preparations (9–11). These are all based on the practical application of seven species of bacteria (*Agrobacterium radiobacter*, *Bacillus subtilis*, *Burkholderia cepacia*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Streptomyces griseoviridis*, *Streptomyces lydicus*) and more than 10 species of fungi (*Ampelomyces quisqualis*, *Candida oleophila*, *Coniothyrium minitans*, *Fusarium oxysporum*, *Gliocladium virens*, *Phlebia gigantea*, *Pythium oligandrum*, *Trichoderma harzianum*, and other *Trichoderma* species). The current market for biological agents is estimated at only $500 million, which is about 1% of the world's total output for crop protection. The largest share of this market involves biopesticides marketed for insect control (mainly products based on *Bacillus thuringiensis*

41

control should become possible. Transgenic BCAs therefore offer the potential of substantially reducing the amount of chemical fungicides required to produce crops protected from diseases and pests. A combination of transgenic BCAs and transgenic plants resistant to pathogens and pests would appear to yield a very environmentally friendly and efficient strategy of plant protection as we begin the third millennium—when chemistry will meet ecology.

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42