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The Use of a Gamma-irradiated Mutants of *F. solani* f.sp. *phaseoli* with Reduced Pathogenicity for the Biological Control of *Fusarium* Root Rot of Bean (*Phaseolus vulgaris*) in Field Conditions

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ABSTRACT

***Fusarium solani* was isolated from diseased roots of common bean (*Phaseolus vulgaris*) grown in fields which were severely infected with Fusarium root rot of bean in Markazi Province of Iran. Specific PCR primer pairs were used for the identification and verification of *F. solani* (Mart.) f.sp. *phaseoli* (Burkholder) W.C. Snyder and N.H. Hans. Spore suspensions of *F. solani* f.sp. *phaseoli* were subjected to gamma irradiation at 130 Gy using Cobalt-60, and 700 mutants were generated. All 700 mutagenized colonies were tested in the greenhouse for reduced pathogenicity. One mutant (M23) showed the best expected reduction of Fusarium root rot and was chosen to be tested as a biocontrol agent in field experiments. Different concentrations of avirulent mutant (avr-M23) were used against Fusarium root rot in the infested field and their effects on the yield of bean plants were evaluated. The biocontrol effect of avr-M23 on Fusarium root rot was significantly improved with increasing its concentration from 10^3 to 10^9 conidia ml^{-1}. Best yields (no. of pods per plant and 100-seed weight) were obtained at concentrations of 10^6 and 10^9 conidia ml^{-1}. This study confirms that avr-M23 can be used as a biocontrol agent to protect bean plants from Fusarium root rot under field conditions.**

Keywords*:* Biological control, *Fusarium solani*, Gamma irradiation, Pathogenicity, *Phaseolus vulgaris*.

INTRODUCTION

Seed legumes are tremendously important crop plants, which are extensively cultivated in the Middle East, Southern Asia and all over the tropical and subtropical regions (FAO, 2009). Legumes usually have twice and sometimes even three times the protein content of cereals. Among these, the chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.) and lentil (*Lens culinaris*) supply a large quantity of the nutritional protein requirement in many developing countries. With an annual production of about 17 million tons, the bean has also become one of the most important agricultural crop species and an important protein source for human being nourishment, as well (Aziz, 2001).

Root rot of common bean is a soil borne disease that is incited by several fungal pathogens including *Fusarium* spp., *Rhizoctonia solani* Kuhn and *Pythium* spp.

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It happens in all bean growing regions of the world (Spadaro and Gullino, 2005). Root rot caused by *F. solani* (Mart.) f.sp. *phaseoli* (Burkholder) W.C. Snyder and N.H. Hans is a major concern in many bean growing regions leading to huge crop losses and has been seriously considered by plant pathologists for more than three decades (Nelson *et al*., 1981). *F. solani* f.sp. *phaseoli* is the most frequent soil-borne fungal pathogen on bean and causes high economical damages in Iran. Yield losses in severely infested areas may be as high as 85% (Ahari *et al*., 2009). The pathogen is well-known to be very common in soil, is able to survive in infested fields for very long times and is difficult to control (Burke and Hall, 1991).

Intensive use of fungicides has caused severe problems of chemical residues in the environment. Therefore, alternative control methods have been tried. Irradiation cannot eliminate pathogens entirely but it might result in cell injury, and directly damage the chromosomal DNA of living cell (Smith and Pillai, 2004). The destruction of DNA can originate mutagenesis and some genetic traits of pathogens perhaps mutate to be of higher or lower virulence (Barkai-Golan, 2001). Bank and Corrigan showed that irradiation of plant materials is able to delay spoilage by eliminating or sinking plant pathogenicity of microorganisms (Bank and Corrigan, 1995). Mess et al. (1999) showed that, gamma irradiation (at 130 Gy) mutagenesis, using 137Cs generated an *avr*-mutant of *F. oxysporum* f. sp. *lycopersici*. Mutagenized colonies were tested for loss of avirulence on tomato seedlings. One mutant showed the expected loss of avirulence but, surprisingly, also showed reduced pathogenicity toward susceptible tomato plants. In this study, the effects of different concentrations of an avirulent mutant of *F. solani* f.sp. *phaseoli* on biological control of Fusarium root rot and yield of bean plants in field conditions are investigated.

MATERIALS AND METHODS

Isolation, Identification and Purification of the Pathogen

Isolates of *Fusarium* spp. used in this study were obtained from diseased bean roots from commercial bean (Chitti cultivar) fields in the center of Iran (Markazi Province). Isolation of *Fusarium* spp. from samples was done according to isolation procedures for other soil borne pathogens (Caesar *et al.*, 1993). Identification was carried out based on the morphological and molecular characteristics. The isolates were identified morphologically and purified by single spore culture on potato dextrose agar (PDA) by standard methods (Nelson *et al*., 1983). Koch's postulates were demonstrated for the pathogens and proved as the causal agent of root rot of *P. vulgaris*. Also, a set of specific PCR primer pairs was used for the identification of forma specialis. The primer pairs Effp-1 (5-ACCCCGCCCGAGGACTCA-3) and Effp-2 (5-AGACATGAGCGATGAGAGGCA-3) were designed (Meta Bion International AG CO.) to generate a DNA product of 562 bp from the *F. solani* f. sp. *phaseoli* (Filion *et al*., 2003).

Dose Determination and Irradiation Mutagenesis

Conidia scraped from *F.* f.sp. *phaseoli* cultures grown-up for 10 days on PDA plates were counted, diluted, and then plated on separate PDA plates (150 conidia per plate). Plates containing the conidia were irradiated in a Cobalt-60 gamma resource (with dose rate of 0.3 grey/second and specific activity of 2300 curie) at doses of 0, 60, 90, 120, 150 and 180 Gy. The percentage of spore germination after 18 hours as well as the diameter of generated colonies after 10 days were scored. Following dose determination, plates containing the conidia were prepared as

described, irradiated and after 18 hours were transferred to a fresh PDA plate (one conidium per plate) and incubated at room temperature (25±2°C) for 10 days. Conidial suspensions were then prepared (10^6 conidia ml^{-1}) from them.

Selection of Avr-mutants of *F. solani* f.sp. *phaseoli* in Greenhouse

Pathogenicity of irradiated *F. solani* f.sp. *phaseoli* for the identification of avr-mutants was examined in pots (20 cm diameter) in the greenhouse. Conidial suspensions were used to inoculate seeds of bean with each mutant colony. Seed treatment was carried out as seed soaking for three hours in a spore suspension of 10^6 conidia ml^{-1} irradiated *F. solani* f.sp. *phaseoli*. Inoculated seeds were planted (at 1 cm depth) in each pot. The set up of all inoculation trials consisted of a randomized complete block design (RCBD) with six replicates randomized for each treatment and all pots were kept in the greenhouse at 25±2°C (Samavat *et al*., 2011). Root rot symptoms were assessed 30 days later. Infected and uninfected plants were employed to discriminate real avirulent mutants from false positives. Isolation and purification were done as described above. Each experiment was repeated twice. Similar methods were followed for all experiments.

Biocontrol Test

The biocontrol experiment of avirulent mutants of *F. solani* f.sp. *phaseoli* was carried out as follows: seeds were soaked for three hours in a spore suspension of 10^6 conidia/ml avirulent mutants and were transferred to pots previously infested with a wild isolate of *F. solani* f.sp. *phaseoli* (Filion *et al*., 2003). Experiments were carried out as described in the previous section. The percentage of root rot was scored after 30 days. Each experiment was repeated twice. Similar methods were followed for all experiments.

Field Experiment Set up

The avirulent mutant of *F. solani* f. sp. *phaseoli* was cultured on PDA and incubated under fluorescent light for 10 days at room temperature. Spores of the avirulent mutant were washed from the plates with sterile distilled water and the concentrations of the suspensions were adjusted to 10^3, 10^6 and 10^9 conidia ml^{-1} using a spectrophotometer (Valdez and Piccolo, 2006). Seeds of bean were immersed in the spore suspension and were sown (at 1 cm depth) in blocks (10×5 m^2) in a field with history of disease incidence at the National Bean Research Station of Khomein city, Iran, where Fusarium root rot of bean was severe and then a layer of soil (1 cm in thickness) was added on top. The entire experiment was repeated twice, and each experiment consisted of a randomized complete block design with six blocks and four avirulent mutant inoculation treatments randomized among the blocks. Field experiments were repeated twice. The treatments were: (I) avr-mutant at concentration at the concentration of 10^3 conidia ml^{-1}; (II) avr-mutant at the concentration of 10^6 conidia ml^{-1}; (III) avr-mutant at the concentration of 10^9 conidia ml^{-1}; and (IV) water as control.

Assessment of Virulence

Disease severity was evaluated 110 days after inoculation. The plants were removed from the blocks and soil adhering to roots was removed by gently shaking and by dipping the roots in water. The severity of root rot was visually scored by evaluating necrotic lesions on the roots and hypocotyls using a rating scale of 0-6 described by Anthony *et al*. (1998).

Evaluation of the Plant Yield

The performance of the plants was assessed after 110 days by means of the following variables: (a) number of pods per plant; (b) 100-seed weight (grams); and (c) plant yield (kilogram per hectare). In assessing variable "a", p/pl was counted on ten plants chosen randomly out of each row, then calculating the average per plant. Variable "b" was measured by weighing 100 dry seeds from each plant in the row. Variables b and c correspond to bean yield components (Nelson *et al*., 2009**).**

Statistical Analysis

All statistical analyses were performed using Duncan's multiple range test ($P \leq 0.05$), by MSTATC Version 1.42.

RESULTS

Pathogen Identification

Colonies grown on PDA became cream 10 days after incubation, and produced micro and macro conidia. The pathogens were identified as *F. solani*, based on the characteristics described by Nelson *et al*. (1983). Based on the Koch's postulates, *F. solani* isolates were pathogenic to all tested bean cultivars. PCR assays using oligonucleotide primer pairs Effp-1/Effp-2 for *F. solani* f. sp. *phaseoli* detected the individual estimated amplicons of 562 bp from DNA extracted from pathogen samples, confirming the presence of forma specialis (Figure 1). No amplification product was obtained from other forma specialis.

Dose Determination and Irradiation Mutagenesis

Gamma irradiation was followed to produce avirulent mutants. Spores of *F. solani* f. sp. *phaseoli* were irradiated at different doses before germination on PDA plates. The

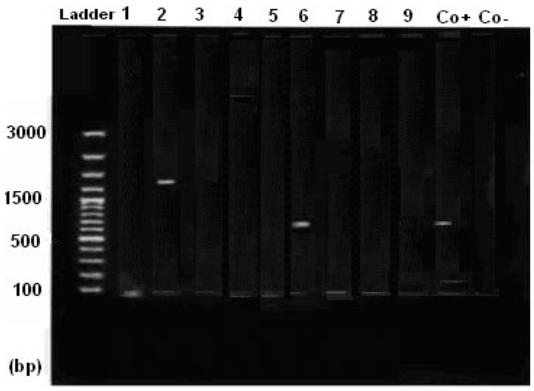

Figure 1. Electrophoresis pattern of DNA fragments amplified by RAPD-PCR with specific *Fusarium solani* primer pair. **-** numbers (1-9): Number of *Fusarium solani* isolates, Co (+,-): Positive and negative control.

comparison of the percentage of spores germination and diameter of colony in different dose rates, showed a linear relationship between survival, diameter and exposure. On the basis of 50% spore germination and maintaining hyphal growth ability, an exposure dosage of 130 Gy was determined for irradiation mutagenesis (Table 1). Plates containing the conidia were prepared as described, irradiated at 130 Gy and each generated conidia (after 18 hours) was transferred to a fresh PDA plate (one conidi per plate) and incubated for 10 days from which, the conidial suspension (10^6 conidia ml^{-1}) was then prepared.

Selection of Avr-mutants in Greenhouse

In a large-scale experiment to generate and select avr-mutants, a total of 700 colonies that survived the gamma irradiation and selection on PDA medium were examined for avirulence on seeds of bean. In two cases, plants did not show *Fusarium* root rot symptoms. From such un-infected plants, the pathogen was recovered, purified, and tested again on plants. Two avirulent mutants were identified that illustrated non-pathogenicity

Table 1. Mean of percentage of spore germination (after 18 hours) and colony diameter (after 10 days) of *F. solani* f. sp. *phaseoli* exposed to radiation of different dose rates

Colony diameter (cm)	Spore germination (%)	Dose rate (Gy)
7.7 a	96.3 a*	0
7.1 ab	93.2 a	60
7.0 b	81.3 b	90
6.9 b	59.6 c	120
6.0 c	54.3 d	150
5.5 c	51.6 d	180

* Data followed by different letters differ significantly at $P \leq 0.05$ (Duncan's multiple range test).

(Figure 2). These mutants were assigned as M22 and M23.

BiocontrolTest

The biocontrol test using the avirulent mutants was carried out. Experiments were repeated twice, and the results were all analogous. The results of these experiments are shown in Table 2. No significant difference in root rots between non-inoculated (control) and inoculated seeds (M22 and M23 treated) was observed. Therefore, mutants did not show any infection symptoms on the bean plants. Roots were not greatly infected by M22+wt (the mutant of M22 with the wild type) and M23+wt (the mutant of M23 with the wild type), resulting in a significant reduction of root rot. M23+wt infects roots, although less than M22+wt (Table 2). According to the achieved results, M23 was more effective in biological experiments.

Table 2. Mean of percentage of root rot 30 days after inoculation of seeds infected by the wild type of *F. solani* f.sp. *phaseoli* (Wt), sterile soil (control), the mutant of M22 (M22), the mutant of M23 (M23), the mutant of M22 with the wild type (M22+Wt) and the mutant of M23 with the wild type (M23+Wt).

Treatments	Percentage of root rot
Control	0 d*
Wt	77.5 a
M22	2.5 d
M23	2.5 d
M22+Wt	58.7 b
M23+Wt	22.5 c

* Data followed by different letters differ significantly at $P \leq 0.05$ (Duncan's multiple range test).

Biocontrol of Fusarium Root Rot in Field

In this experiment, three concentrations of avr-M23 spores (10^3, 10^6 and 10^9 conidia/ml) were evaluated for their ability to suppress Fusarium root rot of bean plants, grown in naturally infested soils in the field 110 days after sowing. Percentages of root rot in seeds colonized with M23 at concentrations of 10^6 and 10^9 conidia ml^{-1} were 39.5% and 38.8%, respectively whereas it was 67.3% in the control. In contrast, the percentage of root rot at the concentration of 10^3 conidia ml^{-1} was not significantly lower than that of the control (59.6% compared to 67.3%). Results

Figure 2. The comparison between wild type and avr- mutants. K12: Wild type isolate, 22 and 23: Avr-mutant isolates.

showed that the percentage of root rot was significantly decreased at both concentrations (10^6 and 10^9 conidia ml^{-1}) in comparison with that at 10^3 conidia ml^{-1}. Concentrations of 10^6 and 10^9 conidia ml^{-1} did not give complete protection (until maturity) against root rot in these infested soils, but they significantly reduced the root rot severity in the bean plants (Table 3).

Effect of concentrations (106 and 109 conidia ml^{-1}) on bean plants reached the best yield results (higher p/pl and 100 seed weight values) the best compensation among yield components: higher p/pl and 100 seed weight values (Table 4). Number of p/pl was evaluated for the performance of the plants after 110 days. When analyzed through the Duncan's multiple range test, the means of this variable showed that high performance was achieved at concentrations of 10^6 and 10^9 conidia ml^{-1}, even if they were not significantly different. Avr-M23 at the concentration of 10^3 conidia ml^{-1}, showed no significant difference from the control. The highest 100-seed weight scores were reached by using of concentrations of 10^6 and 10^9 conidia ml^{-1}, with no significant difference among them. The concentration of 10^3 conidia ml^{-1}, did not have a good effect on the performance of 100-seed weight, and showed no significant difference from the control (Table 4).

Table 3. Mean of percentage of root rot 110 days after seed treatment with different concentrations of avr-M23 and water (control) in Fusarium_infested soil.

Concentration (conidia ml^{-1})	Percentage of root rot
Control	67.3[a*]
10^3	59.6 [a]
10^6	39.5 [b]
10^9	38.8 [b]

*Data followed by different letters differ significantly at $P \leq 0.05$ (Duncan's multiple range test).

DISCUSSION

On the basis of gene-for-gene hypothesis, a race-specific resistance response depends on the presence of both a pathogen avirulent gene and a corresponding resistance gene in the plant (Flor 1971). For many bacterial avirulent genes, it has been found that they are involved in the pathogenicity of the pathogen (Dangl, 1994; Leach and White, 1996; Vivian and Gibbon, 1997). In our experiments, these mutant isolates were significantly less pathogenic compared with the wild type pathogenic isolates. The virulence of pathogens probably changes if the genetic mutation occurs in the genes related to pathogenicity of fungi after gamma treatment. This could involve single mutation in a pathogenicity-avirulent gene that results in a more efficient pathogenicity factor capable to circumvent plant recognition. The mutant recognized in our gamma irradiation screen was altered in both avirulence and pathogenicity. The modifications in pathogenicity and avirulent could perhaps be justified if pathogenicity and avirulent are determined by the same gene (Mes *et al*., 1999). Destruction of such a gene could result in a non-pathogenic mutant. Our inoculation of bean seeds with avr-M23 significantly reduced the incidence

Table 4. Means of the effects of three concentrations of avr-M23 and one control on the performance of bean plants in field condition.

Concentrations	P/Pl	100-seed weight (g)	P/Y(kg ha^{-1})
10^3 conidia/ml	7.83 [b]	32.0 [b*]	2161.6 [b]
10^6 conidia/ml	11.0 [a]	42.16 [a]	3090.0 [a]
10^9 conidia/ml	11.16 [a]	45.16 [a]	3083.6 [a]
control	7.33 [b]	30.0 [b]	2028.3 [b]

P/Pl: Number of pods per plant, P/Y: Plant yield, control: water.
* Data followed by different letters differ significantly at $P \leq 0.05$ (Duncan's multiple range test).

of Fusarium root rot in naturally infested soils. The mutant of *F. solani* f.sp. *phaseoli* described here shows the change in the avirulent gene. Our results confirm the conclusion of Mes and co-workers who investigated gamma-irradiated mutant of *F. oxysporum* f. sp. *lycopersici* that showed reduced pathogenicity toward susceptible tomato plants (Mes *et al*., 1999).

Also, the results demonstrate the capacity of avr-M23 to gain biocontrol effect. Root rot symptoms were significantly reduced in the inoculated bean plants predisposed to M23 isolate. These results indicate that avr-M23 induced changes that protected the plants and significantly reduced the root rot caused by *F. solani* f.sp. *phaseoli* in bean plants. The reduction was related to the population density of avr-M23 on the roots. The protection exerted by the mutant at concentrations of 10^6 and 10^9 conidia ml^{-1} against *F. solani* f.sp. *phaseoli* was significantly more pronounced than that at the concentration of 10^3 conidia/ml. According to Tellier and Brown (2008), this difference may be due to several mechanisms exerted by a biocontrol agent which may have an additive effect in plant protection. The significant relationship between increasing of avr-M23 population and reduction of root rot demonstrated that competition for nutrient and niche at the infection site might be the most important probable mechanism involved.

On the basis of field experiments results, the use of the root-colonizing avr-M23 at concentrations of 10^6 or 10^9 conidia ml^{-1} to potentially biocontrol Fusarium root rot for improving yield of bean plants, is suggested. Our results indicate that p/pl and 100-seed weight are the most closely associated indicators of crop yield itself, because other plant parameters used for determination of yield performance were highly variable and were therefore disregarded. 100-seed weight is the most important criterion in determining the yield of bean plants (Nelson *et al.*, 2009). Nevertheless, in a yield study carried out on climbing bean, high p/pl values failed to show any relation with higher productivity (Nelson *et al.*, 2009). In the present work, the selection of suitable concentrations of spore was based on the analysis of the number of pods per plant, 100-seed weight and plant yield (Table 4). This led to choosing concentrations of 10^6 or 10^9 as the best which attained the highest yield performance (3,090.0 and 3,083.6 kg ha^{-1}, respectively).

In general, Fusarium root rot severity was greater on bean plants inoculated with low concentration of avr-M23 isolate (suspension 10^3 conidia ml^{-1}) grown in *F. solani* f.sp. *phaseoli* infested fields. It seems that, determination of *F. solani* f.sp. *phaseoli* population density in soil is necessary before using avr-M23 as a biocontrol agent. Understanding the modes of interaction between avr-M23 and the wild type is necessary for biological control of Fusarium root rot in fields with a history of *F. solani* f.sp. *phaseoli* infestation. With this aim, further analysis and assessment of molecular pathways are in progress to visualize genomic changes in the used mutant under greenhouse conditions. As a result, pure cultures of the wild type and mutant of *F. solani* f.sp. *phaseoli* are kept under accession number of "IRAN 1669 C" and "IRAN 1670 C" respectively, at the culture collection of Ministry of Agriculture, Iranian Research Institute of Plant Protection.

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کاهش بیماری‌زایی موتانت *F. solani* f.sp. *phaseoli* با استفاده از اشعه گاما به منظور کنترل بیولوژیکی بیماری پوسیدگی ریشه لوبیا *Phaseolus vulgaris* در شرایط مزرعه

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چکیده

قارچ *Fusarium solani* از ریشه بیمار گیاهان لوبیا (*Phaseolus vulgaris*) کشت شده در مزارع به شدت آلوده به بیماری پوسیدگی ریشه لوبیا استان مرکزی، جداسازی گردید. به منظور شناسایی و تائید فرم مخصوص لوبیا (*F. solani* f.sp. *phaseoli*) از پرایمر اختصاصی استفاده گردید. سوسپانسیون اسپور *F. solani* f.sp. *phaseoli* تحت دز ۱۳۰ گری با منشا کبالت ۶۰ قرار گرفت که نهایتا ۷۰۰ اسپور موتانت تولید شد. همه موتانت‌ها به منظور کاهش بیماری‌زایی، پس از کشت مجدد و تشکیل کلونی مورد آزمایش گلخانه‌ای قرار گرفتند. در نهایت یک موتانت (M23) غیربیماری‌زا به عنوان عامل بیوکنترل تحت آزمایش‌های مزرعه‌ای قرار گرفت. بدین منظور، غلظت‌های مختلفی از تک موتانت غیربیماری‌زا به منظور بررسی تاثیر غلظت‌ها بر عامل بیماری پوسیدگی ریشه لوبیا تهیه و اثر آنها بر روی عملکرد گیاه لوبیا مورد ارزیابی قرار گرفت. نتایج نشان دادند که اثر بیوکنترلی موتانت غیربیماری‌زا با افزایش غلظت از 10^3 به 10^9 اسپور در هر میلی‌لیتر بهبود یافت. همچنین، بهترین عملکرد گیاه (تعداد غلاف در هر گیاه و وزن ۱۰۰ دانه) در غلظت‌های 10^6 و 10^9 اسپور در هر میلی‌لیتر به دست آمد. نتایج این تحقیق، موتانت غیر بیماری زای M23 را به عنوان عامل کنترل بیولوژیک در کنترل بیماری پوسیدگی ریشه لوبیا تحت شرایط مزرعه تائید نمود.