Current Research in Environmental & Applied Mycology (Journal of Fungal Biology)
8(1): 69–74 (2018) ISSN 2229-2225

www.creamjournal.org **Article**

Doi 10.5943/cream/8/1/5

Formulated *Sarocladium oryzae* suppress common bean root rot caused by *Rhizoctonia solani*

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Guimarães RA, Barbosa ET, Silva-Lobo VL, Côrtes MVCB 2018 – Formulated *Sarocladium oryzae* suppress common bean root rot caused by *Rhizoctonia solani*). Current Research in Environmental & Applied Mycology (Journal of Fungal Biology) 8(1), 69–74, Doi 10.5943/cream/8/1/5

Abstract

Applying a prototype biopesticide containing *Sarocladium oryzae* as a biocontrol agent at the time of sowing significantly reduced common bean root rot disease caused by the soil-borne fungi *Rhizoctonia solani* with *in vivo* experiments. The results suggest that there is a possibility of using *Sarocladium oryzae* as part of a more economically and environmentally sustainable root rot disease management.

Key words – Biopesticide – Biocontrol – Cerulenin – Soil-borne pathogen

Introduction

The common bean (*Phaseolus vulgaris* L.) is the most important legume food crop in Latin America (Godoy-Lutz et al. 2008). In the last four years, Mercosur countries produced together at least 3.06 million of tons/year average. Brazil is the producing leader as well the consumer, with 90% of participation in production and consumption (Conab 2017).

Rhizoctonia solani Kuhn, one of the most investigated soil-borne pathogens, causes bean root rot, which is a limiting factor in the commercial production of beans (Paula Jr. et al. 2008). The disease management usually uses a combination of cultural, chemical and resistant cultivars. However, there is limited information for applied research concerning the use of biological agents to suppress root rot disease, a low-cost and environment safe alternative for promoting a more sustainable agriculture.

The fungus *Sarocladium oryzae* has been known to be antagonist against some microorganisms due to production of antimicrobial metabolite cerulenin (Gnanamanickam & Mew 1991, Côrtes et al. 2014, Liu et al. 2017). However, information on potential of using *S. oryzae* as a biocontrol agent of soil-borne diseases remains to be determined. The objective was to study the application of *Sarocladium oryzae*, in a formulation, as suppressor of common bean root rot.

Materials & Methods

Isolates

The strains *Sarocladium oryzae* BRM 6461 and *Rhizoctonia solani* BRM 29725 used is the experiments are both part of Embrapa's Culture Collection.

Submitted 5 December 2017, Accepted 30 January 2018, Published 7 February 2018
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Production of *Sarocladium oryzae* biomass

Sarocladium oryzae BRM 6461 was grown in Petri-dishes containing potato dextrose agar medium (PDA) and incubated at 25 ± 2°C. After 10 days, 5 mm diameter mycelium discs were inoculated into 500mL conical flasks containing 200 mL of sterile liquid culture medium (1% glucose, 3% glycerol, 0.5% peptone, and 0.2% sodium chloride). Flasks were incubated for eight days by continuous shaking on an orbital rotary shaker (150 rpm) at 25 ± 2°C (Masuma et al. 1982). At the end of the incubation period the cells were harvested by filtration.

Biopesticide prototype production - *S. oryzae* formulation

The fungi biomass was formulated, leading to a prototype *S. oryzae* biopesticide. Formulation was produced by immobilization of *S. oryzae* biomass containing mycelia and spores in calcium alginate beads. Moist biomass was resuspended in sterile distilled water to the final ratio of 1% ($g.mL^{-1}$). The suspension was added to a solution of sodium alginate at a concentration ranging from 3.0% in the ratio 1:1 (volume: volume). The suspension produced was kept under constant stirring until total uniformity and dripped through a syringe in a solution of 0.1 M calcium chloride. The beads were maintained for at least 30 minutes in the 0.1 M calcium chloride solution at a temperature between 4°C and 15ºC for their maturation. After this time the beads were washed with NaCl 0.9% solution and used immediately. Prototype was submitted to a quality control taking account the uniformity of the product and viability of fungi. For quality control criteria, at least 20 random produced beads diameter were measured. Their diameters should be homogeneous (2.0 mm to 5.0 mm), whose maximum acceptable variation is 5%, more or less, guaranteeing the uniformity of action of the product. Also for quality control criteria, after immobilization of the fungus, the spheres were tested for viability, at least 20 beads were randomly chosen and inoculated at PDA and incubated at 25°C for seven days. The mycelial development, should be at least 90% to consider the process efficient.

Partial characterization of *S. oryzae* active metabolite

The crude extract obtained during the *S. oryzae* biomass production (described above) was filtered at 0.22 um. 20.0 uL were eluted at 1 $mL.min^{-1}$ in a reverse -phase HPLC system (Perkin Elmer Flexar). The system was composed by a C18 150 mm x 4.6 mm x 5 um column, maintained at 40ºC. A gradient of 15% acetonitrile in water to 100% acetonitrile was developed over 15 min. Both solutions contained trifluoracetic acid 0.005%. Detection and quantification was done with a UV detector at 210 nm (Bills et al. 2004). Cerulenin (major related *S. oryzae* active metabolite) was identified and quantified by co-injection of authentic sample (Sigma-Aldrich, St. Louis,MO, USA)

Thin layer chromatography (TLC) was done in silica gel G60 20 × 20 cm plates previously activated at 130°C for 25 minutes. Plates were developed with solvent system of diethyl ester-acetic acid (100:0.5) at 25°C. The spots were detected by iodine vapor (Sakthivel et al. 2002).

Evaluation of *Sarocladium oryzae* metabolite inhibiting *R. solani* mycelial growth and sclerotium production

Rhizoctonia solani BRM 29725 was grown in PDA at 25 ± 2°C for 48 hours. 5 mm diameter *R. solani* discs were positioned 35 mm from sterile paper filter discs (7 mm diameter) containing 25, 50, 75 and 100 µL of filtered extract of *Sarocladium oryzae* (produced as described above) on a 90 mm Petri-dish containing PDA. The plates were incubated at 25 ± 2°C for five days to measure mycelial development and ten days for sclerotium quantification. Mycelial development was quantified measuring de distance between the extreme mycelia and the center of a filter paper disc containing the extract, subtracting of 35 mm. Sclerotium quantification was done by gently agitation of the Petri dish with 5 mL of distilled water and observation under a 10× light microscope.

***Rhizoctonia solani* inoculum preparation**

Rhizoctonia solani BRM 29725, previously confirmed as highly aggressive to common bean cultivar BRS Pérola (data not shown), was grown in PDA at 25 ± 2°C for 48 hours. Inoculum was prepared with addition of *R. solani* mycelial discs (5 mm) transferred from the Petri-dishes to a plastic bag with sorghum grains according to Trindade et al. (1997). The inoculum was mixed with sterile vermiculite in a proportion of 1:1000 (g.mL-1) and disposed in 500 mL vessels according to Hanson (2003), Chaudhary et al. (2006).

***In vivo* evaluation of formulated *Sarocladium oryzae* suppressing root rot**

Five seeds of common bean cultivar BRS Pérola were planted per vessel and four vessels per treatment. The formulated containing *S. oryzae* or not) were placed at the time of sowing, in the same level of the seeds. The experiment was conducted using a completely randomized design under controlled conditions of temperature (20 ± 2°C). The following treatments were included: T1 = non-infested vermiculite; T2 = non-infested vermiculite + 10 g formulated product without *S. oryzae*; T3 = non-infested vermiculite + 10.0 g of formulated product with *S. oryzae*; T4 = infested vermiculite with *R. solani* inoculum (control); T5 = infested vermiculite with *R. solani* inoculum + 10.0 g formulated product without *S. oryzae*; T6 = infested vermiculite with *R. solani* inoculum + 10.0 g of formulated product with *S. oryzae*. Fifteen days after planting, the disease evaluation score (DES) was made using a 10 grade (0-9) visual rating scale according to Schoonhoven & Pastor-Corrales (1987). The germination rate (GER) was accessed either. Measures of plant high (PH), root length (RL) and plant total length (PTL) were made using a calibrated ruler.

Statistics analysis

In vitro experiments had three replicates and data on quantification of cerulenin, mycelial development and sclerotium inhibition were submitted to analysis of variance using statistical program SASM-Agri, version 3.2.4 and means were compared by Tukey test ($P<0.05$).

In vivo experiments had four replications and data on germination rate, suppression of root rot, plant high, root length and plant total length were submitted to analysis of variance using statistical program SASM-Agri, version 3.2.4 and means were compared by Duncan test ($P<0.05$).

Results

Evaluation of *Sarocladium oryzae* metabolite inhibiting *Rhizoctonia solani* mycelial growth, sclerotium production and its partial characterization

Analysis of extract resulted of *Sarocladium oryzae* biomass production showed the presence of approximately 127 $\mu g.mL^{-1}$ cerulenin. *In vitro* tests showed extract containing metabolites was able to inhibit 100% of *Rhizoctonia solani* mycelial development when applied without dilution. The inhibition power decreases together with higher extract concentrations. The results show reduction of *R. solani* sclerotium production also depends on concentration of extract, being totally reduced at higher concentrations (Fig. 1a, Table 1). Cerulenin was detected in both TLC and HPLC methods. Besides, helvolic acid was not detected. TLC shows cerulenin suffered degradation during the process of biomass production until time of analyzes (Fig. 1b).

Biopesticide prototype production - *Sarocladium oryzae* formulation

It was produced approximately 5g of *Sarocladium oryzae* biomass, including mycelia and spores. The homogeneity of biopesticide prototype, formulated *S. oryzae*, was approximately 94%, with a *S. oryzae* viability of 100%.

***In vivo* evaluation of formulated *Sarocladium oryzae* suppressing root rot**

Difference between T6 (infested vermiculite with *Rhizoctonia solani* inoculum + 10.0 g of formulated product with *Sarocladium oryzae*) and T4 (infested vermiculite with *Rhizoctonia solani* inoculum - control) was significant in relation to all analyzed parameters: GER, DES, PH, RL and TPL (Table 2, Fig. 2a). Additionally, difference between T6 (infested vermiculite with *R. solani*

inoculum + 10.0 g of formulated product with *Sarocladium oryzae*) and T1 (non-infested vermiculite) was not significant in relation to RL. Difference between T4 and T5 was not significant in relation to GER, DES and PH, showing formulated product without *S. oryzae* does not interfere in root rot suppression or at plant development (Table 2, Fig. 2b).

Table 1 Influence of *Sarocladium oryzae* metabolite in *Rhizoctonia solani* mycelial development and sclerotium production.

Treatments	***MDM*[1]**	***SP*[2]**
Control (no-extract)	35.0a	112.3a
25 µL extract	10.7b	37.5b
50 µL extract	7.9bc	7.3c
75 µL extract	4.6c	6.5c
100 µL extract	0.0d	1.5d

[1]Mycelial Development Measure (MDM) in millimeters. [2]Sclerotia Production (SP) in unities. [3]Means followed by the same letters in column do not differ statistically according to the Tukey test (P<0.05).

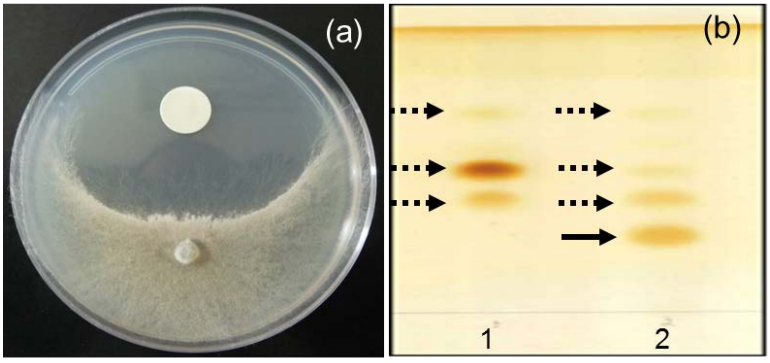

Fig. 1 – a *Sarocladium oryzae* metabolites inhibiting *Rhizoctonia solani* in PDA plates. b Thin layer chromatography profiles: (1) standard cerulenin (2) extract of *S. oryzae* biomass production. Continuous arrow indicates residual culture media present on extract and dashed arrow indicates cerulenin and their residual active, or not, degrading products.

Table 2 Efficiency of *Sarocladium oryzae* formulated (biopesticide prototype) in suppressing common bean root rot.

Treatments	***GER*[1]**	***DES*[2]**	***PH*[3]**	***RL*[3]**	***TPL*[3]**
T1: non-infested vermiculite	1.0a	1.07c	18.3a	17.3a	35.7a
T2: formulation without *S. oryzae* + non-inf. vermiculite	1.0a	1.07c	19.2a	17.1a	36.3a
T3: formulation with *S. oryzae* + non-inf. vermiculite	1.0a	1.00c	18.6a	17.1a	35.7a
T4: control (*R. solani* infested vermiculite)	0.60b	7.33a	9.0c	12.8c	21.8d
T5: formulation without *S. oryzae* + infested vermiculite	0.53b	7.07a	10.5c	14.8b	25.4c
T6: formulation with *S. oryzae* + infested vermiculite	0.93a	3.46b	13.9b	16.3a	30.2b

[1]Germination rate (GER). [2]Root rot disease score (DES) using a 10 grade scale (0-9) according to Schoonhoven and Pastor-Corrales (1987). [3]Means measures of PH (plant high), RL (root length) and TPL (total plant length) in cm. [4]Means followed by the same letters in column do not differ statistically according to the Duncan test (P<0.05).

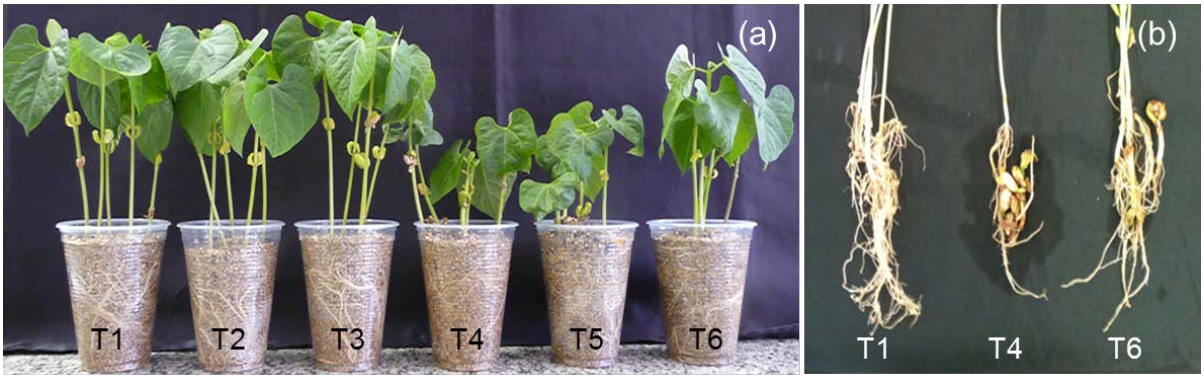

Fig. 2 – a common bean plants 15 days after planting. T1 – non-infested vermiculite; T2 – non-infested vermiculite + 10.0 g formulated product without *Sarocladium. oryzae*; T3 - non-infested vermiculite + 10.0 g of formulated product with *S. oryzae*; T4 - infested vermiculite with *Rhizoctonia solani* inoculum (positive control); T5 – infested vermiculite with *R. solani* inoculum + 10.0 g formulated product without *S. oryzae*; T6 – infested vermiculite with *R. solani* inoculum + 10.0 g of formulated product with *S. oryzae*. b common beans roots: healthy (T1), rotten (T4), and covered with injuries but not rotten (T6).

Discussion

Sarocladium oryzae is frequently reported as a rice pathogen, as others species of genus *Sarocladium*. However, some strains of *Sarocladium* sp. show ability to inhibit a diversity of plant pathogens including *Colletotrichum gloeosporioides, Fusarium oxysporum* f.sp. *Cubense*, *Gloeosporium musarum*, *Corynespora cassicola*, *Magnaporthe oryzae*, *Bipolaris oryzae*, *Colletotrichum falcatum* and *Drechslera* sp. within *vitro* experiments (Liu et al. 2017). Guimarães et al. (2017) showed the suppression of leaf blast disease in rice applying a suspension of *Sarocladium oryzae* spores (not aggressive to the used rice cultivar) or metabolic extract, at *in vivo* experiments.

Antimicrobial activity using *Sarocladium* sp. seems to be agro-industrially promising. In general, this species easily produces large number of spores, with low nutrient requirements, in submerse fermentation that makes process less expensive (data not shown). Due to the known pathogenicity of *Sarocladium* sp. to grasses species, the application of these fungi in rice diseases management, wheat or brachiaria may be risky. On the other hand, it is valid explore the potential of *Sarocladium* sp. as biocontrol agents against pathogens from other plant groups.

This work demonstrates for the first time the potential of *Sarocladium oryzae* in significantly reducing common bean root rot caused by *Rhizoctonia solani* at *in vivo* experiments. According to Schoonhoven & Pastor-Corrales (1987), T4 (positive control) disease evaluation scored (DES) 7.33 implying more than 50% of root is covered with injuries and rotten tissues, compared to T6 (treatment with formulated *Sarocladium oryzae*) DES scored 3.46 implying approximately only 10% of root covered with necrotic injuries, without rotten tissues. This is a reduction of at least 400% of disease severity. In addition, the other parameters analyzed (GER, DES, PH, RL and TPL) were significantly different to these treatments, reinforcing the DES results. Previous works showed suppression of beans root rot using strains of *Pseudomonas* sp. (D'aes et al. 2011) and *Glomus intraradices*, *Azotobacter chroococcum* and *Trichoderma harzianium* (Matloob & Juber 2013), with similar efficiency results on the present work.

Trichoderma sp. are the classic biocontrol agents used in management of bean diseases. In Brazil, there are only four biological products legally registered for bean root rot control caused by *Rhizoctonia solani*. All the products contain *Trichoderma harzianum* or *T. asperellum*. Development of products based on *Sarocladium oryzae*, or other potential bioagent, is strategic to develop an economically and environmentally sustainable disease management. The next step of this research is the development of appropriate formulation to increase the efficiency of *S. oryzae* and sequential field tests.

Acknowledgements

We thank Dr. Murillo Lobo Jr. and Dr. Anne S. Prabhu for technical support.

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