



Biocontrol of *Rhizoctonia solani* causing stem canker disease of potato using rhizosphere mold fungi

Adel K. Madbouly^{1*}, Angela Boari², Maurizio Vurro², Hassan M. Gebreel¹ and Mohamed A. Abouzeid³

¹Department of Microbiology, Faculty of Science, University of Ain Shams, 11566 Abbassia, Cairo, Egypt

²Istituto di Scienze delle Produzioni Alimentari, Consiglio Nazionale delle Ricerche (CNR), Via Amendola 122/O, I-70125, Bari, Italy

³Preparatory year, Taibah University, AL-Madinah ALMonowarah, Saudi Arabia

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Abstract

The aim of the present work is to obtain effective rhizosphere fungal bio-control agents (bio-fungicides) that can antagonize *Rhizoctonia solani*; the fungal causal agent of stem canker disease of potato (*Solanum tuberosum* L.) and to improve the growth of the potato plant. A total of eleven different isolates of mold fungi were isolated from the rhizosphere soil of potato plants collected from three different potato cultivation areas in El-Monofia governorate-Egypt. Three isolates belong to the genus of *Fusarium* and three to *Aspergillus*, whereas the other five isolates belong to the genera of *Penicillium*, *Absidia*, *Mucor*, *Gliocladium* and *Alternaria*. In dual culture technique on Sabouraud dextrose agar media, three species (*Gliocladium virens*; *Penicillium oxalicum* and *Trichoderma viride*), completely surrounded the pathogen colonies and prevented their radial spread. *In vivo* results of antagonism in greenhouse were very promising as growth of potato plant was highly promoted by the three bio-agents compared with positive and non-infested control plants. Studies on soil population dynamics of selected *Gliocladium virens* showed a gradual decrease in CFU with increasing periods of incubation. Maize kernels and wheat bran were the best solid substrates for the mass production of *Gliocladium virens* conidia especially at 30% initial moisture level. The highest percentage of viable conidia of formulated *Gliocladium virens* was obtained on storage at 4°C. *Gliocladium virens* showed promising results of antagonizing *R. solani* *in vitro* and improving potato growth under greenhouse conditions, subsequently, could be mass produced and formulated to be used as an effective biofungicide.

* Corresponding Author: Adel K. Madbouly ✉ adelkamelmadbouly@yahoo.com

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important crops for local consumption and exportation in Egypt, the total cultivated area of potato is about 220 thousand acres. Egypt is one of the top ten potato exporting countries in the world and is number one in Africa because of its favorable climate; coupled with the presence of loamy soils, particularly suitable for the crop. The exportation of potato in Egypt at 2012\ 2013 reached 101.209.414 tons (Anonymous, 2013).

Stem-canker of potato caused by *Rhizoctonia solani* Kuhn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is one of the most important fungal diseases that cause great reduction in both quality and quantity of tuber yield (Weinhold *et al.*, 1978, Escande and Echandi, 1991, Jeger *et al.*, 1996). *R. solani* as a soil borne pathogen (Campion *et al.*, 2003), is the causal agent of the so called *Rhizoctonia* disease complex of potato (Wilson *et al.*, 2008a) and is responsible for delayed emergence; lesions on stems (stem-canker); stolons and sclerotial formation on potato tubers (black scurf) (Anderson, 1982). Attacks on stolons may induce the development of miss-shaped tubers (Escande and Echandi, 1991, Jeger *et al.*, 1996). A post-harvest *R. solani* disease symptom was recently described by (Buskila *et al.*, 2011). Infection of tubers by *R. solani* AG 3 before skin-set, was shown to be the cause of dark skin spots, likely due to an 'oversuberization' response of the tuber. This can reduce the value of potato varieties that are washed before sale. Stem-canker disease was managed by cultural practices such as crop rotation and chemical fungicides (Parry, 1990). Fungal disease control through the use of fungicides is hazardous and toxic to both people; domestic animals and leads to environmental pollution, however, biological control is an innovative; cost effective and eco-friendly approach (Abdel-Kader *et al.*, 2012). Whipps, (2001) confirmed that mold fungi have much greater potential than bacteria to grow and spread through soil and in the rhizosphere, hence could be used as bio-control agents. A previous study by Wilson *et al.*

(2008a) demonstrated that, biological control of *Rhizoctonia* diseases using bioagents such as *Trichoderma harzianum* or *Trichoderma (Gliocladium) virens*; have successfully suppressed *R. solani* in greenhouse and under field conditions, through preventing or lowering the incidence of infection. Only a few formulations of the fungal bioagents are commercially available because of their short shelf-life and high cost of their mass production (Duan *et al.*, 2008). This study aimed to evaluate the potential of some potato rhizosphere mold fungi for antagonizing stem-canker pathogen (*R. solani*) *in vitro*, improving potato growth under green-house conditions (*in-vivo*), studying the soil population dynamics of the promising bioagent and determining its shelf-life after formulation and storage.

Material and methods

Isolation of mold fungi from the rhizosphere of potato plants

Healthy potato plants with their intact roots and adhering soil were collected from potato fields at El-Monofia governorate, Egypt. Ten grams of soil was dispersed into sterile 0.1% (w/v) agar in dist. water, shaken for 30 min and left to settle down. Isolation was made using the dilution plate method on Sabouraud dextrose agar medium (SDA) (Difco laboratories, Detroit, MI, USA). After incubation at 28 °C for 4–6 days, colonies were counted and expressed as CFU g⁻¹ dry rhizosphere soil. Fungal isolates were all obtained as single spore colonies stored at 4 °C and sub-cultured on SDA slants. A *Trichoderma viride* isolate of known antifungal activity against *R. solani* was provided by Institute of Science and Food Production, Bari-Italy, used as a reference isolate for comparison of results.

Identification of fungal isolates

All fungal isolates were identified following the taxonomic keys of Booth, (1971) for the genus *Fusarium*; Barnett and Hunter, (1998) for the genera *Gliocladium*, *Penicillium* and *Absidia*; Simmons, (1995), Simmons, (1999), Andersen *et al.* (2001) for the genus *Alternaria*; Klich and Pitt, (1988), Pitt and

Hocking, (1997), Klich, (2002) and Samson *et al.* (2002) for the genus *Aspergillus*.

Isolation and identification of R. solani, the fungal causal agent of potato stem-canker disease

Ten diseased potato plants showing typical symptoms of stem-canker were collected from potato fields of El-Monofia governorate, Egypt. Small pieces of underground stem were excised, surface-disinfested in 1% NaOCl for 30 sec, rinsed with dist. water then placed in Petri dishes with 2% antibiotic water agar (AWA) and SDA media. After incubation for 72 h at 25 °C, hyphae were transferred into SDA slants and kept at 4 °C. Identification of stem-canker causal agent was based on morphological and microscopic characteristics described by (Parmeter and Whitney, 1970).

Pathogenicity of R. solani in the green-house

This test was conducted in the green-house following the procedure modified by Carling and Leiner, (1990), to assess the virulence of the isolate and ensure that it is the causal agent of stem canker disease.

In vitro detection of antifungal activity of fungal isolates

All fungal isolates were tested as antagonists of the pathogenic *R. solani* according to the methods described by (Wilson *et al.*, 2008a). The ability of the antagonists to inhibit the growth (antagonize) of *R. solani* were detected *in-vitro* on Hussein's fish meal extract agar (HFMEA) as it enhances the ability of the isolates to produce antifungal inhibitory compounds (El-Tarabily, 1996a, El-Tarabily, 1996b). Antagonists were streaked to one side of HFMEA plates and then incubated for 4 days to allow the production and diffusion of metabolites into the agar. An agar disk (6 mm diameter) containing *R. solani* mycelia were placed onto the opposite side of the antagonist inoculated plates. *R. solani* mycelial disks were placed on un-inoculated HFMEA separately as control. Plates were incubated in the dark at 25 °C, and were examined for inhibition of pathogen growth after 2–7 days of incubation. The level of inhibition

was determined according to Yuen and Crawford (1995).

Briefly, the level of inhibition (ΔY) was defined as the subtraction of pathogen growth radius (\hat{Y} in cm) of a control culture from the distance of growth of pathogen in the direction of the antagonist colony (Y in cm), where $\Delta Y = \hat{Y} - Y$. Inhibition was indicated when mycelial growth of *R. solani* in the direction of the antagonist colony was retarded. The active isolates showing antifungal potential against *R. solani* were selected and further tested also through the dual culture technique on SDA. SDA medium was inoculated with two disks (5 mm diameter each) of fungal antagonist; disks were positioned on both sides of 5 mm diameter disk of the pathogen. Distance between disks were approximately 5 cm. Cultures were grown at $25 \pm 2^\circ\text{C}$ in the dark. Inhibition of pathogen growth in direction of antagonist was determined within 2-6 days of incubation (Mcquilken *et al.*, 2001).

In vivo antagonistic potential of fungal isolates against R. solani

Preparation of pathogen inoculum

R. solani was grown on SDA medium at 25 °C for 7 days in the dark. Crashed maize seed-based inoculum were prepared by adding 100 g of crashed seeds with 30 ml of dist. water into 250 ml conical flasks and kept overnight. Flasks were steamed without pressure for 10 min and then autoclaved at 121 °C for 30 min on three consecutive days as described by (Wong *et al.*, 1984). Under aseptic conditions, the maize seeds were then inoculated with eight agar plugs (2 mm diameter each) from actively growing margins of *R. solani* colonies. Flasks were incubated at 25 °C in the dark for one week and were occasionally shaken to ensure uniformity of colonization. Maize seeds free of inoculum and which had been autoclaved twice served as control. Small amounts of the colonized and non-colonized maize seeds were plated onto SDA medium to confirm the presence or absence of target pathogen.

Preparation of antagonistic fungal inocula

Inoculum of the fungal antagonists (*Gliocladium virens*, *Trichoderma viride* and *Penicillium oxalicum*) was prepared on crashed maize seeds in the same way described before for the pathogen inoculum. The used concentrations of antagonist's inoculum were around 12.7×10^6 CFU g^{-1} soil for *P. oxalicum* 5×10^5 CFU g^{-1} soil for *Trichoderma viride* and 5.15×10^5 CFU/g soil for *Gliocladium virens*.

Soil infestation

Ten free draining pots were used for each antagonistic isolate; each containing 3–4 Kg of non-sterile clay soil. One hundred grams of crashed maize seeds infested with *R. solani* was dispersed through the lower quarter of soil in each pot, left for 2 days. Two hundred grams of crashed maize seed inoculum of each antagonist were dispersed in the upper quarter of soil in each pot. Pots containing soil treated with the pathogen only served as positive controls whereas those containing soil free of pathogen and antagonist served as negative controls. After adding the antagonist's inoculum, two potato seed pieces (variety *Spunta*), were sown in each pot in the upper quarter of soil, pots were watered every day.

After 4–5 weeks, several parameters (shoot length, shoot fresh weight, number of leaflets/plant, and visual symptoms e.g. yellowing and necrosis) were used to assess the effect of antagonists on growth of potato plants. The pathogen and antagonists were re-isolated from the lower and upper soil layers; respectively, to ensure their presence and effect on potato plant growth.

Soil population dynamics of the fungal antagonist

This experiment was carried out on the selected isolate of *Gliocladium virens*. A soil sample was collected from a field located in Altamura (province of Bari, Italy) with a water capacity of 31.9%. The soil samples were dried at 35°C for about one week to constant weight then sifted through 4 × 4 cm mesh sieve. Low and high inoculum levels of the antagonist isolate were prepared in 500-ml jars containing 150 g (dry weight) of non-sterile soil. An appropriate

volume of a conidial suspension of the bio-control agent in sterile dist. water was mixed thoroughly with soil in the jars to obtain a final concentration of 10^3 CFU g^{-1} soil (low inoculum level) or 10^6 CFU g^{-1} soil (high inoculum level). The jars were closed loosely with their screw lids, and incubated at 25 °C.

Sampling from soil was carried out after 1, 15, 30, 60 and 90 days of incubation for determination of population level (CFU g^{-1}) using the dilution plate method. At each sampling time, two soil samples (5 g/each) were taken from each jar, one was oven dried at 150 °C for 48 h to determine the soil dry weight whereas the other sample was introduced into a centrifuge tube containing 45 ml of sterile dist. water and shaken for 1 h at 200 rpm to obtain an even suspension. A series of 1/10 dilutions were prepared with sterile dist. water and plated on *Trichoderma* selective medium (TSM) for *Gliocladium virens*. Colonies were counted after incubation at 25 °C for 3–6 days and CFU g^{-1} of dry soil was calculated, based on soil dwt. previously determined, the experiment was carried out in triplicate and repeated twice.

Mass production of Gliocladium virens conidia in solid state fermentation

Wheat, wheat bran, maize, and oat seeds were used as solid substrates. Batches of each substrate (100 g) were placed in 500-mL flasks with an initial moisture content of 15% and 30%; flasks were left overnight, and then sterilized at 121 °C for 1 h. The substrates were inoculated with a conidial suspension (10^5 conidia g substrate $^{-1}$) of *Gliocladium virens* and flasks were incubated at 25 °C for 5 weeks. The conidial production was estimated every week till the end of incubation period. One g was taken from each substrate and a series of 1/10 dilutions were made, then 100 μ l of this suspension was spread on SDA plates using sterile glass rod, plates were incubated at 25 °C for 2–6 days. Colony count of antagonist was calculated and CFU g^{-1} solid substrate was estimated.

Estimation of shelf-life of formulated Gliocladium virens

One hundred g of maize seeds were placed in 500-ml conical flasks with initial moisture content of 30%, left overnight then sterilized at 121 °C for 1 h. Maize seeds were inoculated with conidial suspension (10 ml) of *Gliocladium virens*, incubated at 25 °C for 1 week in darkness. After incubation, maize seeds were placed in plastic plates, dried at 30 °C for 48 h. Samples (20 g) of dried maize seeds were placed in falcon tubes then stored at -20 °C, 4 °C and 25 °C (3 replicates for each storage temperature). After 1, 2, 5 and 6 weeks of storage, 1 g sample of stored maize seeds was suspended in sterile water for determining the percentage of viable conidia according to (de Cal *et al.*, 1988).

Statistical analysis

All treatments were replicated three times, data were reported as mean \pm SE (standard error) and subjected to statistical analysis by ANOVA and Tukey's HSD.

Results

Isolation and identification of rhizosphere mold fungi

A total of eleven different species of mold fungi were obtained on the isolation medium. These fungal isolates were identified mycologically as: *Fusarium sambucinum*, *Fusarium solani*, *Fusarium oxysporum*, *Penicillium oxalicum*, *Gliocladium virens*, *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus niger*, *Mucor* spp., *Absidia* spp. and *Alternaria* spp. All these isolates were tested for antifungal potency against *R. solani*.

Isolation and identification of R. solani

Ten isolates of *R. solani* were recovered on 2% AWA and SDA media from stem lesions of the diseased potato plants showing typical symptoms of stem canker, no other fungal isolates were recovered on isolation plates of these two isolation media. Re-inoculation of the isolated pathogen into potato stem produced typical symptoms of stem canker thus verifying Koch's postulates. Isolates of *R. solani* showed rapid growth on culture medium and covered

Petri dishes within two days as described by (Anderson 1982 and Ogoshi 1985). The presence of multinucleate cells, branching near the distal septum of cells as well as constriction of the branch near the point of origin, was observed.

Pathogenicity of R. solani in the green-house

About 95% of infested potato plants showed typical symptoms of stem canker disease, such as dwarfism; yellowing; discoloration of stem bases and curling of leaves. In addition, small green tubers (aerial tubers) were noticed on the base of stems at the soil surface, whereas; non-infested plants did not show any symptoms of stem-canker. *R. solani* was re-isolated from the soil and underground stolons of infested plants, thus verifying Koch's postulates.

In vitro detection of antifungal potency of the fungal isolates

Detection of *in vitro* antifungal potential of the fungal isolates against *R. solani* on (HFMEA) demonstrated a moderate inhibitory level (ΔY) ranging between 30-35%. The most active fungal species (*Gliocladium virens*, *P. oxalicum* and *Trichoderma viride*) were selected and further examined for *in vitro* antagonistic activity against *R. solani* using the dual culture technique. The remaining isolates showed weak inhibitory level ($\Delta Y = 5-10\%$), thus were excluded from any further research. Results of dual culture assay showed that the selected species surrounded the pathogen colony completely and prevented it from any radial spread. Thus we observed that the antagonism of these fungal species against *R. solani* was not due to antibiosis but due to competition for space and nutrients. Similar results were obtained by the reference isolate of *Trichoderma viride*, which showed coiling of its hyphae around hyphae of *R. solani* (mycoparasitism) as shown in (Fig. 1).

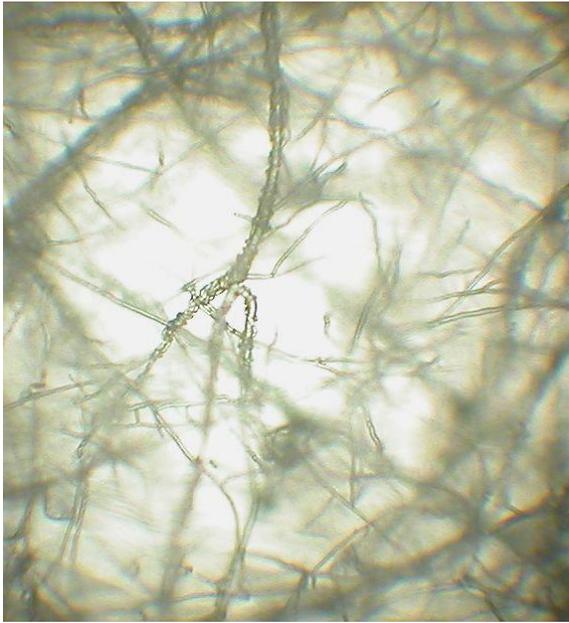


Fig. 1. Coiling of *Trichoderma viride* hyphae around *Rhizoctonia solani* hyphae (mycoparasitism)

In vivo antagonistic activity of selected fungal species against *R. solani*

Treatment of soil with *Gliocladium virens*, *P. oxalicum* and *Trichoderma viride* alone gave the highest shoot length; largest number of leaflets and fresh wt. of shoot compared with pathogenic *R. solani* treated and control plants (Table 1). They also caused significant promotion of potato growth in presence of *R. solani* compared with the controls treatments. It is important to add that potato plants grown in *Gliocladium virens* and *Trichoderma viride* treated soils did not show any visual symptoms of stem-canker. However, *Gliocladium virens* isolate gave the best results of antagonizing *R. solani* and causing appreciable promotion of potato growth (acting as an effective bio-fungicide); consequently this isolate only was selected for further assays, whereas the remaining two isolates of (*Trichoderma viride* and *P. oxalicum*) were excluded from further research.

Table 1. Effect of soil treatments with fungal antagonists on stem canker disease symptoms and potato plant growth in the greenhouse

Soil Treatments	Parameters of plant growth assessment			
	Length of shoot (cm)	Number of leaflets	Fresh wt. of shoot (g)	Visual symptoms of stem canker
<i>Gliocladium virens</i>	76.2 ^a ± 0.21	126 ^b ± 0.11	30.01 ^c ± 0.32	-
<i>Penicillium oxalicum</i>	64 ^a ± 1.04	94 ^b ± 1.03	27 ^c ± 1.13	-
<i>Trichoderma viride</i>	63 ^a ± 0.85	119 ^b ± 0.19	26 ^c ± 1.00	-
<i>R. solani</i> + <i>Gliocladium virens</i>	69 ^a ± 0.41	115 ^b ± 1.27	24 ^c ± 0.64	-
<i>R. solani</i> + <i>Penicillium oxalicum</i>	62 ^a ± 0.93	90 ^b ± 1.03	24 ^c ± 1.20	yellow lower leaves
<i>R. solani</i> + <i>Trichoderma viride</i>	59.1 ^a ± 0.56	106 ^b ± 0.87	23 ^c ± 0.22	-
<i>R. solani</i>	53.4 ^a ± 1.03	71 ^b ± 1.32	20.6 ^c ± 1.00	yellow, necrotic leaves and cankers on stem bases
Non treated control soil	57 ^a ± 0.99	80 ^b ± 0.41	24.75 ^c ± 0.75	-

Values are the mean of 20 plants, ± standard error.

The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.

Soil population dynamics of Gliocladium virens

At high initial inoculum level of *Gliocladium virens* (10⁶ CFU g⁻¹ soil), there was a gradual decrease in CFU/ g soil during incubation. The CFU after one day after inoculation was (31× 10⁵ CFU g⁻¹ soil) and decreased continually till the third month of

incubation reaching 2.45× 10³ CFU g⁻¹ soil. At low initial inoculum level (10³ CFU g⁻¹ soil), there was a gradual decrease in population level till complete disappearance after 3 months of incubation (Table 2).

Table 2. Soil population dynamics of *Gliocladium virens* at two inoculum levels

Inoculum level (CFU\ g soil)	CFU of <i>Gliocladium virens</i> / g dry wt. of soil				
	Period of incubation				
	1 day	15 days	1-Month	2-Months	3-Months
10 ⁶	31.37×10 ^{5a} ± 0.68	18.50×10 ^{3b} ± 1.29	15.75×10 ^{3c} ± 0.28	5.94×10 ^{3d} ± 1.34	2.45×10 ^{3e} ± 0.71
10 ³	13.23×10 ^{2a} ± 1.08	13.51×10 ^{2b} ± 0.11	7.08×10 ^{2c} ± 0.99	0.55×10 ^{2d} ± 1.37	-

Values are the mean of five replicates, ± standard error.

- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.

Mass production of *Gliocladium virens* conidia

As summarized in (Tables 3a, b, c and d), at 15% initial moisture level; the maximum population (CFU) of *Gliocladium virens* was obtained at the third week of incubation for maize seeds; wheat bran and wheat seeds and at the fourth week when using oat seeds; whereas further incubation was accompanied by a CFU decline. At the initial moisture level of 30%,

maximum CFU was obtained after 2 weeks of incubation for maize seeds and wheat bran and after 3 weeks for oat and wheat seeds. Further incubation was accompanied by a decline in population level till the fourth and fifth week of incubation for oat and wheat seeds.

Table 3a. Mass production of *Gliocladium virens* conidia on maize seeds at two moisture levels

Moisture level	CFU of <i>Gliocladium virens</i> / g fresh wt. of maize seeds				
	Period of incubation				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
15%	74.7×10 ^{5a} ± 0.73	1.4×10 ^{7b} ± 1.05	1.68×10 ^{7c} ± 0.45	11.6×10 ^{6d} ± 1.04	81.4×10 ^{5e} ± 0.77
30%	239.3×10 ^{5a} ± 0.75	4.49×10 ^{7b} ± 0.90	1.83×10 ^{7c} ± 0.87	18.8×10 ^{6d} ± 0.88	22.6×10 ^{6e} ± 0.21

Values are the mean of five replicates, ± standard error.

- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.

Table 3b. Mass production of *Gliocladium virens* conidia on oat seeds at two moisture levels

Moisture level	CFU of <i>Gliocladium virens</i> / g fresh wt. of oat seeds				
	Period of incubation				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
15%	73.5×10 ^{5a} ± 0.11	256×10 ^{5b} ± 1.91	21×10 ^{6c} ± 1.33	22×10 ^{6d} ± 1.49	224×10 ^{5e} ± 1.02
30%	153×10 ^{5a} ± 0.68	38×10 ^{6b} ± 0.89	39×10 ^{6c} ± 0.38	24×10 ^{6d} ± 0.37	250×10 ^{5e} ± 0.11

Values are the mean of five replicates, ± standard error.

- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.

Table 3c. Mass production of *Gliocladium virens* conidia on wheat bran at two moisture levels

Moisture level	CFU of <i>Gliocladium virens</i> / g fresh wt. of wheat bran				
	Period of incubation				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
15%	221.3×10 ^{6a} ± 1.98	356×10 ^{6b} ± 0.76	7.59×10 ^{7c} ± 0.99	179.5×10 ^{6d} ± 1.98	93.7×10 ^{6e} ± 0.54
30%	245.4×10 ^{6a} ± 0.88	45×10 ^{7b} ± 0.88	11.3×10 ^{7c} ± 1.32	236.6×10 ^{6d} ± 1.31	247.9×10 ^{6e} ± 1.09

Values are the mean of five replicates, ± standard error.

- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.

Table 3d. Mass production of *Gliocladium virens* conidia on wheat seeds at two moisture levels

Moisture level	CFU of <i>Gliocladium virens</i> / g fresh wt. of wheat seeds				
	Period of incubation				
	1 week	2 weeks	3 weeks	4 weeks	5 weeks
15%	75×10 ^{5a} ± 1.99	108.7×10 ^{5b} ± 0.81	2.8×10 ^{6c} ± 0.19	146.8×10 ^{5d} ± 0.78	129×10 ^{5e} ± 1.29
30%	143.2×10 ^{5a} ± 0.67	36.6×10 ^{6b} ± 0.10	11.2×10 ^{7c} ± 1.00	32.7×10 ^{6d} ± 0.89	15.8×10 ^{6e} ± 1.29

Values are the mean of five replicates, ± standard error.

• The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.

Estimation of shelf-life of formulated *Gliocladium virens*

The percentage of viable conidia of formulated *Gliocladium virens* at different storage temperatures

are shown in (Fig. 2). Storage at 4°C gave the highest percentages of germinable (viable) conidia (95%) of *Gliocladium virens* for up to 6 weeks.

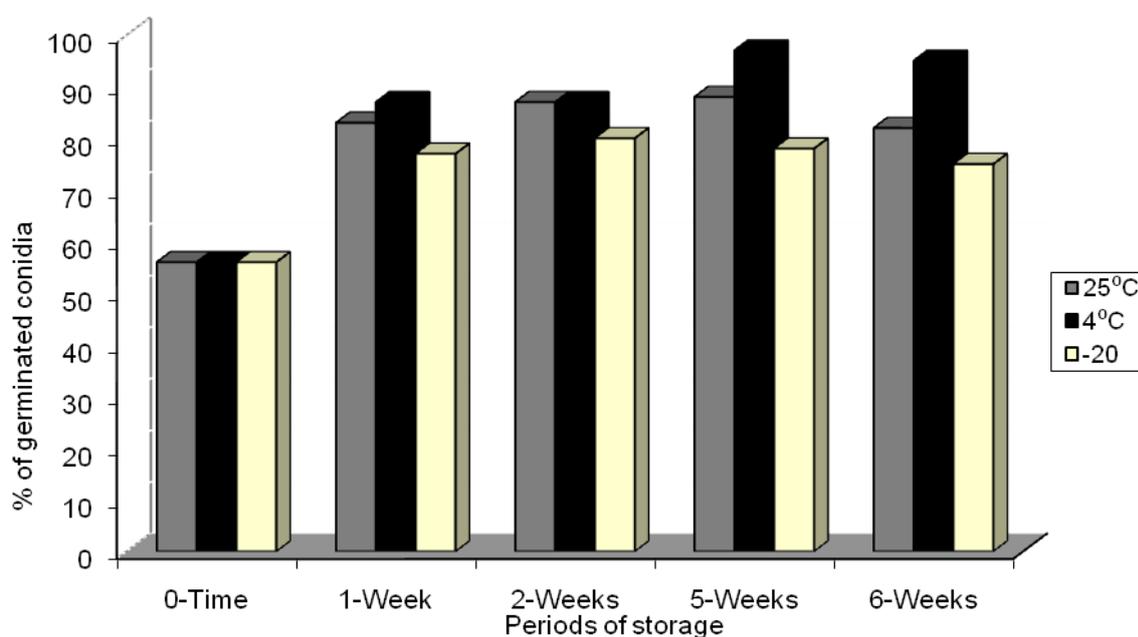


Fig. 2. Percentages of germination of formulated conidia of *Gliocladium virens* after storage at -20°C, 4°C and 25°C for different periods

Discussion

Rhizoctonia stem canker is caused by strains of the fungus *R. solani* and occurs wherever potatoes are grown. *In vitro*, *Trichoderma viride*, *P. oxalicum* and *Gliocladium virens* showed moderate inhibitory activity (30-35%) on HFMEA medium, whereas, the inhibitory activity of the other isolates on HFMEA medium ranged between 5 and 10%, thus were excluded from further research.

In dual culture technique, *P. oxalicum* and *Gliocladium virens* succeeded to surround the pathogen colony completely and prevented it from any radial spread. This means that the tested antagonists were unable to produce antifungal antibiotics but were able to compete with the pathogen for nutrients (especially carbon) and for space. However, *Trichoderma viride* showed mycoparasitic activity against *R. solani*, as its hyphae showed complete coiling around that of *R. solani*.

During the *in vivo* assay in the greenhouse, antagonists were inoculated into the soil in concentrations double that of *R. solani*; as density of the pathogen inoculum was unknown and to make their concentration close to the capacity limit of the soil as described previously by (Alabouvette *et al.*, 1997).

In the greenhouse, the growth of potato was promoted by the three used fungal bio-agents (at different degrees) compared with that of positive and non-infested control plants. This promotion might be due to solubilization of phosphates and micronutrients that could enhance plant growth as described earlier by (Altomare *et al.*, 1999). One another possible mechanism suggested by Sneh *et al.* (1998) for protection of potato plants against the pathogen; was the colonization of their roots and hypocotyls which worked as a masking barrier at recognition and infection sites, thus consequently prevented host penetration by the pathogen.

According to Diallo *et al.* (2011), some organisms promote growth by enhancing nutrient uptake (biofertilising) or abiotic/biotic stress tolerance, which may make host plants more able to cope with infection; others synthesize phytohormones which directly stimulate plant growth. Among the tested antagonists, *Gliocladium virens* gave appreciable plant growth promotion and therefore was selected for further assays.

Studies of soil population dynamics of *Gliocladium virens* showed a gradual decrease in CFU with increasing incubation periods. This might be due to the poor competition with soil microflora, thus conidia of *Gliocladium virens* died as a result of depletion of nutrients and water with increased periods of incubation. Maize kernels and wheat bran were the best solid substrates for the mass production of antagonist's conidia in accordance with by Babu *et al.* (2004). At initial moisture level of 30%, 10–1000 folds increase of antagonist's conidia were obtained more than that at 15%; because water causes swelling of the substrate and facilitates utilization of the

medium by the organisms and is necessary for the metabolic activities of the fungus as confirmed previously by Ridder *et al.* (1998).

As stated by Harman *et al.* (1991), the biomass used for biological control should be capable of being dried with retention of a high level of germinable propagules; be insensitive to environmental fluctuations (e.g. temperature and humidity) and possess a long shelf life. Storage of formulated *Gliocladium virens* at 4°C scored the highest percentage of germinable conidia. This longer shelf-life at low temperature could be attributed to the lower metabolic activity of the fungal propagules (Elzein *et al.*, 2004a). In accordance with Abdel-Kader *et al.* (2012), the use of stored formulated bio-agents might be considered as safe; cheap; avoid environmental pollution and easily applied.

Conclusion

Gliocladium virens acting as a bio-fungicide could be of help in the management of *R. solani* stem canker disease of potato and promotes its growth. The application of this bio-agent in the potato field soil should be repeated during the growing season for maintaining of an adequate population level, however, the fungal population might be more stable in the presence of plants, when they are able to colonize the rhizosphere. Maize seeds or wheat bran with moisture level of 30% could be suitable for fungal mass production and formulation; these should be stored at 4°C to increase their shelf life till delivery to the potato field.

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