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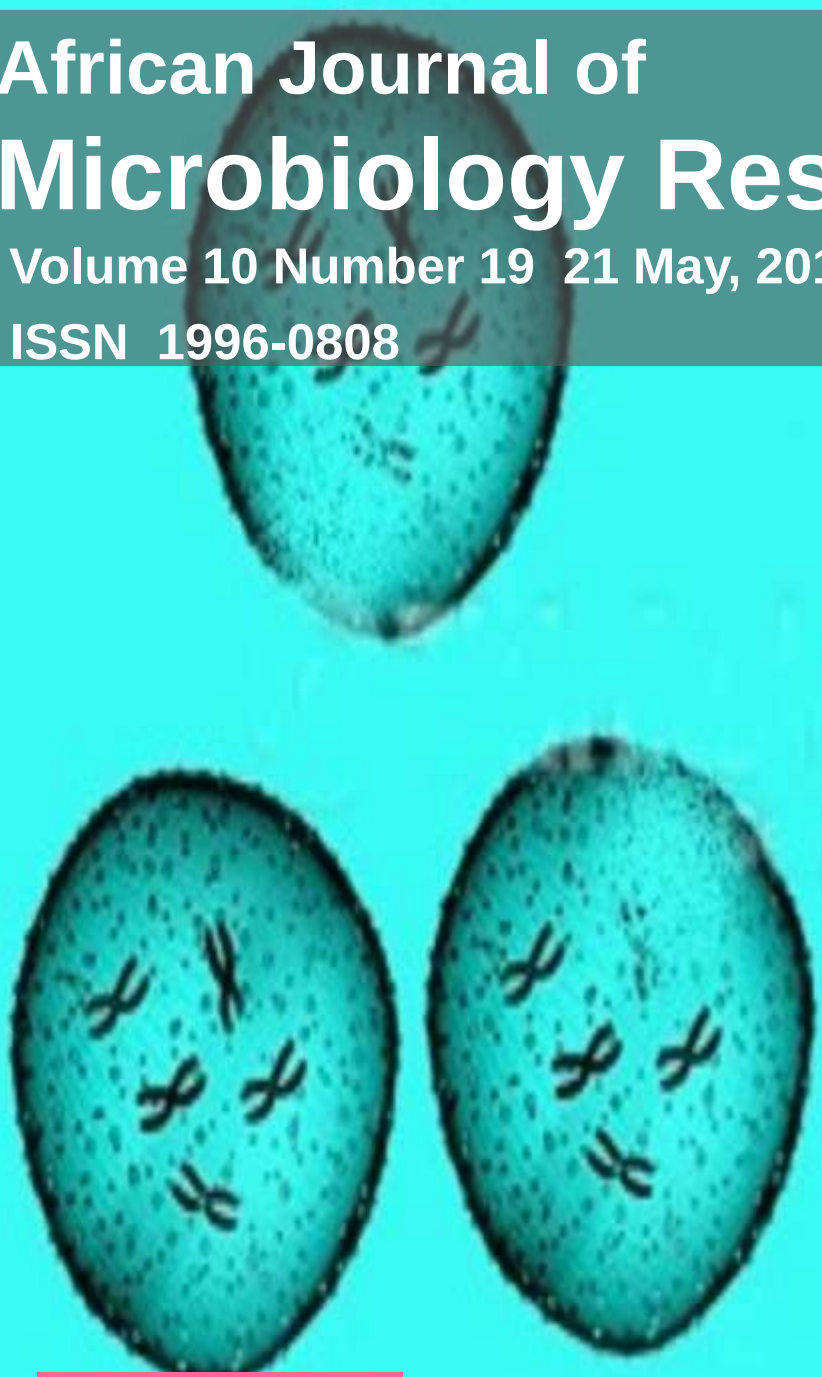

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Full Length Research Paper

Effects of growth-promoting endophytic *Methylobacterium* on development of *Citrus* rootstocks

Andréa Cristina Bogas[1], Carlos Ivan Aguilar-Vildoso[2], Aline Aparecida Camargo-Neves[3] and Welington Luiz Araújo[3]*

[1]Department of Genetics, University of São Paulo, ESALQ/USP, Av. Pádua Dias, 11, 13418-900, Piracicaba, SP, Brazil.
[2]Institute of Biodiversity and Forests, Federal University of Western Pará, IBEF/UFOPA, Rua Vera Paz s/n, 68035-110, Santarém, PA, Brazil.
[3]LABMEM/NAP-BIOP, Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 1374 -Ed. Biomédicas II, Cidade Universitária, 05508-900, São Paulo, SP, Brazil.

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Endophytic *Methylobacterium* spp. were inoculated on citrus seed and evaluated for their ability to promote growth of *Citrus limonia* and *Citrus sunki* seedlings under commercial nursery conditions. The germination rate and seedlings growth differed according to the combination between *Methylobacterium* species and citrus rootstock, showing that the interaction depends on their compatibility. *Methylobacterium* had no effect on germination of both rootstocks, except AR 1.6/2 that reduced the germination of *C. limonia*. On the other hand, some strains from citrus significantly promoted biomass production and height of aerial part of both rootstocks. The pathway of Indole-3-acetic acid (IAA) biosynthesis was identified in *M. mesophilicum* SR1.6/6 genome and this ability was confirmed in culture medium, suggesting that this mechanism is probably involved in growth promotion observed in present study. Recovery of strains in culture medium and ARDRA analysis confirmed the endophytic colonization of rootstocks by *Methylobacterium*. Our analyses of *C. limonia* and *C. sunki* seeds revealed that *Methylobacterium* is not vertically transferred to citrus plants. These results suggest that *Methylobacterium* can endophytically colonize the plant and have a potential for plant growth promotion under commercial nursery conditions. However, this growth promoting effect depends on specific interactions between *Methylobacterium* and citrus species.

Key words: *Methylobacterium*, *Citrus*, plant-bacteria interaction, growth promotion, Indole-3-acetic acid (IAA) pathway.

INTRODUCTION

Methylobacterium spp. are pink-pigmented facultative methylotrophic (PPFM) bacteria able to metabolize one-carbon compounds (C1), such as methanol, as well as C2, C3 and C4 compounds (Toyama et al., 1998;

*Corresponding author. E-mail: wlaraujo@usp.br.

Lidstrom, 2001). Member of this genus are ubiquitous in natural environments and have been isolated from soil, air, freshwater, sediments and plant surface (Gallego et al., 2005; Weon et al., 2008; Veyisoglu et al., 2013; Madhaiyan and Poonguzhali, 2014). In addition, *Methylobacterium* species have been found in symbiotic association with plants, colonizing the inner tissues (Bulgari et al., 2014; Madhaiyan et al., 2015).

The establishment of *Methylobacterium* in association with the host is thought to directly influence seed germination and plant growth by mechanisms, such as cytokinin and auxin synthesis (Omer et al., 2004; Tani et al., 2012; Eevers et al., 2015), nitrogen fixation (Sy et al., 2001, Madhaiyan et al., 2009; Madhaiyan et al., 2014) and plant protection (Ardanov et al., 2012; Yim et al., 2014). In this context, *Methylobacterium* seems to be an important bacterial group to be employed for improvement of crop productivity.

Nowadays, the availability of genetic tools such as the genome sequencing and studies of central metabolism have attracted attention to the genetic manipulation of *Methylobacterium* for the production of various bioproducts (Fitzgerald and Lidstrom, 2003; Choi et al., 2008, Sonntag et al., 2015) as well as for symbiotic control of phytopathogens (Gai et al., 2009; Ferreira Filho et al., 2012). The occurrence of new pests and diseases has affected the yield and production cost of several crops around the world. However, the economic importance of crops such as *Citrus* requires changes in the management and treatment of plants and use of new technologies to improve the productivity and quality of fruits (Duenhas et al., 2002; Donadio, 2011).

Therefore, bacteria associated with plants play an important role in the adaptation of their hosts, either in natural environments or in stressful conditions, and may be used as a strategy to promote better association between some crops and the environment (Hallmann et al., 1997; Sturz and Nowak, 2000; Bhattacharyya and Jha, 2012). In this way, the use of plant growth-promoting bacteria could be a possibility to improve the productivity and the quality of citrus orchards, reducing the use of chemical inputs, generating economic and ecological benefits, since these products might often affect the environment negatively.

Endophytic *Methylobacterium* has been isolated from citrus plants (Araújo et al., 2002) and previous studies have proposed an interaction between these bacteria and *Xylella fastidiosa* (Lacava et al., 2004; Araújo et al., 2002).

In citrus, one possibility to improve the productivity of the orchards could be through the inoculation of plant growth promotion endophyte (PGPE) into the rootstocks thereby, accelerating growth and consequently reducing the time that the plant remains in nursery. Therefore, in the present study we analyzed the effects of *Methylobacterium* species on seed germination and growth of *C. limonia* and *C. sunki* under commercial nursery conditions.

MATERIALS AND METHODS

Bacterial strains, culture conditions and plant material

In the present study, the effect of *Methylobacterium mesophilicum* (SR1.6/6, SR1.6/13, ER1/21, AR5/1), *M. extorquens* (AR1.6/2, AR1.6/11) and *M. radiotolerans* (AR1.6/4) isolated from *Citrus sinensis*; *M. mesophilicum* (PR1/3) and *M. zatmanii* (PR3/8) isolated from *Citrus reticulate*; *M. fujisawaense* (D5) isolated from *Saccharum officinarum* and *M. hispanicum* (TP4/2) isolated from *Capsicum annuum* were evaluated on the citrus growth promotion. For this, the bacteria were cultivated at 28°C in CHOI medium containing ($g\ L^{-1}$) $(NH_4)_2SO_4$, KH_2PO_4, $Na_2HPO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, and trace elements, supplemented with methanol 0.5% (v/v) as carbon source (Choi et al., 1989). The experiments were performed in greenhouse under commercial conditions for rootstocks (Rangpur lime - *Citrus limonia* and Sunki Tangerine - *Citrus sunki*) production (certified seeds, sterilized substrate, protected environment from insect vectors) at Horticitrus - Seedling Nursery, Cordeirópolis, SP, Brazil (22°28'59.9"S and 47°26'52.1"W).

In vivo assays

Evaluation of the effects of Methylobacterium spp. on seedlings growth of Citrus rootstocks

For seedlings production, the bark seeds (stored for 4 months in cold chamber at 5/8°C) was removed and the seeds treated with bacteria (10^8 CFU mL^{-1}) or CHOI medium (control 1), for 5 h at 28°C with agitation by an orbital shaker at 80 rpm, and planted in dibble tubes (3 × 12cm; 50 cm^3) containing commercial substrate pH 6.0 (Golden Mix 11, Amafibra, Holambra, SP, Brazil). Each treatment was composed of 4 replicates, where each replicate consisted of 10 seedlings (40 seedlings per treatment). The germination rate was estimated as the number of seedling growth per replicate.

After germination, only one seedling was replanted per dibble tube containing substrate pH 6.0 (Golden Mix 11, Amafibra, Holambra, SP, Brasil) and maintained in greenhouse under controlled relative humidity (70%) and temperature ranging from 19 to 40°C.

The effects of the endophytic *Methylobacterium* spp. on seedling growth were compared to the effects of the controls (CHOI and without inoculation). The seed germination rate was evaluated 30 days after inoculation of bacterial strains. The plant height was evaluated after 30, 90 and 120 days after germination, while the seedlings biomass (shoot and root) were evaluated only after 120 days.

Isolation of Methylobacterium from seeds and inoculated Citrus rootstocks

The bacterial communities inside citrus seeds were evaluated from fresh seeds and those stored for 4 months in cold chamber at 5/8°C, the latter usually used for rootstocks production. For this, 100 seeds from each citrus species were surface disinfected (70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl^-) for 3 min, 70% ethanol for 30 s, two rinses in sterilized distilled water for 1 min) and triturated in sterile PBS solution containing ($g\ L^{-1}$) Na_2HPO_4, 1.44; KH_2PO_4, 0.24; KCl, 0.20; NaCl, 8.00; pH 7.4. Serial dilution was plated on CHOI medium amended with 50 µg mL^{-1} of the fungicide carbendazim and incubated at 28°C for 15

days. After growth, colonies were picked out, purified by streaking on CHOI agar medium and stored for further evaluation.

The citrus rootstocks are used for grafting 120 days after seed planting. Therefore, after this time we evaluated the presence of inoculated *Methylobacterium,* in order to assess the potential of these bacteria to be transmitted aerial plant from inoculated rootstock, increasing the plant growth promotion. For this 10 seedlings per treatment were sampled, roots and shoots were separated and surface disinfection was performed as following: 70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl^-) for 3 min, 70% ethanol for 30 s, two rinses in sterilized distilled water for 1 min.

Both roots and shoots tissues were cut in small pieces, triturated in sterile phosphate buffered saline (PBS) solution and incubated at 28°C for 1 h under agitation (100 rpm). Appropriated dilutions (10^{-1}, 10^{-2}, 10^{-3}) were plated onto CHOI medium supplemented with 50 µg mL^{-1} of the fungicide carbendazim and incubated at 28°C for 15 days. The disinfection process was checked by plating aliquots of the sterile distilled water, used in the last wash, on CHOI and incubated under the same conditions. Colonies were purified by streaking in CHOI medium and stored in 70% glycerol solution at -80°C for further analysis.

Amplified ribosomal DNA restriction analysis (ARDRA) and molecular identification of endophytic bacteria

The bacterial community isolated from seeds was identified by 16S rRNA gene sequencing using colony-PCR. After growth on culture medium, the isolates were transferred to a tube containing 200 µL of sterilized ultra pure water. The bacterial suspension was used as the source of DNA in PCR reactions. Primers PO27F (5´-GAGAGTTTGATCCTGGCTCAG -3´) and 1387R (5´-CGGTGTGTACAAGGCCCGGGAACG - 3´) (Heuer and Smalla, 1997) were used in 50 µl PCR reaction containing 10 X buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3) (Fermentas Life Sciences, Brazil), 0.2 mM dNTP, 3.75 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Fermentas Life Sciences, Brazil), 0.2 µM of primers and 2 µl of the boiled cells (15 min at 90°C in 80 µl H_2O). Amplifications were performed in thermal cycler (PTC 200, MJ Research - USA) programmed for an initial denaturation (94°C for 4 min) followed by 35 cycles of 94°C for 30 s, 62.5°C for 1 min, 72°C for 1 min and a final extension of 10 min at 72°C. A negative control (PCR mixture without bacterial DNA) was included in all analysis. The PCR fragments were purified with polyethylene glycol (PEG) (20% PEG 8000; 2.5 mM NaCl) and sequenced at Human Genome Research Center (HGRC), (Institute of Biosciences, University of São Paulo, São Paulo, SP, Brazil). The nucleotide sequences were compared with sequences information available in the GenBank database, by BLASTn.

In order to compare with inoculated bacteria, the isolates obtained from seedlings rootstock shoot were identified by ARDRA technique and 16S rRNA gene sequencing. For this, the 16S rRNA gene was amplified by *Methylobacterium* colony-PCR using PO27F and 1387R primers as described above. The PCR products were digested with 2 units of *Alu*I restriction enzyme (Life Technologies, Brazil) according to the manufacture´s recommendations. The reaction mixture was incubated at 37°C for 1 h. The restriction patterns were examined using 2.5% (w/v) agarose gel and stained with ethidium bromide.

In vitro assays for production of plant growth promoting substances

Auxin production

Auxin production (IAA) was evaluated by the colorimetric method as described by Gordon and Weber (1951) with modifications. For this, the bacteria were grown in 10% TSB medium (10 mL) amended with 5 mM of L-tryptophan and incubated in the dark at 28°C for 72 h. Cells were harvested by centrifugation (8 000 x g for 5 min), the supernatant (900 µl) was treated with 600 µl of Salkowski reagent (50 mL of perchloric acid (35%) and 1 mL of $FeCl_3$ solution (0.5 M) and incubated for 30 min at room temperature in the dark. IAA was quantified using a spectrophotometer (Pharmacia Biotech Ultroespec 3000) at 530 nm of absorbance. The readings were normalized by using the standard curve with different concentrations of IAA (µg mL^{-1}). All analysis was performed in triplicate.

Biological nitrogen fixation

The ability to fix N_2 in *Methylobacterium* strains was evaluated in strains able to improve plant growth. The strains were inoculated in nitrogen-free semi-solid NFb medium (Döbereiner et al., 1995) and, after growth the bacterial cells were re-inoculated in nitrogen-free NFb medium. This procedure was repeated three times and strains able to form a growth pellicle in this medium were considered able to fix nitrogen.

Phosphorus solubilization

The ability to solubilize phosphorus was assayed according to Verma et al. (2001) with modifications. Ten microliters containing about 10^8 CFU mL^{-1} of bacterial strains grown in CHOI medium were inoculated onto agar medium containing inorganic phosphate (agar, 15 g; glucose, 10 g; NH_4Cl, 5.0 g; NaCl, 1.0 g; $MgSO_4.7H_2O$, 1 g; $Ca_3(HPO_4)_2$, 0.8 g) in a final volume of 1 L filled with distilled water, pH 7.2. Bacteria were incubated at 28°C for 7 days. The ability to solubilize inorganic phosphate was characterized by a clear halo around bacterial colonies. All analysis was performed in triplicate using a *Burkholderia seminalis* (TC3.4.2R3) as a positive control.

Screening of genes associated to nitrogen fixation, IAA biosynthesis and ACC deaminase in *Methylobacterium mesophilicum* SR1.6/6

M. mesophilicum SR1.6/6 genome was published as announcement (Almeida et al., 2013) under access ANPA01000000 in NCBI (www.ncbi.nlm.nih.gov/) thus some genomic re-annotation of genes that encode enzymes related to nitrogen fixation, IAA biosynthesis and ACC deaminase were made in order to confirm the physiological results approached in this present work. Artemis software (Rutherford et al., 2000) was used to overview the genome annotation as well as KEGG: Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and Biocyc (http://biocyc.org/) to study target pathways, such as IAA biosynthesis and ACC deaminase.

Statistical analysis

The ESTAT (Barbosa et al., 1992) statistical package was used for ANOVA followed by 2×2 factorial design (plant height × month) and Tukey's test at 5% probability level for means comparison.

RESULTS

Checking the occurrence of *Methylobacterium* spp. in rootstocks seeds

No bacteria were recovered from fresh seeds indicating that in citrus cultivable endophytic bacteria are not

Table 1. Effect of *Methylobacterium* strains on seed germination and growth of *Citrus limonia* under commercial nursery conditions.

Bacterial species	Strains	Seed Germination (%)	Plant height (cm)			Root dry biomass (g)	Root fresh biomass (g)	Shoot dry biomass (g)	Shoot fresh biomass (g)
			30 days	90 days	120 days				
Control		71.1^{ab}	7.62^{a}	19.01^{abc}	23.42^{de}	0.40^{bc}	1.62^{c}	1.23^{abcd}	3.66^{ab}
CHOI control		73.5^{a}	8.81^{a}	18.74^{bc}	24.37^{bcd}	0.39^{bc}	1.56^{c}	1.16^{bcd}	3.55^{ab}
M. mesophilicum	SR1.6/6	69.2^{ab}	8.19^{a}	20.48^{ab}	24.42^{bcd}	0.48^{a}*	2.07^{a}*	1.40^{ab}	4.16^{a}
	AR5/1	68.6^{ab}	8.15^{a}	19.57^{abc}	25.70^{abc}	0.34^{c}	1.54^{c}	1.14^{bcd}	3.59^{ab}
	SR1.6/13	62.8^{b}	8.27^{a}	18.75^{bc}	23.63^{cd}e	0.37^{bc}	1.70^{bc}	1.09^{cb}	3.72^{ab}
	ER1/21	70.8^{ab}	8.19^{a}	18.50^{bc}	24.02^{cd}	0.32^{c}	1.56^{c}	1.04^{cd}	3.74^{ab}
	PR1/3	69.4^{ab}	8.69^{a}	19.67^{abc}	23.84^{cd}	0.40^{bc}	1.69^{bc}	1.27^{abc}	4.01^{a}
M. extorquens	AR1.6/2	47.85^{c}*	8.46^{a}	19.55^{abc}	23.18^{de}	0.41^{bc}	1.59^{c}	1.24^{abcd}	3.83^{ab}
	AR1.6/11	63.4^{b}	7.83^{a}	21.02^{a}*	26.56^{a}*	0.45^{ab}	2.00^{ab}*	1.40^{ab}	4.32^{a}
M. radiotolerans	AR1.6/4	67.9^{ab}	8.10^{a}	20.29^{ab}	26.41^{ab}	0.43^{abc}	1.71^{bc}	1.27^{abc}	3.96^{ab}
M. zatmanii	PR3/8	68.7^{ab}	9.13^{a}	19.24^{abc}	25.21^{abcd}	0.33^{c}	1.50^{d}	1.11^{cd}	3.41^{ab}
M. fujisawaense	D5	70.1^{ab}	8.47^{a}	17.90^{c}	23.93^{cd}	0.39^{bc}	1.61^{c}	1.14^{bcd}	3.97^{ab}
M. hispanicum	TP4/2	68.5^{ab}	8.60^{a}	18.57^{bc}	21.73^{e}	0.39^{bc}	1.63^{c}	0.99^{d}	3.06^{b}

*Means followed by the same letter show no significant differences by the Tukey's test at $p \leq 0.05$.

vertically transferred from plant to plant. However, bacteria were isolated from seeds stored for 4 months. This community ranged from $0.81.10^3$ to $0.76.10^1$ UFC g^{-1} for *C. limonia* and *C. sunki*, respectively. Based on 16S rRNA gene sequencing, the microbial community from stored seeds of *C. limonia* and *C. sunki* was composed of *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Streptomyces* and *Mycobaterium*. Isolates close related (pink pigmented) to *Methylobacterium* spp. were not found, indicating that this group is not able to colonize the seeds under these conditions. No differences in bacterial communities were observed between *C. limonia* and *C. sunki.*

Effects of *Methylobacterium* spp. on seed germination and seedlings growth of *C. limonia*

The seed germination ranged from 47.85 to 73.5%. The inoculation of *M. extorquens* AR1.6/2 reduced the seed germination, while the others strains have no effect on this plant growth parameter (Table 1). However, the presence of this *M. extorquens* AR1.6/2 strain has no negative effect on plant growth (Table 1) indicating that this bacterium could be inoculated only after seed germination.

M. extorquens AR1.6/11 and *M. mesophilicum* SR1.6/6 inoculation resulted in plant growth promotion. The strain AR1.6/11 was able to increase plant height after 90 and 120 and root fresh biomass at 120 days, while the strain SR1.6/6 increased root dry and fresh biomass at 120 days (Table 1). The TP4/2 strain promoted a negative effect on height plant 120 days after inoculation (Table 1). Based on these results, the strains AR1.6/11, AR1.6/2, SR1.6/6 and TP 4/2 were selected for further analysis in *C. sunki*. The colonization of rootstocks seedlings by AR1.6/11, AR1.6/2 and SR1.6/6 was evaluated 120 days after inoculation. Except for AR1.6/11 that was recovered only from *C. limonia* roots, all strains were re-isolated from shoots and roots tissues of both rootstocks at 10^2 CFU g^{-1} of plant tissue. The identity of the strains was successfully confirmed by ARDRA technique with *Alu*I restriction enzyme.

Effects of *Methylobacterium* spp. on seed germination and seedlings growth of *C. sunki*

Unlike our finds in *C. limonia*, the strain AR1.6/2 has no negative effect on germination of *C. sunki* seeds (Table 2). All treatments induced seed germination when compared with the control, but this effect was due to the CHOI medium, since the germination rate between CHOI medium and bacterial inoculated seeds were not significantly different (Table 2). The strains SR1.6/6, AR1.6/2 and AR1.6/11 increased the plant height after 120 days. In addition, AR1.6/2 strain enhanced shoot fresh biomass (Table 2). *C. sunki* inoculated with

Table 2. Effect of *Methylobacterium* strains on seed germination and growth of *Citrus sunki* under commercial nursery conditions.

Bacterial species	Strains	Germination (%)	Plant height (cm)			Root dry biomass (g)	Root fresh biomass (g)	Shoot dry biomass (g)	Shoot fresh biomass (g)	IAA production ($mg.ml^{-1}$)
			30 days	90 days	120 days					
Control		61.7[b]	5.05[a]	11.07[c]	19.82[b]	0.21[a]	1.02[ab]	1.20[ab]	3.40[c]	
CHOI control		80.7[a]	5.40[a]	12.17[bc]	19.71[b]	0.15[b]	0,93[ab]c	1.13[b]c	3.38[c]	
M. mesophilicum	SR 1.6/6	80.0[a]	5.40[a]	12.72[ab]	21.37[a]*	0.21[a]	1.05[a]	1.22[ab]	3.80[ab]	2.3
M. extorquens	AR 1.6/11	81.0[a]	5.25[a]	12.87[a]*	20.45[a]*	0.19[ab]	0.83[b]c	1.21[ab]	3.66[abc]	1.8
M. extorquens	AR 1.6/2	83.7[a]	5.57[a]	12.60[ab]	21.22[a]*	0.19[ab]	1.00[ab]	1.29[a]	3.90[a]*	2.1
M. hispanicum	TP 4/2	79.2[a]	5.57[a]	12.32[bc]	19.55[b]	0.18[ab]	0.92[ab]c	1.05c	3.36c	

*Means followed by the same letter show no significant differences by the Tukey's test at $p \leq 0.05$.

TP4/2 strain no presented negative effect on height plant as observed to *C. limonia* (Table 2).

In vitro plant growth promoting traits

Bacterial strains that exhibited positive effect on seedlings growth were evaluated *in vitro* for their ability to synthesize indole-3-acetic acid (IAA), phosphorus solubilization and N_2 fixation. The strains SR 1.6/6, AR 1.6/2, and AR 1.6/11 produced IAA at 2.3, 2.1 and 1.8 mg ml^{-1} (Table 2), respectively. A clear zone halo around the colonies was not observed, indicating that these strains are not able to solubilize inorganic phosphorus. Although a growth had been observed in nitrogen-free medium after the first bacterial inoculation, it was not observed after successive inoculation in this medium, indicating that these strains were not able to fix nitrogen.

M. mesophilicum SR 1.6/6, which had the genome published (Almeida et al., 2013) was one of the most effective strain in promoting height plant and root biomass. We screened this genome and found at least 22 genes potentially related to IAA synthesis. According to these information, there are three possible pathways in *M. mesophilicum* SR 1.6/6 for the production of indole-acetic acid, (1) from idole-3-acetonitrile, (2) from indole-3-acetamide, (3) tryptamine (Figure 1). In the first route, the Indole-3-acetonitrile is catalyzed to Indole-acetic acid by Nitrilase/cyanide hydratase and N-acyltransferase. Through the second route, Indole-3-acetamide can be synthetized from Indole-3-acetonitrile by a nitrile-hydratase (subunit alpha and beta), and then to IAA by amidase. In the third route, IAA could be synthetized via tryptamine and indoleacetaldehyde. We did not find any Trp decaborxylase and tryptophan-2-monooxygenase enzymes to metabolize L-Tryptophan in *M. mesophilicum* SR 1.6/6 genome.

In addition, an evaluation of the SR1.6/6 genome showed that *nif* and *fix* gene clusters are not present in this strain, but a gene that encodes an ACC deaminase is present in the *M. mesophilicum* SR 1.6/6 genome, indicating that the suppression of stress response could be a mechanism present in these endophytic bacteria related to plant growth promotion.

DISCUSSION

In sustainable agriculture the application of growth-promoting bacteria may increase productivity and quality of the crop, reducing the costs associated with the use of chemicals and the environmental impacts. Previously studies had shown that *Methylobacterium* species have the ability to induce shifts in physiological traits of their hosts, promoting positive, negative or any effect on seed germination and/or on plant development (Holland and Polacco, 1994, Abanda-Nkpwatt et al., 2006; Lee et al., 2011; Pohjanen et al., 2014). The specificity of plant-bacteria interaction has been considered an important factor for the generation of these different effects on plant host and the success of this approach for plant production.

Our results revealed that some *Methylobacterium* strains originally isolated from *Citrus sinensis* promoted significantly the height and biomass of *C. limonia* and *C. sunki*, as already described in other plant species with native *Methylobacterium* (Madhaiyan et al., 2005; Lee et al., 2006; Madhaiyan et al., 2015). In fact, some authors emphasize the need to employ native isolates or isolates adapted to their hosts, justifying the higher capacity of colonization and lower risk in the introduction of exogenous microorganisms in the plants (Enebak et al., 1998; Khalid et al., 2004). However, as we observed, bacterial strains isolated from *Citrus,* such as

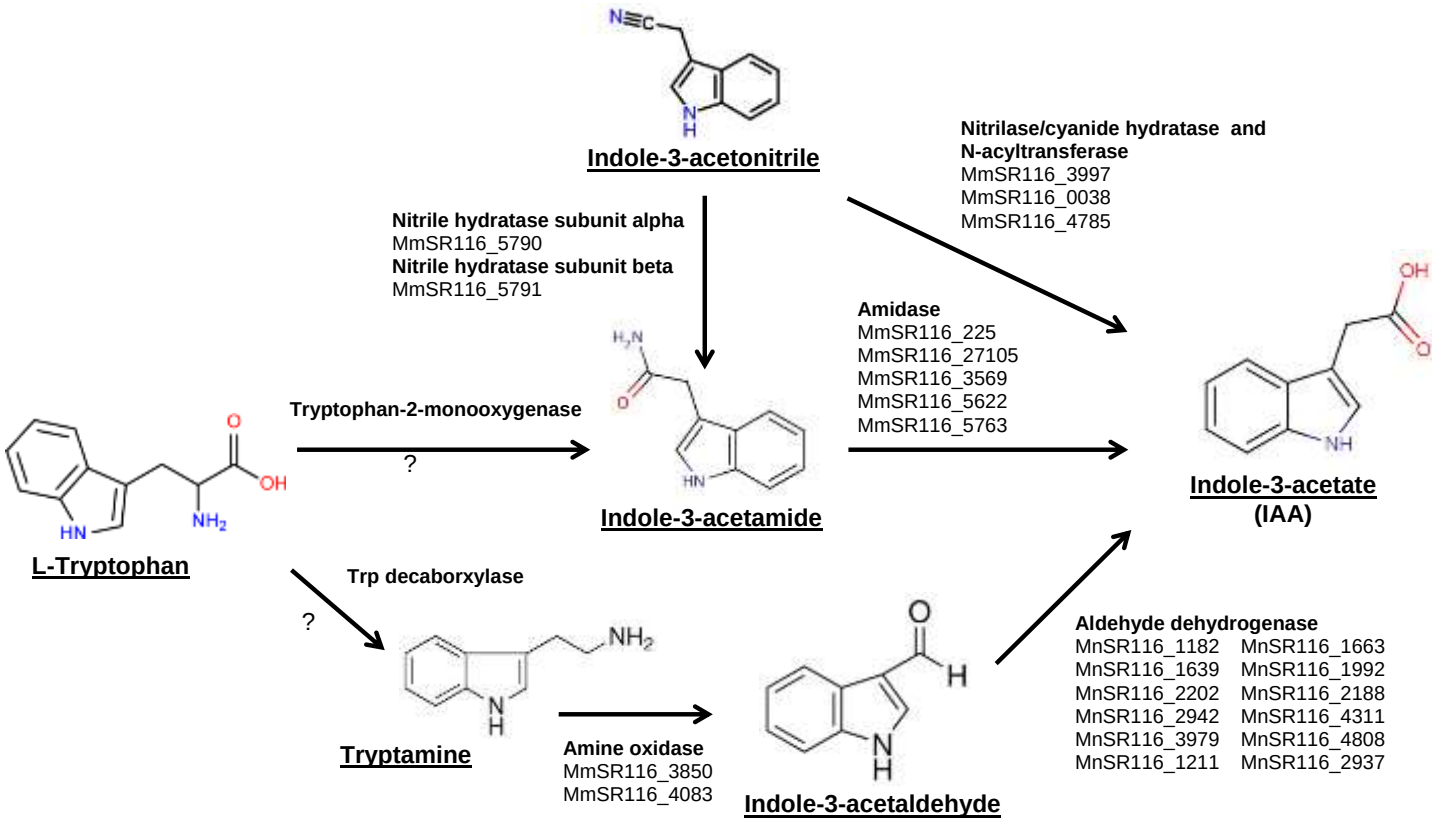

Figure 1. Predicted IAA biosynthesis pathways in *M. mesophilicum* SR 1.6/6.

AR1.6/2 induced negative effects on seed germination of *C. limonia,* but had no effect on plant growth of both *C. limonia* and *C. sunki.* In addition, this strain promoted the plant height and enhanced the shoot biomass.

During plant colonization, the cross-talking between bacteria and the host seems to has a key function (Rosenblueth and Martinez-Romero, 2006), which suggest that plants can communicate specifically to attract microorganisms for their own ecological and evolutionary benefits (Compant et al., 2005). Thus, endophytes may differentially express genes required to colonize the host and modulate plant genes stimulating plant growth (Rosenblueth and Martinez-Romero, 2006). In many cases, the plant responses to endophytes seem to be conditioned by the plant genotype (Nowak, 1998; Rosenblueth and Martinez-Romero, 2006). Besides, the environmental conditions and balance between other microorganisms can also be decisive for the final effect (Azevedo, 1998; Andrews and Harris, 2000; Montesinos et al., 2002).

Plant growth promotion by *Methylobacterium* isolates has been attributed to the nitrogen fixation and production of phytohormones such as auxins and gibberellins (Sy et al., 2001; Madhaiyan et al., 2014; Eevers et al., 2015).

In the present study, all *Methylobacterium* strains with potential to promote plant growth were able to synthetized IAA, but could not to solubilize phosphorus and growth in nitrogen-free medium. This result suggested that, although many factors, such as species-specific recognition may be related to the ability of bacteria to promote plant growth, the citrus growth promotion, based on height and biomass analysis seems to be only related to IAA production and ACC deaminase. These results were confirmed by the analysis of the genome of SR1.6/6 strain. Besides, a gene that encodes an ACC deaminase is present in the *M. mesophilicum* SR1.6/6 genome, indicating it could act in the suppression of the stress response resulting in plant growth promotion. The IAA plant hormone, under biotic and abiotic stress conditions, is able to activate the enzyme 1-aminocyclopropane 1-carboxylate (ACC) synthase, which synthetize ACC that is converted to ethylene by the enzyme ACC oxidase (Wang et al., 2002). The enzyme ACC deaminase, present in many endophytes, is known to compete with ACC oxidase, modulating the ethylene levels in plants, reducing the stress response triggered by ethylene and promoting plant growth under stress conditions (Hardoim et al., 2008).

The inoculated *Methylobacterium* strains were not present in seeds, but were able to colonize roots and

shoots endophytically after seed inoculation, as evident by their recovery from seedlings obtained from inoculated seeds. Generally, in experiments carried out in greenhouses the inoculated bacteria are subject to competition with other microorganisms present in the soil or inside the plant, as described previously (Omer et al., 2004), suggesting that the evaluated *Methylobacterium* strains present competitiveness to establish inside the plant even under this environment condition. The AR 1.6/11 was recovered only from root of inoculated plants, suggesting that the ability to colonize the plant shoot is not necessary for promoting the plant growth, since this strain was able to increase the plant height.

Our results obtained by *Methylobacterium* seed inoculation highlights the potential use of these bacteria to stimulate seed germination, plant height and biomass production of *C. limonia* and *C. sunki* under commercial nursery conditions. We observed that the non-specificity of bacteria-plant interaction could lead to an undesired effect on the plant. Thus, is very important to conduct a selection of strains in a breeding program and being careful to not extrapolate the results produced on one plant to another host. Future proposals may be the development of a bacterial consortium with specific strains that could be effective in citrus growth promotion and protection generating a low-cost and environmentally safe product.

Conflict of Interests

The authors have not declared any conflict of interests.

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Removal capacity of faecal pathogens from wastewater by four wetland vegetation: *Typha latifolia, Cyperus papyrus, Cyperus alternifolius* and *Phragmites australis*

H. J. Kipasika[1], J. Buza [1], W. A. Smith[2] and K. N. Njau[1]*

[1]Nelson Mandela African Institution of Science and Technology, P. O. Box 447 Arusha, Tanzania.
[2]One Health Institute, School of Veterinary Medicine, University of California Davis, CA, USA.

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The ability of four wetland vegetation: *Typha latifolia, Cyperus papyrus, Cyperus alternifolius* and *Phragmites mauritianus* in removing pathogenic and indicator microorganisms in the wetlands were studied in bucket experiments. The findings suggested that vegetated systems can effectively reduce faecal pathogens in wastewater. Both *Salmonella* species and *Escherichia coli* removal efficiencies were above 98%. This proved the positive use of plants in bacteria removal from wastewater. Nevertheless, removal of faecal bacteria differed significantly between macrophytes where *C. alternifolius* and *T. latifolia* were the most effective followed by *C. papyrus* and the least was *P. mauritianus*. The study also observed no significant difference between planted and unplanted buckets. The effect of physicochemical parameters such as dissolved oxygen, pH, temperature and salinity were thought to influence the bacterial removal.

Key words: Constructed wetland, *Typha latifolia*, *Cyperus papyrus*, *Cyperus alternifolius*, *Phragmites mauritianus*, *Salmonella* species, *Escherichia coli*.

INTRODUCTION

Macrophytes play an important role in maintaining the wetland ecosystem. They have the capacity to improve water quality by removing faecal pathogens present-in with wastewater. The influence is principally explained by supply of oxygen to the roots, which plays a crucial role in the activity and type of metabolism performed by microorganisms in the root zone (Stottmeister et al., 2003), especially the grazing predators like protozoan, nematodes and zooplankton and lytic bacteria and viruses (Vymazal, 2005). Another potential source of removal is the adsorption by bio-films on the rock media and plant roots (Stevik et al., 2004; Stott and Tanner, 2005). Macrophytes also affect faecal pathogens by excreting toxic antimicrobial substances from their roots (Sundaravadivel and Vigneswaran, 2001; Stottmeister et al., 2003). The roots which grow vertically and horizontally favour the removal by enhancing hydraulic pathways and increase the contact time (Vymazal et al., 1998; Stottmeister et al., 2003). Other mechanisms reducing microbial contaminants in vegetated systems are natural

*Corresponding author. E-mail: karoli.njau@nm-aist.ac.tz.

Table 1. Characteristic of the selected macrophytes and their use in CWs of Sub-Saharan Africa.

Macrophytes	Common name	General characteristic	Maximum water depth	pH tolerance	% reduction of faecal indicator bacteria	Use in CWs of sub-Saharan Africa
Typha latifolia	Cattail	Grow aggressively with large biomass	30 cm (Reed, 1993)	3.0-8.5 (Davis, 1995)	91% (Mashauri et al., 2000)	Tanzania (Mashauri et al., 2000; Njau et al., 2011).
Cyperus papyrus		Unique due to its C_4 photosynthetic pathway (Mnaya et al., 2007)	-	-	>98% (Kansiime and Mwesigye, 2012; Abou-Elela et al., 2014)	Kenya (Vymazal, 2013); Ethiopia (Tadesse, 2010); Uganda (Okurut et al., 1999; Kansiime and Mwesigye, 2012).
Cyperus alternifolius	Umbrella sedge	-Strong-growing rhizomes which quickly establish a large clump -Positive visual impact	-	-	90% (Leto et al., 2013)	-
Phragmites mauritianus	Reed	-Highly invasive with poor wildlife value -Not recommended for storm-water wetlands (Davis, 1995)	60 cm (Reed, 1993)	3.7- 8.0 (Davis, 1995)	>96% (Reinoso et al., 2008; Abou-Elela et al., 2014)	Sudan (Vymazal, 2013); Tanzania (Njau et al., 2011; Mairi et al., 2013)

temperature, unfavourable pH, and presence of toxic chemicals (Vymazal et al., 1998; Stevik et al., 2004) and sedimentation (Stott, 2003) as well as pathogen-sediments interaction (Searcy et al., 2006). Removal may also depend on water type and salinity which significantly affect pathogens settling (Hogan et al., 2013).

The contribution of UV light may not be effective in some wetlands due to shadowing effects of full growing macrophytes which protect pathogens from effective exposure (Naja and Volesky, 2011) or presence of substrate in case of sub-surface wetland systems.

Nevertheless, various studies have demonstrated that the effect of macrophytes on pathogen reduction may be irrelevant when compared with unplanted beds (Sleytr et al., 2007; Mburu et al., 2008; Torrens et al., 2009). The comparative effect brought by unplanted system was described by a tracer test study from Torrens et al. (2009), who discovered there was enough oxygen transfer in unplanted system facilitated by batch loading and diffusion process from the air. Although, the discovery provides clues on observed differences, which earlier sought to be unclear, yet the information is insufficient, because other factors such as macrophyte type and system design might also contribute. Macrophytes differ and their efficiency on pollutant removal is not similar; varying across plant species and with plant phenology (Fu et al., 2002). As reviewed by Faulwetter et al. (2009), root oxygen release and the diversity of the rhizosphere microbial community differs according to macrophyte species and on environmental conditions. Hogan et al. (2013) cited that different aquatic plants may vary in the ability to remove parasites due to distinct surface properties, unique biofilms, and differential effects on water flow and drag. Therefore, the choice of the macrophytes is of particularly important aspect in the design of a constructed wetland. The choice should include several factors, such as geographical distribution, climate and habitat conditions, wastewater composition, availability of the plants, long term maintenance, agronomic management costs, and the project aims (Leto et al., 2013).

In this study, four types of macrophytes: *Typha latifolia, Cyperus papyrus, Cyperus alternifolius* and *Phragmites mauritianus* commonly occurring in natural wetlands of Tanzania were selected to evaluate how vegetation type affects the removal of faecal indicator bacteria and pathogens under controlled laboratory conditions.

The evaluated plants are capable of surviving and proliferating in extreme tropical climates (Katsenovich et al., 2009). Characteristics and the use of the selected macrophytes in constructed wetlands (CWs) of sub-Saharan Africa are described in Table 1.

METHODOLOGY

Experimental site and design

The set-up was conducted at the Nelson Mandela African

Figure 1. Buckets experimental set-up with various macrophytes at NM-AIST, Tanzania.

Institution of Science and Technology (NM-AIST) in Arusha, Tanzania. The area is at an altitude of 1,400 m above sea level on the slopes of Mount Meru (Latitude 03° 24' S, Longitude 036° 47' E). The region is characterized with distinct wet and dry season and cool, dry air for much of the year. The temperature ranges between 13 and 30°C with an average of around 25°. The experiment was performed in a greenhouse with an area of 120 m^2. Inside the greenhouse, the temperature was averaged 27°C during the study period. Fifteen circular buckets made of plastic (PVC), with dimension 80 cm long and 54 diameter were filled with graded gravel, granite type, with size 12 to 20 mm to cover a depth of 60 cm. All the gravel was thoroughly washed with tap water to remove silt and debris before use. The porosity of the media was 0.35. Twelve buckets were planted with four wetland-macrophytes, each in triplicates, and three buckets were left unplanted to stand as controls.

Planting and macrophytes growing

Four types of wetland vegetation were studied: *T. latifolia, C. papyrus, C. alternifolius* and *P. mauritianus* (Figure 1). These plants were preferred based on the criteria of locally and widely distributed, easily propagated and able to tolerate waterlogged-anoxic and hyper-eutrophic conditions (Thomas et al., 1995). The plants were transferred from the surrounding natural marshes and planted on the same day in the buckets. Each plant composed of a piece of rhizome cut into lengths of 8 inches (20 cm) or two to three nodes. The cuts were evenly spaced in the buckets at densities of 4, 4, 4 and 8 cuts per bucket for *T. latifolia, C. papyrus*, *C. alternifolius* and *P. mauritianus*, respectively. After planting, the buckets were filled and kept in irrigation with hydroponic nutrient solution (Hoagland) to almost 10 cm beneath the gravel layer. The systems were emptied and refilled with the new nutrient solution once a week. Six months later, before sampling, tape water replaced the hydroponic nutrient solution as the medium solution.

Organisms

Escherichia coli (strain K-12) and *Salmonella enteric Serovar Typhimurium* labelled with green fluorescent protein (GFP) were used in this study. Both *E. coli* and *GFP Salmonella* were prepared by incubating broth with isolated colony on rotating shaker at 37°C, 120 rpm for 12 to 16 h. After a late log-phase, a measure of 10^8 cells per millilitre was obtained from Optical Density (OD) measurement using a Spectrophotometer (UNICO 2800 UV/VIS) set at OD_{600} and dilution reads 0.1. The broth for culturing *E. coli* and *GFP Salmonella* comprised of tryptic soy broth (TSB; Difco Laboratories, France) and Luria-Bertani broth (LB; Difco Laboratories, France) mixed with antibiotic Carbenicillin (Fisher

Table 2. Average value of physical and chemical parameters.

Vegetation type	No. of samples	Temperature (°C)		pH		DO (mg/L)		Salinity (ppt.)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Typha latifolia	18	24.6	0.30	6.95	0.03	1.43	0.19	0.47	0.01
Cyperus papyrus	18	24.8	0.16	6.74	0.02	1.30	0.10	0.50	0.02
Cyperus alternifolius	18	24.4	0.18	7.09	0.02	1.26	0.14	0.53	0.02
Phragmites mauritianus	18	25.0	0.26	7.18	0.07	1.19	0.08	0.50	0.05
Unplanted (control)	18	24.7	0.20	8.08	0.09	2.07	0.66	0.40	0.00

BioReagent), respectively.

Sampling and analysis

The amount of 5 × 10^8 cells per millilitre for both *Salmonella* spp. and *E. coli* were mixed together with 500 L of water in a plastic tank and then introduced to the buckets by using a hosepipe. Samples were collected in each bucket at the bottom tap in the interval of 6, 12, 24, 48, and 72 h. They were collected in sterile glass bottles and kept in an ice-packed cooler and transported to the laboratory at NM–AIST. They were processed within 4 h of collection. Both samples of *E. coli* and GFP *Salmonella* were analysed using membrane filtration protocol in accordance with conventional methods (Standard Methods for Examination of Water and Wastewater - (APHA, 1998)). The membranes for *E. coli* were placed in plates with Hach's m-ColiBlue24 Broth and incubated at 35°C for 24 h for complete enumeration. *E. coli* colonies appeared blue. The membranes of *GFP Salmonella* were plated in LB Carb+ and incubated at 37°C for 24 h followed by verifying their green autofluorescence colonies under UV illumination (Cole-Palmer UV-Transilluminator). Physical/Chemical parameters such as DO, pH, and temperature were measured directly by using a Multi-Parameter Digital Meter (Thermo Scientific Orion 4 Star) and recorded onsite.

Statistical analysis

Data were processed by using the Origin Version 8 software to obtain the trends in the concentration and the IBM-SPSS Version 20 for comparison and testing significance under one-way analysis of Variance (ANOVA). Comparison was considered significantly different at $p < 0.05$.

Evaluation of the results

It is assumed that the bucket experiments can be modelled as batch reactors; however, mixing was very poor due to lack of stirring or flowing of water. In this regard, faecal bacterial removal is modelled based on a first order kinetics model where removal depends on influent concentrations, retention or travel time, and a first order rate constant (Vymazal, 2005; Kadlec and Wallace, 2008). The first-order reaction equation is described as:

$$\ln\frac{C_t}{C_0} = -k \times \mathrm{HRT}$$

where C_t is the microbial concentration at a given time (cfu/100 ml), C_0 is the initial microbial concentration (cfu/100 ml), k is the first-order rate constant (h^{-1}) and HRT is the hydraulic retention time (h). Since all parameters are known, the observed values of k as the slope of the regression line were obtained by plotting $\ln\frac{C_t}{C_0}$ against the values of HRT. Comparing k- values for various macrophyte species helps to determine the different removal rate constants for each species.

RESULTS AND DISCUSSION

Effect of physicochemical parameters on reduction of *Salmonell*a spp. and *E. coli*

Table 2 summarises the average value of physical and chemical parameters in the planted and unplanted buckets. Temperatures in all buckets were almost identical, ranging between 24 and 25°C. The highest temperature was observed in *P. mauritianus* (25.0± 0.2°C) and the lowest was in *C. alternifolius* (24.4± 0.18°C). The pH in planted buckets was almost neutral while the unplanted buckets were alkaline. Very little variation in DO was observed in the planted systems (1.2 to 1.4 mg/L). The highest oxygen concentration was observed in unplanted system (2.07 ± 0.66). All experimental buckets had low salinity (<1 ppt.).

Usually, the removal of bacteria and other pollutants depend on biological and chemical reactions occurring at specific physicochemical environmental parameters such as temperature, pH and dissolved oxygen (Naja and Volesky, 2011). Temperature in all systems was almost the same with average values ranging from 24 to 25°C. This range normally favours the elimination of micro-organisms in porous media. The review done by Kristian et al. (2004) in a survival experiment using *Pseudomonas* spp. in soils revealed no difference between 5 and 15°C, but a significant reduction of the bacterial numbers at 25°C. Temperature appeared to be a factor with a positive influence upon the behaviour and removal of bacteria in surface and subsurface flow CW in Leon, Spain (Molleda et al., 2008).

The survival of most bacteria decreases at both low

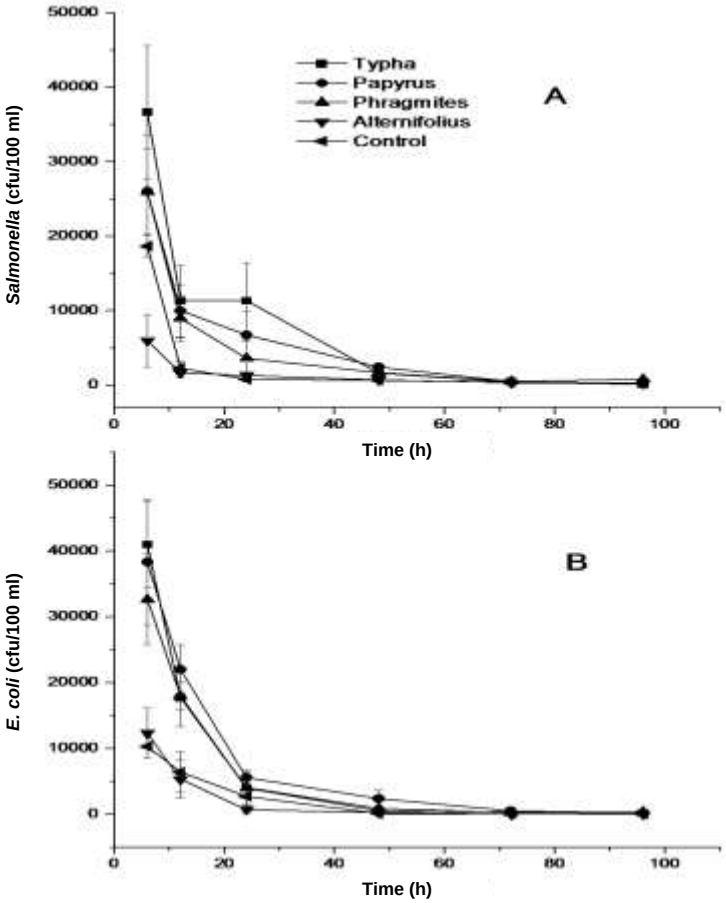

Figure 2. Concentrations of *Salmonella* spp. (A) and *E. coli* (B) per 100 ml volume of water plotted over time from buckets experiments planted with *T. latifolia, C. papyrus, C. alternifolius* and *P. mauritianus* and in unplanted buckets that stands as a control. Three replicates under each wetland condition were tested to evaluate the effect of vegetation on the removal of faecal pathogen and indicator bacteria from the system.

and high pH (Mawdsley et al., 1995; Stevik et al., 2004). The survival of *Salmonella* spp. and *E. coli* was found to be optimal when pH falls between 5 and 6.4 (Stevik et al., 2004). Therefore, as the average pH was around neutral and alkaline in planted and unplanted systems respectively, a considerable reduction of both *Salmonella* spp. and *E. coli* can be expected.

Most of enteric bacteria such as *Salmonella* spp. and *E. coli* are facultative or obligate anaerobes and thus the presence of oxygen creates unfavourable growth conditions (Vymazal, 2005). The presence of oxygen also facilitates the survival of predators for bacteria such as protozoans and lytic bacteria and viruses (Vymazal, 2005). However, this experiment observed less DO in planted than unplanted systems (Table 2). This was in contrast to other findings where the values of DO were greater in the planted systems due to the release of oxygen through roots into the rhizosphere (Stottmeister et al., 2003). It may be that more oxygen in planted systems was utilized for decomposition of organic matter released from root decay and droppings from macrophytes (Hench et al., 2003; Kyambadde et al., 2004). Similarly, the absence of macrophytes, in unplanted systems, encouraged greater atmospheric aeration in the substrate which facilitates, in some cases, the growth of algae with significant release of oxygen during algal photosynthesis (Leto et al., 2013). This increases pH as well as the carbonate-bicarbonate equilibrium is destabilised (Mashauri et al., 2000).

Effect of macrophytes on reduction of *Salmonella* spp. and *E. coli*

Figure 2 shows the trends on the removal of *Salmonella* spp. and *E. coli*. The overall removal for both *Salmonella*

Table 3. Effect of macrophytes on reduction of *Salmonella* spp. and *E. coli*.

Vegetation type	No. of samples	*Salmonella*				*E. coli*			
		k	SE	R^2	% Removal	k	SE	R^2	% Removal
Typha latifolia	36	0.055	0.006	0.946	99.04	0.057	0.009	0.878	99.59
Cyperus papyrus	36	0.045	0.006	0.927	98.75	0.055	0.005	0.964	99.39
Cyperus alternifolius	36	0.051	0.006	0.933	99.31	0.080	0.007	0.967	99.89
Phragmites mauritianus	36	0.046	0.007	0.889	98.55	0.053	0.010	0.841	98.88
Unplanted (control)	36	0.067	0.012	0.865	99.97	0.071	0.012	0.878	99.61

and *E. coli* was above 98% (Table 3). *C. alternifolius* achieved the greatest removal for both bacteria followed by *T. latifolia, C. papyrus* and the least was *P. mauritianus*. The same trend was also observed along the values of kinetic rate constant (k). When statistically tested, the vegetation types differed significantly in the removal of faecal bacteria ($p < 0.05$). A plot of fitted regression line for all treatments showed a very good linear correlation with R^2 above 0.84 (Table 3).

Both *Salmonella* spp. and *E. coli* were reduced in all treatment systems by almost two orders of magnitude (99%). These reductions are in line with other reports on removal of faecal coliforms in wetlands (Okurut et al., 1999; Molleda et al., 2008). The reductions were encouraging since the average concentrations for both *Salmonella* spp. and *E. coli* after 4 days retention time were below 400 cfu/100 ml. *C. alternifolius* has the least concentration (*Salmonella* 55 cfu/100 ml; *E. coli* 10 cfu/100 ml) and the *P. mauritianus* observed the highest (*Salmonella* 400 cfu/100 ml; *E. coli* 350 cfu/100 ml). Findings from various operating systems suggested that removal efficiency of faecal pathogens in planted systems is primarily influenced by hydraulic characteristics and presence of vegetation (Vymazal et al., 1998; Stottmeister et al., 2003; Vymazal, 2005). The effect of retention time can simply be explained; the longer the hydraulic retention time, the longer the bacteria are exposed to unfavourable conditions (Vymazal, 2005).

Despite the high removal of faecal bacteria during the 4 days of operation, substantial differences in performance were noted between the vegetation. When the comparison was established between kinetic rate constant (k), *C. alternifolius* outperformed other vegetation. The effect is probably caused by relative high pH, DO and salinity (Table 2) as compared to other vegetated systems. *T. latifolia* and *C. papyrus* appeared the second and third, respectively, and the least was *P. mauritianus*. Abou-Elela et al. (2014) found that *C. papyrus* which grows and distributes more widely in the bed was more effective in the removal of bacterial indicators as compared to *P. mauritianus.* Higher removal of faecal indicator bacteria was observed in *T. latifolia* as compared to *P. mauritianus* (Kaseva, 2004). In contrast to this study, Leto et al. (2013) and Katsenovich et al. (2009) observed better removal of faecal indicator bacteria in *T. latifolia* as compared to *C. alternifolius.* It was explained that significantly higher yields of biomass above and below the ground contributed to the highest performance in *T. latifolia* (Leto et al., 2013).

Planted vs. unplanted treatments system

Comparing the rate of removal between planted and unplanted systems, there were no significant differences ($p<0.05$) on removing both *Salmonella* and *E. coli*. This can be explained by relative high DO and pH, which facilitates the reduction of bacteria in unplanted as compared to planted systems. Similar to these findings, several studies had also experienced no significant difference between planted and unplanted systems for removal of indicators organisms and pathogens (Sleytr et al., 2007; Mburu et al., 2008; Torrens et al., 2009). The comparable effect might also be attributed to diffusion of oxygen from the air (Torrens et al., 2009) and exposure to UV light (Sleytr et al., 2007) in unplanted systems.

Conclusion

Findings of the present study suggested that vegetated systems can effectively reduce faecal pathogens in wastewater. Both *Salmonella* spp. and *E. coli* removal rates were above 98%. This indicates the positive use of plants in bacteria removal from wastewater. Removal of faecal bacteria differed significantly between macrophytes where the comparison of the rate constants showed that *C. alternifolius* and *T. latifolia* were the most effective followed by *C. papyrus* and the least was *P. mauritianus.* On the other hand, no significant difference was observed between planted units compared with the unplanted ones. The observed physicochemical parameters such as DO, pH, temperature and salinity were thought to influence the differences. However, the results of pot/ bucket experiments might not reflect the actual field conditions due to several factors such as growing conditions, choice of the type of pot, application of

nutrients and general adaptation of bacteria. Therefore, the present study recommends further researches to be conducted in the field on evaluating the removal efficiency of faecal pathogens using different macrophytes.

Conflicts of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Molecular characterization and virulence of *Beauveria bassiana* and *Metarhizium anisopliae* against *Galleria mellonella* (Lepidoptera: Pyralidae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae

Margy A. Esparza Mora[1]*, Julie G. Chacón-Orozco[2], Ricardo Harakava[3], Janaina Ribeiro Costa Rouws[4] and Marcelo E. Fraga[5]

[1]Plant Health and Applied Biotechnology Program - Instituto de Ciência Biológica e da Saúde - Federal Rural University of Rio de Janeiro - BR 465 Km 07 - ZIP. 23890-000, Seropédica - RJ, Brazil.
[2]Health, Food Safety and Environmental in Agribusiness - Instituto Biológico - ZIP. 04014-002, São Paulo, SP, Brazil.
[3]Phytopathology and Biochemistry Laboratory, Instituto Biológico, São Paulo, SP, Brazil.
[4]Embrapa Agrobiologia - BR 465, Km 07 - ZIP. 23890-000, Seropedica - RJ, Brazil.
[5]Mycology Laboratory - Research Center in Mycology and Mycotoxicology - Veterinary Institute - Federal Rural University of Rio Janeiro - BR 465 Km 07 - ZIP. 23890-000, Seropédica - RJ, Brazil.

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Entomopathogenic fungi considerably vary in their action and virulence mode. The contamination mainly depends on the adhesion and penetration of the fungus in the host integument. Four isolates from *Beauveria bassiana* and *Metarhizium anisopliae* were molecularly characterized by rDNA-ITS sequencing. Their virulence against last instar larvae of *Galleria mellonella* and *Tenebrio molitor* at four conidia concentrations: 1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia.ml^{-1} were *in vitro* evaluated and the lethal concentrations (LC_{50} and LC_{90}) as lethal time (LT_{50} and LT_{90}) were determined. Sequencing of rDNA-ITS 500 bp fragments allowed the identification of Ma10MI, Ma12MI, Ma58MI and Ma11MI isolates as *M. anisopliae* and Bb11MI, Bb79MI, Bb53MI and Bb27MI isolates as *B. bassiana* by sequence comparison to GenBank. According to the pathogenicity test, *B. bassiana* strains with higher performance for *G. mellonella* were Bb53MI and Bb79MI with $LC_{50}= 3.98\times10^7$ and 1.04×10^7 conidia.ml^{-1} and $LT_{50}= 5.46$ and 5.27 days, respectively, as well as to *T. molitor* was Bb79MI with $LC_{50}= 1.03\times10^7$ conidia.ml^{-1} and $LT_{50}=$ 5.57 days. The *M. anisopliae* strains with the best performance were Ma58MI and Ma10MI both *T. molitor* ($LC_{50}= 1.0\times10^6$ and 1.0×10^7 conidia.ml^{-1} and $LT_{50}= 4.06$ and 5.22 days, respectively) and *G. mellonella* ($LC_{50}= 6.3\times10^7$ and 1.1×10^8 conidia.ml^{-1} and $LT_{50}= 5.18$ and 6.37 days, respectively), these isolates might be considered as new promising candidates for the microbial pest control.

Key words: Pathogenicity, molecular taxonomy, entomopathogenic fungi, microbial control.

INTRODUCTION

It is well known that synthetic insecticides have deleterious effects on the worldwide environment and consequently, attention has addressed to biocontrol agents as suitable alternatives (Safavi et al., 2007). Entomopathogenic fungi are natural common enemies of arthropods, particularly insects, and they might be used in the management of pest populations in agroforestry and urban ecosystems (Lacey and Kaya, 2007). The

most important species, *Beauveria bassiana* (Vuill.) Balsam and *Metarhizium anisopliae* (Metch) Sorok. are associated with the Phylum *Ascomycota*, Order Hypocreales, Families Clavicipitaceae and Cordycipitaceae, respectively (Hibbett et al., 2007). Insect pathogenic fungi have to meet several host challenges producing enough new infectious spores in each generation maintaining viable populations. First, successful transmission often requires the release of massive spore numbers and/or sticky spore surfaces or substances maximizing adhesion in other ways (Vega et al., 2012). Second, spores should germinate and initiate penetration of the solid insect exoskeleton relatively quickly (Cole and Hoch, 1991). Third, the fungal cells must proliferate inside the hemocell, muscles, or other host body tissues collapsing the host immune system in order to the host dies shortly after (Vega et al., 2012). Fourth, the fungal pathogen should manage the host cadaver optimizing spore production and dispersal under prevailing environmental conditions (Roy et al., 2006).

Entomopathogenic fungi act by contact and are able to infect different live stages of insects with piercing-sucking mouthparts. They present horizontal and vertical transmissions, viz., inside the population (among individuals) and over time (across the progenies), respectively. Despite these advantages, a number of factors, such as the difficulty for environmental adaptability, resistance to chemicals, and lower ready-effect compared to the chemicals may limit the use of these fungi for the pest management in greenhouse, and especially, in field (Shah and Pell, 2003; Quesada Moraga et al., 2006a; Wraight et al., 2007). Nevertheless, a wide variability within and among species has been detected in pathogenicity, virulence and ecological features in many entomopathogenic fungi. This variability has been considered and analyzed for selecting candidates for myco-insecticides production (Quesada Moraga et al., 2006a; Wraight et al., 2007).

This study aimed at characterizing and evaluating the pathogenicity of *B. bassiana* and *M. anisopliae* strains isolated from Atlantic Forest soil, Rio de Janeiro, Brazil, against two insect hosts models: *Galleria mellonella* and *Tenebrio molitor,* also comparing the effect of entomopathogenic fungi against Lepidoptera and Coleoptera orders.

MATERIALS AND METHODS

Source of insects and fungal isolates

Four *B. bassiana* (Bb27MI, Bb11MI, Bb79MI and Bb53MI) strains and four *M. anisopliae* (Ma58MI, Ma10MI, Ma11MI and Ma12MI) from Atlantic forest soils in the Rio de Janeiro state, Brazil, were used in the experiment. The fungi were isolated according to the methodology described by Esparza Mora et al. (2016). The strains were maintained at 4°C on potato dextrose agar (PDA). Last instar larvae of *G. mellonella* and *T. molitor* were obtained from the Biological Institute in Campinas - SP. *G. mellonella* was fed by an artificial diet containing 472 g wax pure bee, 96 g skimmed milk powder, 188 g powdered yeast, 385 g maize flour, 160 g soya, 416 g of glycerin, 300 ml water distilled, while *T. molitor* was reared on bread.

Molecular identification of strains

After cultivation, DNA from the strains on PDA media for 7 days at 25 ± 1°C, DNA was extracted according to the CTAB method (Doyle and Doyle, 1987). The polymerase chain reaction (PCR) for amplification of fragment Internal Transcribed spacers (ITS) region was performed by a pair of universal primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990), generating estimated size of 500 bp product. The amplification reaction conditions consisted of 2 min at 94°C followed by 40 cycles of 15 s at 94°C, 30 s at 56°C and 2 min at 72°C with a final extension of 4 min at 72°C. The products were visualized under UV light in 1% agarose gels stained by ethidium bromide. The PCR products were purified by precipitation with polyethylene glycol according to the protocol described by Schmitz and Riesner (2006). The sequencing was performed by chain termination method with 3.1 BigDye (Applied Biosystems) reagent and ABI3500 automatic sequencer (Applied Biosystems). After sequencing, phylogenetic tree with similar sequences of the ITS1 and ITS4 strains regions was completed and sequences of different fungal species were obtained from the GenBank/NCBI, using the Maximum Likelihood method (ML), evaluating the strength of the topology, the tree was assessed by the Jukes-Cantor method with 1000 bootstrap replicates, and phylogenetic analysis was carried out in the Molecular Evolutionary Genetics analysis-MEGA 6.0.

Preparation of inoculum

Each strain was cultivated in Petri dishes containing sterile parboiled rice, maintained at 25 ± 1°C for 8 days. The conidia were collected with by sterile loop and suspended in sterile distilled water containing 0.1% Tween 80, and then agitated in vortex for 3 min. Four *M. anisopliae* and *B. bassiana* conidia concentrations: 1×10^9, 1×10^8, 1×10^7 and 1×10^6 conidia.ml^{-1} were prepared. The treatment control received only sterile distilled water with 0.1% Tween 80. The conidial counts in each suspension with an improved Neubauer Hemacytometer (Marienfeld, Germany) were determined.

Virulence of conidia

Ten (10) *G. mellonella* and *T. molitor* larvae were immersed in 10 ml of fungal suspension for 30 s. Thereafter, the treated insects were placed on filter paper (Whatman No. 1) inside a plastic box (18 × 8.5 × 4.5 cm) with ventilation holes (2 cm^2) on three sides. Food was introduced into the box and the ventilation holes were covered with a metal screen. The set-up was placed inside a climatic chamber at 25 ± 1°C. Three replicates per treatment with 30 species of each insect per repetition were accomplished.

*Corresponding author. E-mail: aleesparza18@hotmail.com.

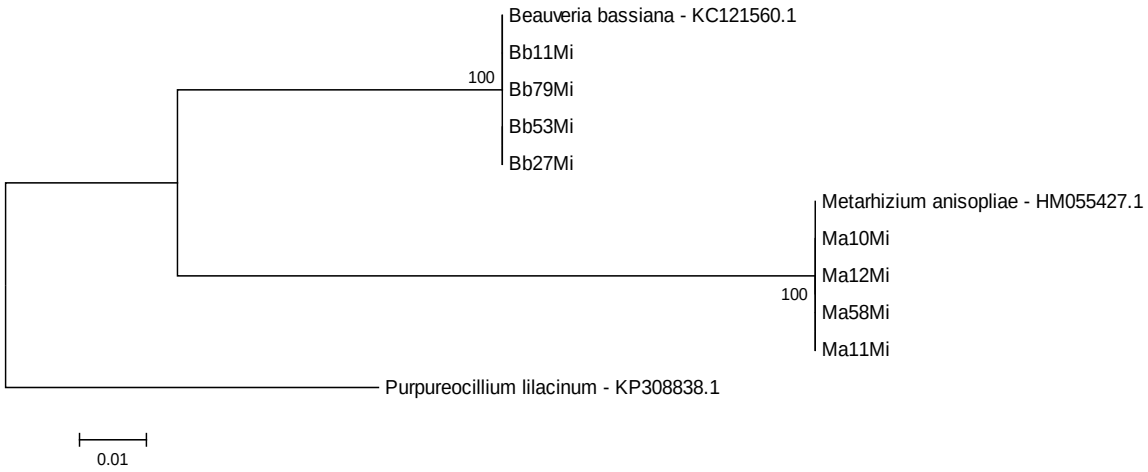

Figure 1. Phylogenetic tree constructed by ML method, Jukes-Cantor showing the relation between the strains of entomopathogenic fungi obtained from Atlantic Forest, and other related species deposited in the GenBank-NCBI. The sequences used are of the ITS regions of rDNA. Built tree in MEGA 6.0 program. The "bootstrap" values for 1,000 repetitions are indicated next to the tree branches. The *Purourеocillium lilacinum* ITS sequences was designated as the outgroup for rooting the tree.

Mortality was daily recorded for 10 days after inoculation. The fungi were re-isolated from dead insect samples selected randomly from each replicate box.

Statistical analysis

The results of *G. mellonella* and *T. molitor* insects mortality according to completely randomized design with three replications in a factorial 4 × 8 corresponding to four concentrations (1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia.ml^{-1}) and 8 species of fungi (Bb11MI, Bb27MI, Bb53MI, Bb79MI, Ma10MI, Ma11MI, Ma12MI and Ma58MI) were analyzed. After the analysis of variance, average between factor levels at 192 h of evaluation by Scott Kant test at 5% probability was compared. Statistical analyses by the SISVAR program (Ferreira, 2011) were performed.

RESULTS

Molecular characterization

The ITS1 – 5.8 – ITS4 rDNA sequences were compared with those available in the GenBank data base for *M. anisopliae* (accession numbers HM055427.1) and *B. bassiana* (KC121560.1). The amplification of the ITS region resulted in a single product for all isolates. The size of the product was about 500 bp.

The alignments and phylogenetic analysis confirmed the taxonomic identity of the strains used in our study. *M. anisopliae* Ma10MI, Ma12MI, Ma58MI and Ma11MI strains grouped from the same species strain sequence from the database, at 100% similarity. Among the *B. bassiana* species, it was observed that the strains Bb11MI, Bb79MI, Bb53MI and Bb27MI composed a grouping with *B. bassiana* strain from GenBank, at 100% similarity (Figure 1).

Evaluation of virulence

According to the Scott Knott test (α = 0.05), the pathogenic strains to *G. mellonella* were: Bb79MI, Bb27MI, Ma10MI and Ma58MI in three (1×10^7, 1×10^8 and 1×10^9 conidia.ml^{-1}) concentrations and Bb53MI (1×10^8 and 1×10^9 conidia.ml^{-1}) moderately; comparing the middle and lower values of larvae mortality at different concentrations, it was observed that Bb11MI, Ma11MI and Ma12MI strains were less pathogenic in 1×10^6 1×10^7 and 1×10^8 conidia.ml^{-1} concentrations (Table 1). Bb27MI and Bb79MI *B. bassiana* strains were more pathogenic than *T. molitor* in 1×10^7, 1×10^8 and 1×10^9 conidia.ml^{-1} concentrations; as well as Ma10MI and Ma58MI *M. anisopliae* strains were more pathogenic than *T. molitor* in 1×10^9, 1×10^7 and 1×10^8 conidia.ml^{-1} concentrations (Table 2).

Bb79MI *B. bassiana*, strain presented the highest pathogenicity on *G. mellonella* larvae with an 80.66% average mortality, $LC_{50} = 1.04\times10^7$ conidia.ml^{-1} and LT_{50}= 5.27 days (Table 3). Ma58MI *M. anisopliae* strain was the most virulent on *G. mellonella* larvae presenting 83.33% mortality, $LC_{50} = 6.31\times10^7$ conidia.ml^{-1} and LT_{50}= 5.18 days. Bb79MI strain presented 78% mortality in *T. molitor*, $LC_{50} = 1.03\times10^7$ conidia.ml^{-1} and $LT_{50} = 5.57$ days. Ma58MI *M. anisopliae* strain presented 82% mortality in *T. molitor,* $LC_{50} = 1.00\times10^6$ conidia.ml^{-1} and $LT_{50} = 4.05$ days.

The LT_{90} values to *B. bassiana* strains on *G. mellonella* larvae ranged from 8.03 to 10.23 days, and for *M. anisopliae* strains on *G. mellonella* larvae ranged from 8.29 to 10.47 days. In *T. molitor* larvae, the LT_{90} *B. bassiana* strains ranged from 9.24 to 10.04 days, in *M. anisopliae* strains the LT_{90} ranged from 7.18 to 11.26 days. The LC_{90} also varied depending upon the insect species and strain. The *B. bassiana* strain presenting the

Table 1. Mortality *G. mellonella* larvae at different conidia concentrations of *B. bassiana* and *M. anisopliae*.

Concentration Conidia.ml^{-1}	Bb11MI	Bb27MI	Bb53MI	Bb79MI	Ma10MI	Ma11MI	Ma12MI	Ma58MI
1×10^{9}	100.0^{Aa}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}
1×10^{8}	93.3^{bB}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	76.7^{bC}	93.3^{bB}	100.0^{aA}
1×10^{7}	83.3^{cB}	100.0^{aA}	96.6^{aA}	100.0^{aA}	100.0^{aA}	63.3^{cD}	70.0^{cC}	100.0^{aA}
1×10^{6}	70.0^{dC}	73.3^{bC}	80.0^{bB}	83.3^{bB}	86.7^{bB}	56.7^{dD}	73.3^{cC}	96.7^{aA}
Control	20.0^{eA}	20.0^{cA}	20.0^{cA}	20.0^{cA}	20.0^{cA}	20.0^{eA}	20.0^{dA}	20.0^{bA}

Averages followed by different letters, small letters on columns and capital letters on lines differ by Scott Knott test at 5%. Mortality average at 8 days.

Table 2. Mortality *T. molitor* larvae at different conidia concentrations of *B. bassiana* and *M. anisopliae*.

Concentration Conidia.ml^{-1}	Bb11MI	Bb27MI	Bb53MI	Bb79MI	Ma10MI	Ma11MI	Ma12MI	Ma58MI
1×10^{9}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}
1×10^{8}	90.0^{bB}	100.0^{aA}	100.0^{aA}	100.0^{aA}	100.0^{aA}	90.0^{bB}	100.0^{aA}	100.0^{aA}
1×10^{7}	86.7^{bC}	100.0^{aA}	93.3^{bB}	100.0^{aA}	100.0^{aA}	70.0^{cD}	83.3^{bC}	100.0^{aA}
1×10^{6}	63.3^{c}E	70.0^{bD}	80.0^{cC}	80.0^{bC}	100.0^{aA}	66.7^{cD}	86.7^{bB}	100.0^{aA}
Control	10.0^{dA}	10.0^{cA}	10.0^{dA}	10.0^{cA}	10.0^{bA}	10.0^{dA}	10.0^{cA}	10.0^{bA}

Averages followed by different letters, small letters on columns and capital letters on lines differ by Scott Knott test at 5%. Mortality average at 8 days.

Table 3. LC_{50} and LT_{50} values of *B. bassiana* and *M. anisopliae* strains against *G. mellonella* and *T. molitor*.

Strain/Insect	LC_{50}	Confidence interval 95%	LT_{50} (days)	Confidence interval 95%
Bb11MI				
G. mellonella	9.12×10^{8}	4.97×10^{6}-2.28×10^{9}	6.99	5.69-7.02
T. molitor	7.94×10^{7}	4.76×10^{6}-2.23×10^{8}	5.42	5.31-5.90
Bb53MI				
G. mellonella	3.98×10^{7}	1.34×10^{7}-2.29×10^{8}	5.46	5.36-6.09
T. molitor	5.87×10^{7}	1.68×10^{7}-2.05×10^{8}	5.73	5.43-6.12
Bb79MI				
G. mellonella	1.04×10^{7}	1.45×10^{6}-5.75×10^{8}	5.27	5.11-5.74
T. molitor	1.03×10^{7}	1.32×10^{6}-4.76×10^{8}	5.57	5.39-6.03
Bb27MI				
G. mellonella	5.53×10^{7}	7.74×10^{6}-2.05×10^{8}	5.56	5.37-5.99
T. molitor	3.98×10^{7}	7.74×10^{6}-2.05×10^{8}	5.83	5.63-6.18
Ma58MI				
G. mellonella	6.31×10^{7}	1.67×10^{7}-2.38×10^{9}	5.18	4.79-5.36
T. molitor	1.00×10^{6}	2.88×10^{5}-3.48×10^{9}	4.06	3.89-4.68
Ma10MI				
G. mellonella	1.10×10^{8}	1.96×10^{7}-1.28×10^{8}	6.37	5.79-6.89
T. molitor	1.00×10^{7}	2.88×10^{6}-3.48×10^{10}	5.22	4.97-5.66
Ma12MI				
G. mellonella	1.59×108	3.93×107-1.21×109	6.17	6.00-6.45
T. molitor	1.13×108	4.60×106-2.77×107	6.59	5.15-5.87
Ma11MI				
G. mellonella	2.12×109	1.28×108-3.51×1010	7.69	6.87-7.91
T. molitor	6.21×108	3.15×106-1.22×109	6.27	6.11-6.46

Table 4. CL_{90} and LT_{90} values of *B. bassiana* and *M. anisopliae* strains against *G. mellonella* and *T. molitor*.

Strain/Insect	LC_{90}	Confidence interval 95%	LT_{90} (days)	Confidence interval 95%
Bb11MI				
G. mellonella	8.31×10^{11}	1.44×10^{10}-4.79×10^{12}	10.23	9.42-10.52
T. molitor	8.46×10^{11}	1.49×10^{11}-4.82×10^{12}	10.04	9.98-10.14
Bb53MI				
G. mellonella	2.15×10^{10}	2.15×10^{10}-6.84×10^{11}	9.38	9.10-9.67
T. molitor	3.16×10^{10}	3.29×10^{9}-2.11×10^{11}	9.48	9.23-9.68
Bb79MI				
G. mellonella	6.31×10^{9}	1.67×10^{8}-2.39×10^{10}	8.03	7.85-8.16
T. molitor	1.00×10^{10}	1.45×10^{9}-6.88×10^{10}	9.42	9.23-9.57
Bb27MI				
G. mellonella	1.00×10^{10}	6.95×10^{9}-1.44×10^{11}	9.19	9.00-9.31
T. molitor	8.34×10^{10}	6.95×10^{10}-1.44×10^{11}	9.68	9.46-9.78
Ma58MI				
G. mellonella	1.00×10^{9}	6.60×10^{8}-1.51×10^{10}	8.29	8.06-8.47
T. molitor	9.90×10^{8}	8.48×10^{8}-1.18×10^{9}	7.18	7.02-7.38
Ma10MI				
G. mellonella	9.98×10^{9}	2.88×10^{9}-3.48×10^{10}	8.51	8.29-8.88
T. molitor	9.98×10^{8}	8.48×10^{8}-1.18×10^{9}	7.80	7.32-8.12
Ma12MI				
G. mellonella	4.38×10^{10}	3.84×10^{9}-1.21×10^{11}	9.20	9.11-9.37
T. molitor	3.56×10^{9}	1.47×10^{9}-1.08×10^{10}	8.41	8.26-8.69
Ma11MI				
G. mellonella	2.88×10^{11}	1.82×10^{10}-4.55×10^{11}	10.47	10.19-10.59
T. molitor	1.26×10^{12}	1.06×10^{12}-1.20×10^{13}	11.26	11.09-11.46

lowest LC_{90} on *G. mellonella* larvae was Bb79MI (6.31×10^{9} conidia.ml^{-1}) and the most LC_{90} was presented by Bb11MI strain (8.31×10^{11} conidia.ml^{-1}). Ma11MI *M. anisopliae* showed the highest LC_{90} (2.88×10^{11} conidia.ml^{-1}) and the lowest LC_{90} was presented by Ma58MI strain (1.00×10^{9} conidia.ml^{-1}) on *G. mellonella* larvae. Finally, for *T. molitor* larvae the LC_{90} varied with the *B. bassiana* strains from 1.00×10^{10} to 8.46×10^{11} conidia.ml^{-1} concentration; in *M. anisopliae* strains the LC_{90} ranged from 9.90×10^{8} to 1.26×10^{12} conidia.ml^{-1} (Table 4).

DISCUSSION

The development of PCR amplification from different rDNA regions has greatly facilitated the fungi taxonomic studies. Alignments and phylogenetic analyses confirmed the *B. bassiana* and *M. anisopliae* strains taxonomic identity. Analysis of ITS-rDNA sequences have been applied to determine the genetic diversity of *M. anisopliae* and *B. bassiana* (Wall et al., 2005; Entz et al., 2005; Becerra et al., 2007; Carneiro et al., 2008; Freed et al., 2011). Thus, Bautista-Galvez et al. (2012), made the genetic characterization of *M. anisopliae* strains obtaining fragments of 600 to 800 bp by PCR amplification from the ITS1-ITS4 rDNA regions. The size of the DNA fragments from *M. anisopliae* strains collected in sugarcane crops were similar to those ones reported for the *M. anisopliae* (L0909) reference strain. Comparison of ITS rRNA obtained from fungal isolates in regarding to the GenBank database (NCBI) indicated that the MM0801, AD0702, AD0803, CD0804, FC0805, FC0706, AS0807, GB0808, L0909, BC0710, SF0811, M370 and M374 strains corresponding to *M. anisopliae* and the JC0816 and AR0814 corresponding to *B. bassiana*. Mergulhão et al. (2014) used the ITS rDNA region, as well as assessed the genetic variability and phylogeny from five *B. bassiana* strains (API 145, API 148, API 223, API 225 and API 226). The ITS region was amplified by the ITS1

and ITS4 primers, wherein amplicon was observed with an average size of 800 bp which was sequenced. The results showed that all strains, except for IPA145, presented themselves as monophyletic group. The IPA145 showed a genetic identity with *B. bassiana* and *Cordyceps bassiana* 99%. The IPA148 was the most genetically distant in regarding to the other ones, at less than 40% similarity.

G. mellonella and *T. molitor* are known to be susceptible to *B. bassiana* and *M. anisopliae*, for this reason they have been used as baits for isolation of entomopathogenic fungi from the soil (Zimmerman, 1986; Bidochka et al., 2002; Montesinos et al., 2011) or, as a probe to pathogenicity of other insect species (Bharadwaj et al., 2011). Mortality and concentrations were considered parameters in the study of the best strains behavior once indicating the pathogen colonization capacity, surpassing all competitor agents present in the insect (Neves, 1998). It was observed that it requires less inoculums amount by Bb79MI *B. bassiana* and Ma58MI *M. anisopliae* treatments to kill the *G. mellonella* and *T. molitor* larvae. Similar results by Garcia et al. (2011) were obtained, evaluating the insecticidal activity from 8 *B. bassiana* strains and from 4 *M. anisopliae* on *Spodoptera frugiperda* and *Epilachna varivestis* larvae at six concentrations (10^4 to 10^9); the BB18 *B. bassiana* strain was more virulent for *E. varivestis* larvae with a 93.3% mortality, LC_{50}= 1.20×10^6 conidia.ml^{-1} and LT_{50}= 5.1 days. Bb42 *B. bassiana* strain presented the highest mortality on *S. frugiperda* larvae (96.6%, LC_{50}= 5.92×10^3 conidia.ml^{-1} and LT_{50}= 3.6 days). Differences among lethal times is a tool widely used in selecting strains, because it is interesting that the fungus quickly eliminate its host, as well (Lohmeyer and Miller, 2006).

A significant interaction was also observed between the conidial concentration and the time. The highest conidia concentrations require less time causing 100% insects mortality. The amount of conidia used should to attain a certain concentration and thus, achieving an efficacious penetration of the fungus on the insect cuticle and causing host death (Zhioua et al., 1997). All strains were able to cause infection and mortality against *G. mellonella* and *T. molitor* by contact. In the *T. molitor* larvae the shortest time to cause the maximum mortality was 6 days at 1×10^9 conidia.ml^{-1} concentration with the Ma12MI, Ma10MI and Ma58MI *M. anisopliae* strains, while for the Bb79MI *B. bassiana* strain the time was 7 days. On *G. mellonella* the shortest time to cause 100% mortality was 6 days with the Bb79MI *B. bassiana* and Ma58MI *M. anisopliae* strains. At the 1×10^9 conidia.ml^{-1} concentration, 100% mortality for the *T. molitor* larvae with the Bb11MI, Bb53MI, Bb72MI *B. bassiana* and Ma11MI of *M. anisopliae* strains was not obtained, as well as for the *G. mellonella* larvae with Bb11MI and Bb27MI *B. bassiana* and Ma12MI and Ma11MI *M. anisopliae* strains. These results are similar to the ones obtained by Khalid et al. (2012), evaluating the virulence of 90 *B. bassiana* and 15 *M. anisopliae* strains on *G. mellonella* larvae using 10^2, 10^3, 10^4, 10^5 and 10^6 conidia.ml^{-1} concentration. The BbaAUMC3076, BbaAUMC3263 and ManAUMC3085 strains caused 100% mortality at concentrations of 5.5×10^6, 5.86×10^5 and 4.8×10^6 conidia.ml^{-1}. The LC_{50} values were 1.43×10^3, 1.04×10^5 and 5.06×10^4 conidia.ml^{-1} Bba3263AUMC, Bba3076AUMC and Man3085AUMC, respectively.

Khosravi et al. (2015), evaluated pathogenicity from *B. bassiana* strains against *Arge rosae* larvae. The bioassay was performed by the immersion method at 2×10^4, 2×10^5, 2×10^6, 2×10^7 and 2×10^8 conidia.ml^{-1} concentrations; IRAN403C strain presented the highest mortality being 70% at 2×10^7 conidia.ml^{-1} concentration, LC_{50} and LT_{50} obtained was 5.54×10^5 conidia.ml^{-1} and 3.92 days at 2×10^8 conidia.ml^{-1} concentration, respectively. However, the findings of this study demonstrated direct proportional relationship between the amount of conidia applied on the insects and mortality from the same ones, so the more diluted suspensions the virulence and pathogenicity were less pronounced, resulting in lower mortality for a longer time than the observed one at higher concentrations. This fact was also observed by other authors studying *M. anisopliae* and *B. bassiana* strains on different insect species (Filho et al., 2002; Silva et al., 2003; Cunha et al., 2008). According to Neves and Alves (2000), as more conidia penetrating, more toxins or enzymes are released, increasing the insect mortality. However, the fungus action speed depends, besides the concentration, of the host species involved (Sosa-Gomez and Moscardi, 1992). According Paccola-Meirelles and Azevedo (1990), St. Leger (1991) and Kleespies and Zimmermann (1998), variation in virulence of entomopathogenic strains is a result of differences in the enzymes and toxins production in conidia germination speed, mechanical activity in the cuticle penetration, colonization capacity and cuticle chemical composition.

It might be concluded that *B. bassiana* and *M. anisopliae* fungi present different capacity cause mortality of the insects, with the Bb53MI and Bb79MI *B. bassiana* strains as the most pathogenic for *G. mellonella*, as well as the Bb79MI *B. bassiana* strain was the most pathogenic for *T. molitor*. Ma58MI and Ma10MI *M. anisopliae* strains presented the highest virulence for *G. mellonella* and *T. molitor*, and also presenting the lowest LC_{50} and LT_{50} values.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Penicillium citrinum VFI-51 as biocontrol agent to control charcoal rot of sorghum (*Sorghum bicolor* (L.) Moench)

Meesala Sreevidya[1,2]* and Subramaniam Gopalakrishnan[2]

[1]Centre for Biotechnology, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad 500 072, Telangana, India.
[2]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Telangana, India.

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In our earlier investigation, a fungal isolate *Penicillium citrinum* VFI-51 and its secondary metabolite was reported to have antagonistic potential against *Botrytis cinerea*, the causative agent of *Botrytis* gray mold disease in chickpea. In the present investigation, *P. citrinum* VFI-51 was further evaluated for its antagonistic potential against *Macrophomina phaseolina*, the causative agent of charcoal rot in sorghum. *P. citrinum* VFI-51 inhibited *M. phaseolina* in both dual culture as well as secondary metabolite production assays. In the *in vivo* blotter paper assay, under light chamber conditions, *P. citrinum* VFI-51 controlled 85% of the charcoal rot disease on the roots when compared to the positive control. Under greenhouse conditions, when *M. phaseolina* was inoculated by tooth pick method in to the stalk of sorghum plant, the charcoal rot disease was controlled by 75% in *P. citrinum* VFI-51 treatment over the positive control. This study demonstrates the biocontrol potential of *P. citrinum* VFI-51 against charcoal rot of sorghum.

Key words: *Macrophomina phaseolina*, charcoal rot, sorghum, *Penicillium citrinum* VFI-51, biocontrol.

INTRODUCTION

Charcoal-rot of sorghum, caused by *Macrophomina phaseolina* (Tassi) Goid., is a root and stalk rot disease observed in most sorghum growing regions and endemic to tropical and temperate regions of the world (Wyllie, 1998). *M. phaseolina* is a soil borne pathogen causing losses up to 64% in southern parts of India, in post rainy sorghum (Das et al., 2008). Improved high-yielding cultivars under good management practices also tend to be susceptible to the disease resulting in high yield losses (Mughogho and Pande, 1984). Symptoms of the

*Corresponding author. E-mail: vidya.meesala@gmail.com Tel: +9140-30703610. Fax: +9140-30713074.

charcoal rot disease includes premature drying of stalks, lodging of plants, soft stalks, root rot and poorly developed panicles with low quality grain formation. The most common indication is lodging of plants on reaching maturity (Uppal et al., 1936). A toxin called "phaseolinone" produced by *M. phaseolina* in the diseased stalk, causes anemia in mice (Bhattacharya et al., 1994). Though chemical control is available for the control of charcoal rot disease, the indiscriminate use of chemicals results in negative impact on nature (Rao et al., 2015). Further, the economic constraints of the small-scale farmers in semi-arid tropics limits them to use, chemical control (Gopalakrishnan et al., 2013).

Biological control can be the safe and alternative method to control this disease as it also contains plant growth-promotion (PGP) traits (Postma et al., 2003). PGP microbes control phytopathogens by producing different compounds such as siderophores, antibiotics, volatile compounds and a group of lytic enzymes such as chitinase, cellulase, lipase and protease (El-Tarabily et al., 2009). They also compete with the pathogen by inducing systemic resistance in plants (Compant et al., 2010). This group of microbes include bacteria such as *Pseudomonas* and *Bacillus*, actinomycetes such as *Streptomyces* and *Nocardia* and fungus such as *Trichoderma* and *Gliocladium* (Ding et al., 2004). The *Streptomyces* strains isolated from vermicompost were proved effective in controlling charcoal rot in sorghum and *Fusarium* wilt in chickpea (Gopalakrishnan et al., 2011a, b). In our previous study, we reported a strain of PGP fungus, *Penicillium citrinum* VFI-51, controlling *Botrytis* gray mold disease in chickpea caused by *Botrytis cinerea* (Sreevidya et al., 2015). In the present study, *P. citrinum* VFI-51 was tested for its ability to control charcoal rot of sorghum under both *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Microorganisms used in the study

A PGP fungus, reported earlier to have biocontrol potential against *Botrytis* gray mold disease in chickpea, *P. citrinum* VFI-51 (GenBank accession number: KM250379), was further studied in the present investigation for its antagonistic potential against charcoal rot in sorghum. The pathogen, *M. phaseolina,* was acquired from cereals pathology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India.

In vitro dual culture and metabolite production assays

The fungus *P. citrinum* VFI-51 was screened for its antagonistic activity against *M. phaseolina* by dual culture assay as per the protocol of Gopalakrishnan et al. (2011b) on glucose casaminoacid yeast extract agar plates. Three replications were maintained for each treatment and control and, the experiment was repeated three times. The plates were incubated at 28 ± 2°C for five days and zone of inhibition was recorded. For metabolite production assay, *P. citrinum* VFI-51 was grown in starch casein broth for five days at 28°C. At the end of incubation, the culture free filtrate of *P. citrinum* VFI-51 was collected and extracted by partitioning against ethyl acetate (EtOAc) and the resultant organic (EtOAc) and aqueous fractions were evaporated on a rotary evaporator and collected in a minimal volume of methanol. Both the fractions were evaluated for their antagonistic potential against *M. phaseolina*. For this, a fungal disc of 6 mm diameter of *M. phaseolina* was bored and kept at center of the potato dextrose agar plate amended with either organic or aqueous fractions (at a concentration of 0.5%). Control plates contained only 0.5% methanol. The plates were incubated at 28 ± 2°C for five days and growth of the pathogen was recorded.

In vivo blotter paper assay

Evaluation of *P. ctrinum* VFI-51 for its efficacy against *M. phaseolina* was done by modified blotter paper method (Nene et al., 1981; Gopalakrishnan et al., 2011a) under light chamber conditions. The sorghum seeds susceptible to charcoal rot (variety R16) were surface sterilized with 2.5% sodium hypochlorite for two minutes and washed thoroughly with sterilized waster. These seeds were sown in pots (12 cm) filled with sterilized vermiculite. The seedlings were collected after two weeks and the roots washed with sterilized water. The pathogen inoculum was prepared by growing *M. phaseolina* in potato dextrose broth (PDB) at 28±2°C for five days and tissumized using tissumizer (Techmar type T 25, Japan). For positive control, the roots of the sorghum seedlings were dipped in *M. phaseolina* inoculum for 30 min and arranged on blotter paper (45 × 25 cm with one fold) placed in a plastic tray, making sure only roots were present in the tray. For treatment, the roots of the sorghum seedlings were dipped in *M. phaseolina* inoculum for 30 min and arranged on blotter paper (45 × 25 cm with one fold) placed in a plastic tray and counter treated with *P. citrinum* VFI-51 (10^{-8} CFU/ml, 1 ml/plant; grown separately in PDB) to the sorghum roots. Ten plants were maintained per replication and three replications were maintained. Negative control was made by dipping the plants in sterile water. The blotter paper was kept moist all the time with sterilized water and incubated at 28 ± 2°C for eight days with a 12-h day length provided by fluorescent lights (120 µ mol m^{-2} s^{-1}). At the end of the incubation, the rotting of roots that indicates disease symptoms of the charcoal-rot were recorded on a 0 to 5 rating scale (0 represents no visible disease symptoms, while 5 represents maximum disease symptoms), and the percentage of infected roots in treatments was calculated by comparing with the control.

Greenhouse study

Evaluation of *P. citrinum* VFI-51 for its efficacy against *M. phaseolina* under greenhouse conditions was done by tooth pick method (Edmunds, 1964). For this, pots (8") were filled with pot mixture containing black soil, sand and farm yard manure (3:2:1). Sorghum seeds (variety B 296) susceptible to charcoal rot were surface sterilized as mentioned earlier and soaked in *P. citrinum* VFI-51 grown in PDB. Three treated seeds were sown per pot but after germination only one plant per pot was maintained. A positive control, infected with *M. phaseolina*, and a negative control, without any inoculation, was also maintained. Each treatment contained 10 replications. Booster dozes of *P. citrinum* VFI-51 were added on 0, 15, 30, 45 and 60 days after sowing by soil application.

For preparing the pathogen inoculum to infect the plant, the *M. phaseolina* was grown on PDA for five days at 28± 2°C. The fungal

Table 1. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - in *in vitro* dual culture and metabolite production assays.

Treatment	Dual culture assay zone of inhibition (cm)	Metabolite production assay Fungal diameter (cm)
P. citrinum VFI-51	1.5	3.4
Control	-	9.0
SE±	0	0.06***

SE = Standard error; ***= statistically significant at 0.001.

Table 2. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - in *in vivo* blotter paper assay.

Treatment	Number of plants infected	% of roots infected	Visual rating
P. citrinum VFI-51	1.7	17	1
Control	10	100	5
SE±	2.4**		

*****Mean of three replications; each replication contains 10 plants; visual rating of 0 to 5 rating scale (0 = no visible symptoms, while 5 represents maximum disease symptoms), SE = Standard error; **= statistically significant at 0.01

spores were scraped and transferred in to a sterilized honey peptone broth. Tooth picks were sterilized by keeping in a glass bottle; the above prepared fungal inoculum was poured in to this bottle up to one fourth of the bottle and incubated until the tooth picks were completely covered by the fungal growth. When the plants reach to flowering stage the plant was infected with the inoculated toothpick at second node from the ground level. After infecting, the plants were grown in stress and drought conditions, irrigation was given to maintain plant viability. At the time of harvesting, the above ground level stalks of the sorghum plants were collected and made transverse cut of the stalk to observe the length of infection and number of nodes infected.

Statistical analysis

Data were analyzed by using analysis of variance (ANOVA) technique, by SAS GLM (General Linear Model) procedure (SAS Inst. 2002-08, SAS V9.3).

RESULTS

In the present investigation, when *P. citrinum* VFI-51 was tested for its antagonistic activity against *M. phaseolina* under *in vitro* conditions, it inhibited *M. phaseolina* in both dual culture as well as secondary metabolite production assays effectively (Table 1). In the *in vivo* blotter paper assay, under light chamber conditions, *P. citrinum* VFI-51 controlled 85% of disease when compared to the positive control (Table 2 and Figure 1). Similarly, under greenhouse conditions, when *M. phaseolina* was inoculated by tooth pick method in to the stalk of sorghum plant, the charcoal rot disease was controlled by 75% over the positive control (Table 3 and Figure 2).

DISCUSSION

In our previous study, we reported the production of citrinin, a secondary metabolite, by *P. citrinum* VFI-51 which was responsible for controlling the *Botrytis* gray mold disease in chickpea. Production of citrinin was also reported by *Aspergillus* spp. and many species of *Penicillium*, including *P. citrinum* (Pitt, 2002). Citrinin is also reported for its antagonistic activity against soil and seed-borne plant pathogenic fungi such as *Sclerotium rolfsii*, *Rhizoctonia solani* and *Sclerotinia minor* (Melouk and Akem, 1987). In the present investigation, the organic fraction of the culture free extract of *P. citrinum* VFI-51 was found to inhibit *M. phaseolina* (Table 1) while in our previous study, the citrinin was extracted from the organic fraction only. Hence, it can be concluded that citrinin may be also responsible for the inhibition of *M. phaseolina.* Though, citrinin is a reported mycotoxin, it is non-phytotoxic and not altering ATPase activity, respiration and photosynthetic rates when applied on sorghum leaves (Damodaran et al., 1975). The LD_{50} of citrinin on various animal models was also very high when compared with the concentrations used for the control of disease *Botrytis* gray mold (Sreevidya et al., 2015).

The control of charcoal rot disease in sorghum by *P. citrinum* VFI-51 could also be due to its capability to produce hydrolytic enzymes. In our previous investigation, *P. citrinum* VFI-51 was reported to produce siderophore, indole acetic acid (IAA), hydrocyanic acid (HCN), lipase, protease and β-1,3-glucanase. Siderophores help plants not only to acquire iron but also

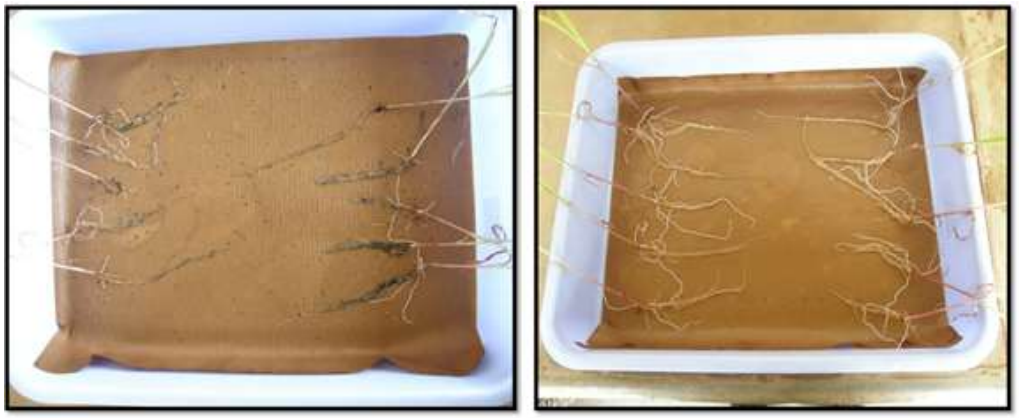

Figure 1. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - in *in vivo* blotter paper assay: **(a)** Positive control; **(b)** treatment.

Table 3. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - under greenhouse conditions

Treatment	Length of infection (cm)*	Infection %	Number of nodes infected*
*P. citrinum*VFI-51	2.4	25	1
Control	9.6	100	5
SE±	0.49***		

***= Mean of three replications; SE= Standard error; ***= statistically significant at 0.001.

Figure 2. Antagonistic activity of *P. citrinum* VFI-51 on *M. phaseolina* - under greenhouse conditions. (a) Negative control, (b) Positive control and (c) Treatment.

helps in disease suppression (Indiragandhi et al., 2008). IAA helps the host plants to stimulate seed germination, root formation and root elongation (Ahemad and Kibret, 2014) whereas HCN was also reported to help in disease suppression (Haas et al., 1991). Microorganisms producing lytic enzymes reported to play not only a role in nutrient mineralization and thus help the plants in growth promotion but also help in lysis of pathogenic fungal cell walls (Lima et al., 1998; Singh et al., 1999). The *Streptomyces* strains containing above mentioned bio-chemical properties were proved effective in controlling the soil-borne pathogens of chickpea and sorghum (Gopalakrishnan et al., 2011a, b). Khan et al. (2008) also reported production of gibberellins GA1, GA3, GA4 and GA7 by *P. citrinum* help in plant growth-promotion.

The *M. phaseolina*, causes the charcoal rot disease in sorghum when the plants are in stressed conditions such as high temperature and low moisture (Das et al., 2008). In our previous study, *P. citrinum* VFI-51 was also reported to tolerate harsh conditions such as high salinity (up to 20% NaCl), high pH (up to pH 11) and high temperatures (up to 40°C) and resistance to fungicides such as Bavistin and Thiram at field application levels (Sreevidya et al., 2015). Hence, *P. citrinum* VFI-51 can be exploited for controlling charcoal rot disease in sorghum.

From this study, it was confirmed that *P. citrinum* VFI-51 was able to control *M. phaseolina* under *in vitro* as well as *in vivo* conditions. Further studies needs to be carried out under on-station field conditions to prove efficacy of *P. citrinum* VFI-51 against charcoal rot disease. Further research also should be carried out to know the effect of citrinin in controlling the charcoal rot disease.

Conflict of Interests

The authors have not declared any conflict of interests.

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