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Biological control of downy mildew of maize caused by *Peronosclerospora sorghi* under environmentally controlled conditions

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Abstract: Downy mildew disease, caused by *Peronosclerospora sorghi*, is one of the most serious diseases of maize. The disease is currently managed by seed treatment with metalaxyl fungicides. However, problems regarding environmental pollution resulting from the use of fungicides and development of fungicide resistance within populations of *P. sorghi* are of increasing concern. Assuming that biological control by means of using antagonistic microorganisms may be an alternative for the management of this disease, the efficacy of biocontrol agents *viz*., *Bacillus subtilis* G1*, Bacillus amyloliquefaciens* B2*, Brevibacillus brevis* 57 and *Pseudomonas fluorescens* Pf1 for the management of downy mildew of maize and for promoting plant growth was evaluated. The results indicated that seed treatment with *B. subtilis* G1 and *B. amyloliquefaciens* B2 significantly (*P* = 0.05) increased the germination percentage and seedling vigour of maize as assessed by roll towel method. Among them, *B. subtilis* G1 was the most effective and recorded 9% and 31% increases in germination percentage and seedling vigour of maize respectively, as compared to the control. A talc- based powder formulation of *B. subtilis* G1 when applied through seed at the rate of 10 g/kg reduced the downy mildew incidence up to 54% under greenhouse conditions. Results of this study suggest that *B. subtilis* G1 is a promising bioagent for the management of downy mildew of maize and for promoting plant growth. This antagonist could be further exploited for commercial scale up for ecofriendly management of downy mildew of maize under localized climatic conditions.

Keywords: *Bacillus subtilis*, Biocontrol, Downy mildew, *Peronosclerospora sorghi*, *Zea mays*

INTRODUCTION

Downy mildew, caused by *Peronosclerospora sorghi* (Weston & Uppal) Shaw, is a disease of worldwide importance on maize (*Zea mays* L.) and sorghum [*Sorghum bicolor* (L.) Moench]. The fungus produces both local and systemic infection in both the crops (Bonman *et al*., 1983) and cause significant yield losses under favourable environmental conditions. Soil -borne oospores or aerially disseminated conidia cause systemic infection, whereas local infection is caused by conidia (Cohen and Sherman, 1977; Ramalingham and Rajasab, 1981; Bock *et al*., 1998). The local infection acts as a source of inoculum for subsequent systemic infection on young plants (Cohen and Sherman, 1977). This disease can occur at any stage of maize development from seedling to harvest, though it primarily infects soon after seedling emergence, until one month after planting. *P. sorghi* can cause significant yield losses under favourable environmental conditions and yields can be reduced up to 100% in susceptible cultivars depending on environmental conditions (Lukman *et al*., 2013). Genetic variabilities among isolates of *P. sorghi* have been reported (Bock *et al*., 2000; Perumal *et al*., 2006; Mathiyazhagan *et al*., 2008; Sireesha and Velazhahan, 2015). This disease is managed primarily with seed treatment with systemic fungicide, metalaxyl (Anahosur and Patil, 1980). But the use of synthetic chemical fungicides may pose threat to the environment by polluting the ecosystem. Further, the seed treatment with fungicides may not protect the crop for a longer period. The development of fungicide resistance within populations of *P. sorghi* has already been reported (Isakeit and Jaster, 2005). Although few resistant genotypes have been identified against this disease (Yen *et al*., 2004; Rashid *et al*., 2013), currently there are no commercially acceptable maize cultivars with adequate level of resistance to *P. sorghi*. Biological control method, by using naturally occurring non-pathogenic, antagonistic microorganisms has been considered as an environmentally safe and sustainable alternative to the use of synthetic chemical fungicides for the management of soil borne diseases. The applied antagonistic microorganisms can compete with the pathogen for nutrients, inhibit multiplication of pathogens by secreting antibiotics or lytic enzymes or reduce pathogen population through hyperparasitism. Sadoma *et al*. (2011) reported that crude culture filtrates of *Trichoderma viride, T. harzianum, Gliocladium virens* and *Bacillus subtilis* inhibited germination of oospores and conidia of *P. sorghi*. Further, the authors reported that seed soaking and spray treatment with crude culture

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filtrates of a combination of *T. viride* with *T. harzianum* or *B. subtilis* effectively reduced the incidence of downy mildew in maize under field conditions. Amin *et al*. (2013) demonstrated that seed treatment followed by soil application at 20 days after sowing with endophytic fungus *Beauveria* sp. effectively controlled downy mildew of maize. In this study, the efficacy of various bacterial antagonists *viz*., *Bacillus subtilis* G-1*, Bacillus amyloliquefaciens* B2*, Brevibacillus brevis* (Bbv) 57 and *Pseudomonas fluorescens* Pf1 selected based on previously determined antagonistic activity against other soilborne fungal pathogens (Meena *et al*., 2001; Shifa *et al*., 2015a) was assessed for suppression of downy mildew of maize caused by *P. sorghi* under environmentally controlled conditions. The aim of the present study was to find out the efficacy of biocontrol agents *viz*., *Bacillus subtilis* G1*, Bacillus amyloliquefaciens* B2*, Brevibacillus brevis* 57 and *Pseudomonas fluorescens* Pf1 for the management of downy mildew of maize and for promoting plant growth was evaluated.

MATERIALS AND METHODS

Bacterial cultures: The bacterial antagonists *viz*., *Bacillus subtilis* G-1*, Bacillus amyloliquefaciens* B2*, Brevibacillus brevis* (Bbv) 57 and *Pseudomonas fluorescens* Pf1 were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. The cultures of *Bacillus* spp. were maintained on nutrient agar (NA) medium and *P. fluorescens* Pf1 was maintained on King's medium B (KMB) (King *et al.,* 1954) under laboratory conditions.

Seed germination and seedling growth bioassay: *B. subtilis* G1*, B. amyloliquefaciens*, *B. brevis* were grown on nutrient broth and *P. fluorescens* Pf1 was grown on Kings' B broth with constant shaking at 150 rpm for 48 hrs at room temperature (26 ± 2°C). The bacterial cells were harvested by centrifugation at 6,000 rpm for 15 min and the bacterial pellet was suspended in 0.01 M phosphate buffer (pH 7.0). The final concentration of the suspension was adjusted to approximately 10^8 CFU/ml (OD_{595} = 0.3) in a spectrophotometer (Thompson, 1996) and amended with 0.2% sterilized carboxymethyl cellulose (CMC) as a sticker.

Maize seeds (cv. CM 500) were surface sterilized with 0.1% mercuric chloride for 5 min and rinsed thoroughly in sterile distilled water. The bacterization of seed was done by soaking surface sterilized maize seeds in bacterial suspensions @ 5 g/25 ml and incubating in a rotary shaker for 6 hrs to facilitate attachment of bacterial cells to the seed coat. The seeds were then dried in shade for 2 hrs and the plant growth promoting activity of the antagonists was assessed based on the seedling vigour following the standard roll towel method (International Seed Testing Association, 1996). The bacterized seeds were placed on coarse blotter paper sheets and covered with a moistened blotter and rolled. The roll was kept on a butter paper sheet and rolled as a bundle and incubated in a growth chamber at 25 °C with 80 % RH. Four replications were maintained for each treatment. The root and shoot lengths of seedlings were measured and the germination percentage was calculated after 7 days. The vigour index was calculated as suggested by Baki and Anderson (1973).

Vigour index = Per cent germination x seedling length (shoot length + root length)

Greenhouse experiments: The talc-based powder formulations of the antagonists were prepared by mixing 400 ml of the bacterial suspension (9×10^8 CFU mL^{-1}) with 1 kg of sterilized talc powder amended with 10 g of carboxymethylcellulose under aseptic conditions (Vidhyasekaran and Muthamilan, 1995). Surface-sterilized maize seeds (cv. CM 500) were treated with talc formulation at a rate of 10 g/kg of seed and sown in 30 cm diameter plastic pots filled with autoclaved soil and sand (2:1). Seeds mock-treated with the powder formulation without bacteria were kept as control. As a check, seeds were treated with Metalaxyl at 2 g kg^{-1} seed. Five seeds were sown in each pot and five pots were kept as one replication. The trial was conducted in a completely randomized design (CRD) with four replications.

In order to prepare pathogen inoculum, maize leaves showing symptoms of downy mildew were collected from the experimental farm of Tamil Nadu Agricultural University, Coimbatore, India. The infected leaves were wiped out with wet absorbent cotton, cut into small pieces of 4-5 cm lengths and placed with their abaxial side facing up in 9 cm diameter Petri dishes lined with wet filter paper on both the sides. The plates were incubated in the dark for 6-7 hrs at 20°C for sporulation (Narayana *et al*., 1995). Conidia were harvested from the surface of leaves by gently washing them into cold distilled water using a camel hair brush. The concentration of conidia was adjusted to 1×10^8conidia mL^{-1} and used as inoculum. At two leaf stage, the seedlings were challenge inoculated by spraying with conidial suspension of *P. sorghi* (4×10^4 spores mL^{-1}). The inoculated seedlings were maintained at 20±2°C temperature with >95% relative humidity. At 30 days after sowing (DAS), plant height and percent disease incidence were measured. The inoculated plants were examined for leaf reaction 7 days after inoculation. Numerical values of 1, 2 and 3 were assigned to resistant (R), intermediate (I) and susceptible (S) leaf reaction classes, respectively as described by Craig (1982).

Data analysis: Arc sine transformation of data on percentage of downy mildew incidence was done and. Duncan's multiple range test (DMRT) was first applied to the transformed values and then transferred to the original means (Gomez and Gomez, 1984). The data were analyzed using AgRes statistical software, version 3.01 (Pascal Int1 Software solutions).

RESULTS AND DISCUSSION

In the present study the biocontrol agents viz., *B. subtilis* G1, *B. amyloliquefaciens* B2, *B. brevis* 57 and *P. fluorescens* Pf1 were tested for their plant growth promoting activity and efficacy in controlling downy mildew of maize under greenhouse conditions. The results indicated that seed treatment with *B. subtilis* G1 and *B. amyloliquefaciens* B2 significantly increased the percent germination and seedling vigour compared to control. Among the biocontrol agents, *B. subtilis* G1 recorded the maximum percent germination (96%) and seedling vigour (4339.2) (Table 1; Fig. 1). However, the fungicide check Metalaxyl recorded the highest germination percentage (97%) and seedling vigour (4573.5). Talc-based powder formulations of the biocontrol agents were prepared and evaluated under greenhouse conditions for their potential in the management of downy mildew of maize. The results indicated that all the tested antagonists significantly (P=0.05) reduced the incidence of downy mildew compared to control (Table 2). Among the biocontrol agents tested, *B. subtilis* G1 was found to be the most effective in reducing the downy mildew disease when used as seed dresser@ 10 g/kg followed by *B. amyloliquefaciens* B2, *B. brevis* 57 and *P. fluorescens* Pf1. Seed treatment with the commercial fungicide Metalaxyl completely eliminated the development of downy mildew in maize seedlings.

Several strains of *B. subtilis* are widely used in agriculture as biopesticides for the management of plant diseases especially those caused by soil-borne plant pathogens (Jayaraj *et al*., 2005; Furuya *et al*., 2011; Yanez-Mendizabal *et al*., 2012; Hu *et al*., 2014; Khabbaz and Abbasi, 2014; Zhao *et al*., 2014). *Bacillus* spp. offer several advantages over other antagonistic microorganisms because they are capable of growing in different environmental conditions due to their ability to produce endospores that can tolerate extreme pH, temperature, and osmotic conditions (Earl *et al*., 2008). Many *B. subtilis* strains when applied through seed bacterization rapidly colonize plant roots and suppress the growth of phytopathogens (Beauregard *et al*., 2013). The mechanisms of plant disease control by *B. subtilis* includes production of antibiotic substances (Stein 2005; Shifa *et al*., 2015b), production of cyclic lipopeptides like iturins, fengycins and surfactins (Ongena and Jacques, 2007), production of hydrolytic enzymes, including β-1,4-*N*-acetyl glucosaminidase (NAGase), chitinase and β-1,3-glucanase (Manjula and Podile, 2005; Liu *et al*., 2011),

Table 1. Effect of seed treatment with biocontrol agents on seed germination and seedling vigour of maize.

Treatments	% Germination*	Shoot length (cm)	Root length (cm)	Seedling vigour
B. subtilis G1	96(78.5)[a]	24.2[a]	21.0[a]	4339.2[a]
B. amyloliquefaciens B2	94(75.82)[a]	23.9[a]	20.3[a]	4163.2[a]
Br. brevis (Bbv) 57	89(70.65)[b]	22.6[b]	19.7[b]	3775.3[b]
P. fluorescens (Pf1)	89(70.65)[b]	20.7[c]	18.2[b]	3471.8[b]
Metalaxyl	97(80.17)[a]	23.9[a]	20.3[a]	4573.5[a]
Untreated Control	88(69.73)[b]	20.3[c]	12.2[c]	3307.0[c]
CD (0.05)	4.48	2.15	1.55	381.5

The root and shoot lengths of seedlings were measured and the germination percentage was calculated 7 days after treatment; *Arc sine transformation of data was done prior to analysis; Four hundred seeds were used per treatment in four replications of 100 seeds each. The data are mean of four replications. Means within a column followed by a common letter are not significantly different (P=0.05) by Duncan's multiple range test.

Table 2. Effect of seed treatment with talc based formulations of biocontrol agents on the incidence of downy mildew of maize under greenhouse conditions.

Treatment	Number of leaves in each reaction class			% of leaves in reaction class			Mean leaf reaction score	% disease incidence
	R	I	S	R	I	S		
Bacillus subtilis G1	40	23	17	50	29	21	1.7	46(42.71)[b]
Bacillus amyloliquefaciens B2	10	16	26	19	31	50	2.3	64(53.13)[c]
Brevibacillus brevis (Bbv) 57	8	22	38	12	32	56	2.4	69(56.17)[c]
Pseudomonas fluorescens Pf1	16	18	24	28	31	41	2.1	73(58.69)[d]
Metalaxyl	85	0	0	100	0	0	1.0	0(0.29)[a]
Untreated Control	7	16	35	12	28	60	2.5	100(89.72)[e]

The bacterial antagonists were applied as seed treatment (10 g/kg) at the time of sowing. The bacterized plants were inoculated with *P. sorghi* at two leaf stage and the incidence of downy mildew was recorded 30 days after sowing;Numerical values of 1, 2, and 3 were assigned to resistant (R), intermediate (I) and susceptible (S) reactions respectively. A mean score was calculated for the leaf reactions of each treatment.;Data are mean of four replications;Data followed by the same letter in a column are not significantly different (P=0.05) from each other according to DMRT.

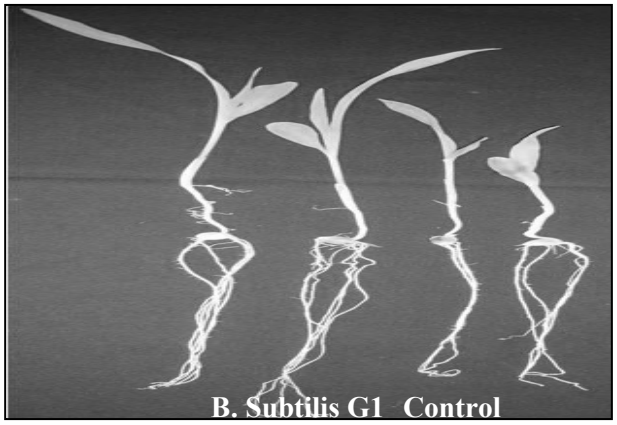

Fig. 1. *Maize seedlings showing enhanced growth upon seed treatment with Bacillus subtilis G1.*

which causes lysis of fungal cell walls and membranes and induction of systemic acquired resistance in plants (Choudhary and Johri, 2009; Lahlali *et al*., 2013; Sivasakthi *et al*., 2014). In addition to direct antagonistic activity against several soil borne fungal pathogens, *B. subtilis* is known to promote plant growth and yield (Kloepper *et al*., 2004; Perez-Garcia *et al*., 2011). The genome of *B. subtilis* strain BAB-1 has been fully sequenced and annotated and genes encoding the antifungal active compound have been identified in the genome (Guo *et al*., 2014). A number of commercial products based on *B. subtilis* including Kodiak (Gufstafson Biologicals, Plano, TX), Serenade (Agraquest Inc., Davis, CA), Subtilex (Becker Underwood, Ames, IA) have been developed for the control of various plant diseases (Schisler *et al*., 2004).

Shifa *et al*. (2015a) recently reported that seed treatment and soil application with the powder formulation of *B. subtilis* strain G1 effectively reduced the incidence of stem rot of groundnut caused by *Sclerotium rolfsii* and increased the pod yield. This strain was compatible with other beneficial rhizobacteria including *Bacillus megaterium* var *phosphaticum* strain PBS, *Rhizobium* strain BMBS, *Azospirillum brasiliense* strain 204 and *Azotobacter chrooccocum* strain AC1. Furthermore, production of 22 different kinds of antibiotics including aldehydes, fatty acids, alkanes, esters and sulphur containing compounds by this antagonistic strain was demonstrated (Shifa *et al*., 2015b). The increase in plant growth due to application of *subtilis* G1 in the present study may be due to plant growth promoting characteristics of *B. subtilis* G1 and the reduction in the incidence of downy mildew may be due to direct antagonistic activity of *B. subtilis* G1 against *P. sorghi*.

Conclusion

In the absence of downy mildew resistant maize cultivars, biological control by means of using antagonistic microorganisms will definitely reduce the environmental pollution and the cost of plant protection measures. The results of this study suggest that *B. subtilis* G1 could be considered as a promising alternative to synthetic chemical fungicides in the management of maize downy mildew and could be successfully exploited as a biocontrol agent within the framework of integrated disease management system.

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