Bacillus amyloliquefaciens (VB7) with diverse antimicrobial peptide genes: A potential antagonist for the management of fairy ring spot in carnations

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Fairy ring spot incited by *Cladosporium echinulatum* is an unexplored yet disastrous disease of carnation. *Bacillus amyloliquefaciens* (VB7) with 10 diverse antimicrobial peptide genes, effectively reduced fairy ring spot intensity to 6.44 (per cent disease index, PDI) and increased flower yield (196.61 Nos/m^2) compared to control (PDI – 60.33; flower yield – 140.70 Nos/m^2). Plants treated with tebuconazole (250 EC, emulsifiable concentrate) and alternated with mancozeb (75% WP, wettable powder) were much effective and reduced disease intensity up to 3.46 PDI. However, flower yield was comparatively lesser to the treatment of *B. amyloliquefaciens* (VB7). Results revealed that *B. amyloliquefaciens* out-performed fungicides in growth promotion.

Keywords: Antimicrobial peptide, *Bacillus amyloliquefaciens* (VB7), *Cladosporium echinulatum*, fairy ring spot.

FLORICULTURE is an emerging venture in India. Globally, carnations have greater stipulation in the cut-flower industry. However, their productivity is hampered due to various diseases. Important diseases of carnations include stem rot (*Sclerotinia sclerotiorum*), wilt (*Fusarium oxysporum* f.sp. *dianthi*), fairy ring spot (*Cladosporium echinulatum*) and blossom blight (*Botrytis cinerea*). Among them, fairy ring spot of carnation is notable and causes considerable damage. In 1870, Berkeley identified and named the causal organism as *Helminthosporium echinulatum*[1]. Later the name was replaced as *Cladosporium echinulatum*[2]. Host range of the pathogen includes carnation, sweet williams, lychnis, saponaria and other members of caryophyllaceae[3]. In India the pathogen was first identified in Himachal Pradesh[4]. Till now there is only one report for the management of fairy ring spot[5]. The study reported that sodium bicarbonate and *T. virens* was effective in reducing disease incidence. However, there were no further studies to support the hypothesis.

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Continuous mono-cropping and indiscriminate use of fungicides have created resurgence among pathogens. At present, it is a difficult challenge to manage diseases under polyhouses. This reveals the significance of biological control for the management of plant diseases under protected cultivation. Efficiency of *Bacillus* species has been well documented against various foliar diseases like sheath blight, bacterial leaf blight in rice[6], *Alternaria* leaf spot in mustard[7] and cucurbit powdery mildew[8]. The antifungal mode of action of the *Bacillus* species is largely influenced by the synthesis of anti-microbial peptides (AMPs). *Bacillus* species has been reported to contain 24 diverse AMP genes responsible for the biosynthesis of antifungal antibiotics like iturin, bacilysin, bacillomycin, fengycin, surfactin, mersacidin, ericin, subtilin, subtilosin and mycosubtilin[9,10]. The AMPs are synthesized through ribosomal synthesis or non-ribosomal synthesis[11,12]. Lantibiotics are a group of ribosomally synthesized peptides containing unusual amino acids and lanthionine[13]. However, post-translational linkage of a thiol to the α-carbon of an amino acid residue responsible for their antimicrobial bioactivities is rare in ribosomal synthesized peptides. This limits their antimicrobial activity. In contrast, the non-ribosomally synthesized AMPs have been reported with significant anti-microbial activity. Ribosomally synthesized AMPs include, bacteriocins, subtilin, ericin, entianin, subtilomycin, mersacidin, amylolysin, lichenicidin, megacins, etc. Ribosomally synthesized AMPs include, iturin, surfactin, fengycin, bacilysin, bacillibactin, zwittermycin, etc.[14]. The present study focuses on the identification of fairy ring spot pathogen and evaluation of the bio-efficacy of *Bacillus* species with diverse AMP genes.

Materials and methods

Survey

Commercially cultivated carnation varieties were surveyed in 2013 for the occurrence of fairy ring spot at Nilgiris and Kothagiri regions of Nilgiris district in Tamil Nadu (India). A grade scale was formulated as follows:

0, No symptom; 1, 0.1–5.0% leaf area affected; 2, 5.1–15.0% leaf area affected; 3, 15.1–30% leaf area affected; 4, 30.1–50% leaf area affected; 5, more than 50% leaf area affected (Figure 1). The percent disease index (PDI) was calculated as per the formula proposed by Datar and Mayee[15].

Pathogenicity and identification

Pathogenicity experiments were executed in 30-day-old, healthy, carnation (gaudina red) cuttings. Conidia were scrapped from infected leaf samples and a suspension was prepared in phosphate buffer (pH 7) @ 4.6×10^4 conidia/ml. The spore suspension was sprayed over healthy carnation cuttings. Control, sprayed with blank phosphate buffer was also maintained[16]. The experiment was replicated thrice with three plants per replication. After inoculation the plants were covered with polythene bags and incubated at ambient environmental conditions inside the polyhouse at Nilgiris. The pathogen was identified based on symptomatology and morphological characteristics[3,17].

Evaluating the efficacy of liquid formulation of Bacillus spp. and fungicides

An experiment was conducted during 2013–14 and 2014–15 in carnation grown under polyhouses at Nilgiris to assess the bio-efficacy of liquid formulations of *Bacillus* spp. and fungicides.

According to our previous studies, *Bacillus amyloliquefaciens* strains VB7 (KJ603234), VB2 (KJ603230), BSC7 (JX036522), *Bacillus subtilis* strains VB9 (KJ603236), VB10 (KJ588182), BS2 (JN873298), *Bacillus cereus* BSC5 (JX036520) and *Bacillus licheniformis* BlTNAU1 (KC540811) were found to perform excellently under carnation ecosystem for the management of *S. sclerotiorum* stem rot. The antagonists have been reported with antimicrobial peptide genes responsible for the biosynthesis of diverse antifungal peptides. Moreover, they were also known to secrete volatile and non-volatile antifungal compounds[18]. Liquid formulations of *Bacillus* species with known antimicrobial peptide gene profile were formulated and 30-day-old rooted cuttings were dipped in the bacterial formulations (2.5×10^{10} cfu/ml) of respective *Bacillus* spp. @ 0.5% (5 ml/litre) prior to planting[18]. The plants were sprayed with liquid formulations of the respective *Bacillus* spp. at weekly intervals for three consecutive weeks. Each treatment was replicated thrice, with a 30-ft long bed per replication. An untreated control bed was also maintained.

Nine different fungicides in fifteen treatment combinations were also tested against fairy spot of carnation. The plants were sprayed at weekly intervals for three consecutive weeks. Each treatment was replicated thrice, with a 30-ft long bed per replication. An untreated control bed was also maintained.

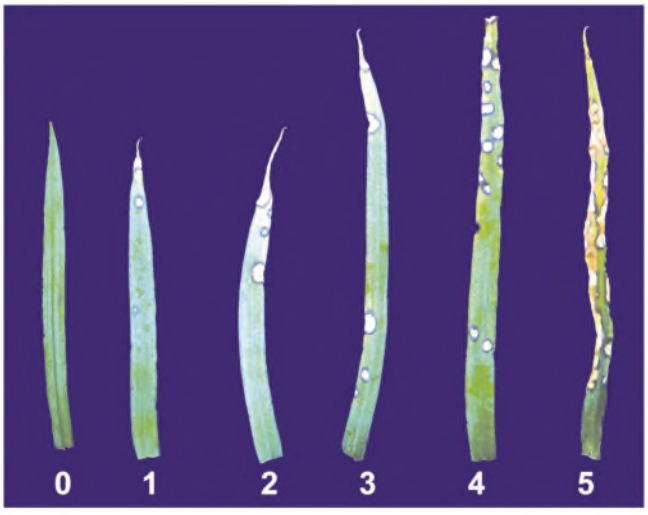

Figure 1. Disease grade scale for fairy ring spot. 0, No symptom; 1, 0.1–5.0% leaf area affected; 2, 5.1–15.0% leaf area affected; 3, 15.1–30.0% leaf area affected; 4, 30.1–50.0% leaf area affected; 5, >50% leaf area affected, complete drying of leaf.

Assessment of the percent fairy ring spot incidence and flower yield

All treatment replications were assessed for fairy ring spot incidence. Fifty plants in each replication were selected randomly in criss-cross manner and observed for disease occurrence, and the mean value was expressed. The total number of flowers harvested up to the first flush in each treatment was recorded/m^2. Flower yield was calculated from 10 different squares selected at random in each treatment replication.

Statistical analysis

Mean differences of the treatment were evaluated with ANOVA by using Duncan's Multiple Range Test at 5% significance[19]. All the data were statistically analysed with IRRISTAT (version. 3/93, Biometrics Unit, International Rice Research Institute) and interpreted.

Results

Survey

A survey indicated that fairy ring spot was severe in Kothagiri (22.93 PDI) followed by Nilgiris (21.15 PDI). At both locations, the variety gaudina red recorded highest PDI (68.2 – Kothagiriand 64.6 – Nilgiris). This indicates

that the climatic conditions prevailing both in Nilgiris and Kothagiri were highly conducive for disease development (Figure 2; Supplementary Table 1). Similar type of disease was observed in sweet williams cultivated in and around carnation farms. Sweet williams are ornamental plants belonging to the same family of carnations. This revealed the possibility of sweet williams (*Dianthus barbatus*) to serve as an alternate host, aiding in the transfer and spread of the disease inoculum.

Symptomatology

The disease was prevalent throughout the stages of crop growth. All the above ground plant parts were severely infected. Disease symptoms initiated as small, pin-head lesions on the leaves, later enlarged as circular to oval spots with dark, purplish margin and grey centre. During rainy seasons, dark brown, spore mass was observed at the centre of the spot. Under severe conditions the spots coalesce and finally lead to complete drying up of the plant. Typical spots were observed in all plant parts including calyx, epicalyx, leaf and petiole. Severe infection on the calyces reduced the quality of marketable blooms (Figure 3). Similar symptoms were also observed in sweet williams (Figure 4).

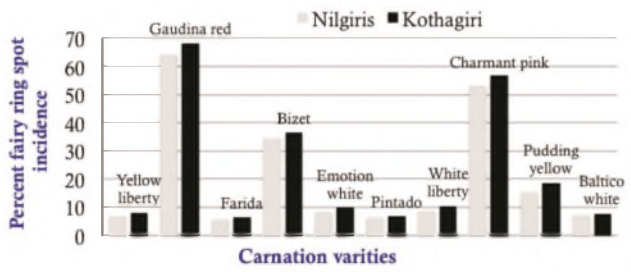

Figure 2. Distribution pattern of fairy ring spot of carnations in various varieties at Nilgiris and Kothagiri, Tamil Nadu, India.

Figure 3. Symptoms of fairy ring spot in carnations. ***a***, Purplish brown necrotic rings with spore mass at the centre; ***b***, Necrotic spots at the calyx chocking flower opening; ***c***, Necrotic spots on the calyx epicalyx; ***d***, Spots on the petiole; ***e***, Infected leaves; ***f***, Necrotic spots on packed marketable blooms.

Pathogenicity and identification

The inoculated plants produced typical symptoms after 15 days. The characteristic symptoms, viz. oval, brown spots with purple margin and grey centre were observed in the inoculated plants. Thus, Koch's postulates were confirmed. The pathogen produced brown, thick-walled, geniculate, conidiophores, with granulation and borne yellowish brown, cylindrical to ellipsoidal or soleiform, echinulated conidia with 1–4 septations measuring 100.75 μm × 31.38 μm at 400× magnification (Figure 5). This morphologically confirmed the identity of the pathogen.

Efficiency of Bacillus spp., and fungicides in the management of fairy ring spot of carnation variety gaudina red

B. amyloliquefaciens – VB7 (KJ603234) was effective in reducing the disease intensity to a greater extent. Plants treated with *B. amyloliquefaciens* (VB7) recorded lowest disease intensity of 6.44 PDI, 20 days after three consecutive sprays. Treatment of plants with fungicides tebuconazole (250 EC) in the first spray, followed by mancozeb (75% WP) was much effective and reduced fairy ring spot intensity to 3.46 PDI. Even though fungicides out-performed *Bacillus* species in disease suppression, plants treated with *B. amyloliquefaciens* (VB7) exhibited greater flower yield (196.61/m^2) compared to fungicides (178.24/m^2) (Figures 6 and 7; Supplementary Tables 2 and 3). Thus growth-promoting activity of the microbe was recorded. The experiment was conducted for two season trails (2013–14 and 2014–15) with data expressed as mean value.

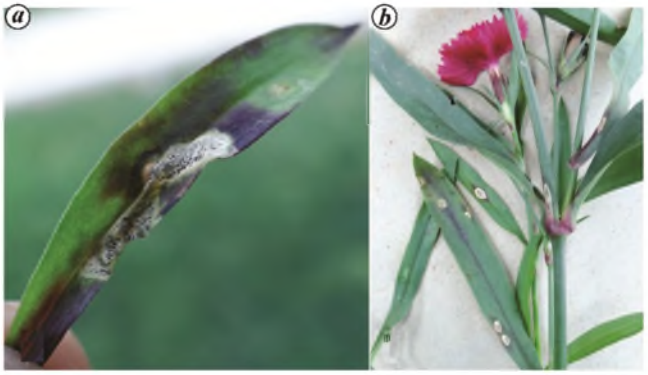

Figure 4. Symptoms of fairy ring spot in sweet williams. ***a***, Necrotic spots with spore mass at the centre on the leaves of sweet williams plant; ***b***, Infected sweet williams plant.

Discussion

Fairy ring spot destroyed approximately 25,000 flowers of carnation varieties, viz. Midas, Nelson and Salamanca at Mucuchies[20]. Even though the destructive nature of fairy ring spot of carnation has not been well explored, recently severe outbreak was observed. The present study revealed PDI >50%, causing serious impact on the economy of the cut flower industry at Nilgiris district.

Symptoms were observed during all stages of the crop growth in all the above-ground parts of the plant. Disease symptoms initiated as small, pinhead, necrotic lesions that later enlarged into circular-oval spots with tan to grey centre with powdery mass of conidiophores and conidia. This brownish growth in the form of dull and dark bands, gives the name 'fairy ring' spot to the disease. The margins of the spots were distinct and purple to dark purplish[4]. Continuous rain coupled with higher relative humidity triggers sporulation at the centre of the spots[17]. In severe cases the spots coalesce and the entire plant may blight and die causing severe losses[21]. The present results corroborated with earlier studies.

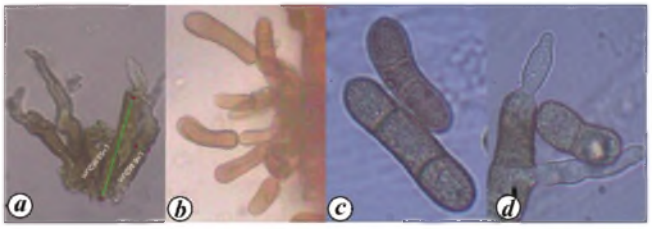

Figure 5. Morphological characteristics of *C. echinulatum*. ***a***, Geniculate conidiophores; ***b***, Geniculate conidiophore bearing yellowish brown, soleiform conidia; ***c***, Echinulate conidia; ***d***, germinating conidia.

The pathogen produced thick-walled, geniculate, brown conidiophores, with granulation (40–200 × 7–10 μm) and bears yellowish brown, 1–4 septated, broadly ellipsoidal to cylindrical or soleiform, echinulated conidia with basal bulbous cell measuring 25–55 × 12–15 μm (ref. 3). Similar to earlier studies, the morphological characteristics of the pathogen were similar and the identity was confirmed as *C. echinulatum*.

Management of plant diseases with alternative eco-friendly approaches is desirable under the present situation. Biocontrol agents promote plant growth and suppress the growth of plant pathogens by the secretion of a variety of extra cellular compounds[22,23]. *Bacillus* species are known to secrete antimicrobial peptides including, fengycin, iturin, bacillomycin, bacilysin, subtilin, mycosubtilin, ericin, subtilosinand surfactin, with broad spectrum anti-microbial activity against foliar, soil-borne and post-harvest diseases[24–27]. Apart from AMPs, *Bacillus* species secreted a variety of secondary metabolites[28]. Aliphatic hydrocarbons secreted by *Bacillus* species have been reported to exhibit antifungal activity against *Phytophthora infestans*[29] and *S. sclerotiorum*[30,31]. Secondary metabolites secreted by *Bacillus* species reduced spore germination and lysed the spores of *Puccinia horianna*[32]. Pentadecenoic acid secreted by *Bacillus* sp. inhibited the mycelial growth of *Fusarium solani*[33]. Heptadecenoic acid is a saturated fatty acid reported with antifungal and antibacterial activity[34]. Octadecenoic acid is a hydroxy fatty acid that interacts with microbial membranes. Moreover, the extract inhibited the growth of *Aspergillus niger* and *Penicillium roqueforti*[35]. Pyrrolo is also known to have antifungal activity[36]. Hexadecenoic acid has been reported with antifungal activity against *Aspergillus*, *Penicillium* and *Fusarium* species[37].

B. amyloliquefaciens (VB7) utilized in the present study has been known to contain 10 AMP genes responsible

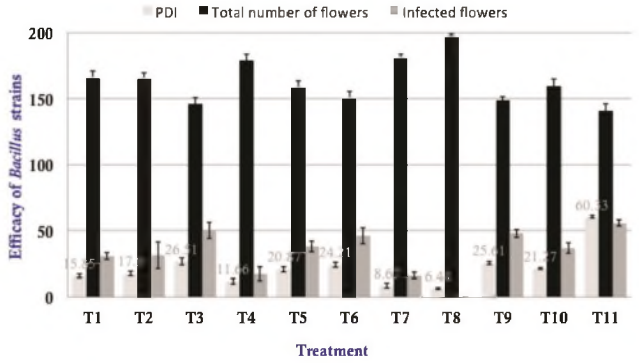

Figure 6. Efficacy of the *Bacillus* species in the management of fairy ring spot and plant growth promotion of carnations under protected cultivation. Data is presented as percentage (PDI) and numbers (yield – number of flowers/m^2). Error bars indicate standard deviation obtained from the replications (150 replicates per treatment – PDI and 30 replicates per treatment – yield). The data represents mean value for two season trials conducted (2013–2014 and 2014–2015). Analysis of variance was performed through DMRT with IRRISTAT (version 3/93, Biometrics unit, International Rice Research Institute).

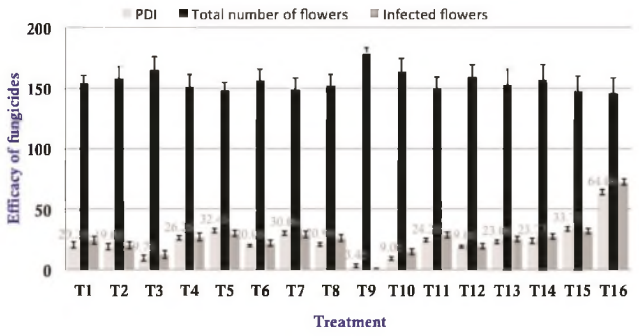

Figure 7. Efficacy of the fungicides in the management of fairy ring spot and plant growth promotion of carnations under protected cultivation. Data is presented as percentage (PDI) and numbers (yield – number of flowers/m^2). Error bars indicate standard deviation obtained from the replications (150 replicates per treatment – PDI and 30 replicates per treatment – yield). The data represents mean value for two season trials conducted (2013–2014 and 2014–2015). Analysis of variance was performed through DMRT with IRRISTAT (version 3/93, Biometrics Unit, International Rice Research Institute).

for the biosynthesis of iturin, bacilysin, bacillomycin, surfactin, subtilin and subtilosin. Moreover GC/MS analysis of the crude secretions of the bacteria revealed the presence of compounds with reported antifungal activity that includes, octadecatrienoic acid, tetradecenoic acid, pyrrolo, pentadecenoic acid, heptadecenoic acid, octadecenoic acid and piperazinedione[18]. The antimicrobial peptides and antifungal secondary metabolites produced by *B. amyloliquefaciens* VB7 (KJ603234) synergistically curtailed fairy ring spot of carnation and the results agree with earlier studies.

Plant growth promotion by bacteria may be direct or indirect[38,39]. Extracellular metabolites such as gibberellins, cytokinins and indole acetic acid are produced by plant growth-promoting rhizobacteria, which in turn increases plant vigour and yield[40,41]. Secretion of plant growth-promoting hormones by bacteria alters the root architecture, increases the production of more root hairs and lateral roots, that in turn increases water and nutrient uptake. This, in turn, promotes plant growth[42]. In relation to the above studies *B. amyloliquefaciens* (VB7) accounted for the management of fairy ring spot of carnation synergistically with multifaceted mode of action and also promoted plant growth.

Conclusion

Apart from the report of Sandoval *et al.*[5], no study has so far been reported for the management of fairy ring spot of carnations. According to the present results, *Bacillus* species with diverse AMP genes are good for the control of fairy ring spot of carnation. Further optimization in the delivery methods would improve the development of cut flower industry.

Conflict of interest: There is no conflict of interest.

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