B. Fungus

Entomopathogenic fungal culture of *B. bassiana* (Meerlust P3/S1) was obtained from the existing cultures in the Horticulture Research Laboratory of Cape Peninsula University of Technology, Bellville campus. Fungal cultures were incubated for 3 weeks in a dark incubation chamber at 25°C. The fungal culture consisted of Potato Dextrose Agar (PDA) supplemented with Streptomycin Sulfate (Sigma-Aldrich), *Streptomyces* sp. and Ampicillin sodium salt (Sigma-Aldrich). Mycelium was grown on the PDA in 9 cm diameter petri-dishes. For preparing media, deionized water was autoclaved for 15 minutes at 121°C. After the incubation period, conidia were harvested by scraping mycelium from the surface of PDA using a sterile scalpel and placed into a Corning® 50 mL centrifuge tube with 10 ml of sterile distilled water containing 0.05% Tween 80 and vortexed for 5 minutes to homogenize the suspension. The conidia concentration was determined using Neubauer haemocytometer with light microscope (400X magnification); the concentration of conidial stock solution was adjusted to 1×10^7 spores/ml. Serial dilution method was performed to obtain different conidial concentrations as follows: 0, 1×10^5 1×10^6 and 1×10^7 spores/ml. Conidial viability was determined by plating the conidial suspension onto potato dextrose agar, incubated at 25 °C for 18-24 hours, and conidial germination enumerated at 400X magnification. Over 90% of the conidial germination was achieved for the *B. bassiana* isolate used in this study.

C. Plants Inoculation

Plants were allowed to establish for a month. Subsequently, they were systematically treated with three different concentrations of *B. bassiana* conidial suspension as described above. Plants in the control treatment were inoculated with sterile distilled water. Individual plants were drenched with 100 ml of the different treatments of conidial suspension every 2 weeks. Temperature and humidity was recorded daily. To determine plant growth in response to the different treatments of endophytic fungi, data on number of leaves and plant height were recorded once a week. Plant height was measured from the soil surface to the tip of the aerial part. At the end of the experiment, aerial part fresh weight, root fresh weight (roots were washed to remove soil), and root length were recorded. To determine dry weights, fresh aerial parts and roots were oven-dried at 35 °C for 7 days before dry weights. The plant growth data collected were analyzed using one-way analysis of variance (ANOVA) and Tukey HSD test was used to separate the means at a level of significance of $P < 0.05$.

D. Assessment of Colonization

The colonization of Leek plants by *B. bassiana* was determined after 28 days of inoculation following methods by Teferea and Vidal [25]. Plants were carefully removed from pots, and the roots were washed with running tap water. Sections (5 mm × 2 mm) of fresh healthy aerial parts and root of plants were cut with sterile blade. The sections were sterilized in 70% ethanol for 30 sections, and then rinsed in sterilize distilled water for 60 seconds. The sterilized samples (sections) were allowed to dry under the laminar flow cabinet. Five samples from each plant were then placed on a Potato Dextrose Agar (PDA). Afterward, they were incubated in the dark at 25 °C for 14 days. Mycelial outgrowth from the sections were examined microscopically. The data on fungal colonization of the tissue were expressed as percentage colonization = 100 × (number of plant pieces colonized/total number of plant pieces) [26].

E. Tissue analysis

Leaf samples were analysed for macro- and micro elements by a commercial laboratory Bemlab (Pty.) Ltd. in Somerset West, South Africa. Leaves were washed with Teepol solution, rinsed with deionized water and dried at 70°C overnight in an oven. The dried leaves were then milled and ashed at 480°C shaken up in a 50:50 HCl (50%) solution for extraction through filter paper (Campbell and Plank, 1998; Miller, 1996). The Potassium (K), Phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na), manganese (Mn), iron (Fe), copper (Cu), zinc (Z) and boron (B) content of the extracts were analysed using Ash method. Total nitrogen (N) content of the leaves will determined through total combustion in a Leco N-analyser. The amounts of N, P, K, Ca and Mg estimated from percentage (%) to mg/kg; 10 000 is used as the conversion factor.

F. Material extracts

Fresh materials of *A. porrum* aerial parts were crushed with a ceramic mortar and pestle for 5 minutes. Extraction was done by transferring 5 g of crushed plant material into a Corning® 50 mL centrifuge tube containing 250 ml of aqueous acetone, tightly capped to avoid evaporation of acetone, and vortexed for 5 min and stored at room temperatures for 22 hours. The supernatant was filtered out with Whatman No. 1 filter paper. The extract was dried for 3 days at room temperature ($26 \pm 2\ ^oC$) with a fan under a fume hood. The quantity of plant extract obtained was recorded after evaporation of the acetone. Weighed extracts were then mixed with water and aqueous acetone in a ratio of 1:3 to obtain a crude extract concentration of 6 mg/ml.

G. Anti-fungal activity

The micro-dilution method [27] was employed with slight modifications in determining the minimum inhibitory concentration (MIC) for the extracts. *A. porrum* extracts were diluted into acetone to obtain a starting concentration of 6 mg/ml. The starting concentration was diluted two fold in each successive serial dilution. Strains of *Fusarium oxysporum* (MTL 211) and *B. bassiana* (Meerlust P3/S1) were obtained from existing cultures in the Horticulture Research Laboratory of Cape Peninsula University of Technology, Bellville campus. The *F. oxysporum* and *B. bassiana* were sub-cultured from stock agar plates and transferred into Nutrient Broth (Merck, South Africa) for four hours. The fungal cultures (100 µl) were added to each well of the 96-well microplates (10^5 cells/ ml). Mancozeb fungicide and Odeon fungicide (Stodels Pty Ltd. Garden Centre, Cape Town, South Africa) were prepared and

served as positive controls, while acetone served as a negative control. Forty microliter (40 µl) of 0.2 mg/ml of p-iodonitrotetrazolium chloride (INT) (Sigma) dissolved in sterile distilled water was added to each microplate well, sealed in a plastic bag and incubated at 37 °C and 100% RH. The MIC values were recorded after 12 and 18 hours. The antifungal bioassay (MIC) consisted of three replicates per treatment.

H. Phytochemical Screening

The total alkaloid assay was based on the methods described by Fadhil and Reza (2007). Dry material of leek plant (100 mg) was extracted with 10 ml of 60% ethanol for 2 hours, and after centrifugation at 4000 g for 10 minutes. The supernatant (2 mL) and atropine standard (2 mL) were mixed with % ml sodium phosphate buffer and 12 mL bromocresol green solution. Afterwards, chloroform (12 mL) was added to the solution and followed by vigorous shaking using a vortex mixer. The absorbance for the test was determined at 417 nm and the concentration of the sample (mg/g) was calculated using standard curve of atropine. The total alkaloid content was expressed as mg of AE/g of extract.

The total flavonol content was evaluated using quercetin 0, 5, 10, 20, 40, and 80 mg/L in 95% ethanol (Sigma-Aldrich, South Africa) as standard. Briefly, crude sample extracts (12.5 µL) was mixed with 12.5 µL of 0.1% hydrochloric acid (HCl) (Merck, South Africa) in 95% ethanol, 225 µL 2% HCl and incubated for 30 min at room temperature. Absorption readings at 360 nm were taken at 25°C temperature (Mazza et al. 1999). Total flavonol content was expressed as mg quercetin equivalent per dry weight (mg QE/g dw).

The total polyphenol content of extract from dry material of leeks were determined with Folin-Ciocalteau assay (Singleton et al. 1999; Swain and Hills, 1959) and adopting methods by Swain and Hills (1959) for using a plate reader. The extract sample (25 µL) was mixed with Folin-Ciocalteu reagent (125 µL) diluted in distilled water (1:10) (Meck, South Africa). Five minutes later, 100 µL (7.5%) aqueous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, South Africa) was added to a 96-well microplate. The plates were incubated for 2 hours at room temperature (25±2 ^{o}C) before the reading of absorbance at 765 nm using Multiskan plate reader (Thermo Electron Corporation, USA). The standard curve was prepared using 0, 20, 50, 100, 250 and 500 mg/L gallic acid in 10% ethanol and the results were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g dw).

I. Statistical analysis

The experimental data collected were analyzed using one-way analysis of variance (ANOVA) and the Tukey test was used to separate the means at a level of significance, P=0.05. These computations were performed using PAST software [28].

III. RESULTS

A. Endophytic tissue colonization of B. bassiana and its effect on the growth of leeks

Drenching leek with *B. bassiana* at conidial suspensions (1x 10^5, 1 x 10^6, and 1×10^7 spores/ml) did not result in tissue colonization; 0% percent fungal colonization was recorded. Generally, exposure to fungi had minimal effects on the fresh and dry weights of aerial parts of leek. However, there were significant differences among treatments in fresh root weight of *A. porrum* (df=3,59 P < 0.01). Control treatment was observed to have the smallest fresh root weight, whereas T1 had the highest fresh weight, see Table I.

TABLE I: EFFECT OF ENTOMOPATHOGENIC FUNGUS- AMENDED MEDIUM ON THE GROWTH PARAMETERS OF *A. PORRUM* AT 10 WEEKS AFTER PLANTING.

Treatments	Control	T1	T2	T3
Dry Root Weight (g)	0.64±0.08a	0.90±0.17a	0.97±0.13a	0.74±0.11a
Dry aerial Weight (g)	3.65±0.32a	4.1±0.38a	3.8±0.16a	3.30±0.44a
Fresh root Weight (g)	6.44±0.53d	11.58±0.69a	9.92±0.88c	10.47±0.93ab
Fresh aerial Weight (g)	27.90±1.08a	33.56±1.52a	32.29±2.02a	31.34±1.49a
Plant height (cm)	38.23±1.44a	43.06±2.0a	41.03±1.9a	41.86±1.72a
Number of leaves	3.53±0.25a	4.13±0.21a	3.8±0.21a	3.6±0.23a

*Means followed by same lowercase letters in the same row are not significantly different following comparison using Tukey test.

B. Macronutrients

While there were no significant differences in the plant tissue levels of P, K, Ca, Mg and Na between fungus and control treatments (df= 1,7 P≥0.05), N tissue contents varied significantly (df=1,7 P≤0.05). The N content obtained was higher in control and significantly lower in T1 (6.5±0.64 mg/kg). Nonetheless, higher levels of P, K, Ca and Na were observed in T1 – Table II.

TABLE II: EFFECT OF ENTOMOPATHOGENIC FUNGUS-AMENDED MEDIUM ON THE MACRONUTRIENT UPTAKE OF *A. PORRUM* AERIAL PARTS.

Nutrients (mg/kg)	Control	T1 (1×10^7 spores/ml)
N	394.25±9.96a	6.5±0.64b
P	40.75±2.49a	44.25±1.84a
K	628±34.07a	640.25±24.61a
Ca	0.40±0.02a	0.42±0.1a
Mg	0.50±0.02a	0.47±0.02a
Na	2228.75±401.92a	2351.5±151.98a

*Means followed by same lowercase letters in the same row are not significantly different following comparison using Tukey test.

C. Micronutrients

Statistically there was a significant difference between treatments of Cu levels (df=1,7; P≤0.005). The Cu levels obtained were higher in T1 (4.5±0.64 g) and low in control (2±0.57 g). However, there was no significant difference between treatments in tissue Mn, Fe, Zn or B level (df=1,7; P≥0.005) (Table III).

TABLE III: TISSUE NUTRIENT CONTENT (MG/KG) IN AERIAL PARTS OF *A. PORRUM FOLLOWING* EXPOSURE TO THREE CONCENTRATIONS OF *B. BASSIANA* CONIDIAL SUSPENSION AND CONTROL AT AFTER 10 WEEKS' POST-TREATMENT.

Nutrients (mg/kg)	Control	T1 (1×10^7 spores/ml)
Mn	47±9.22a	55.5±2.02b
Fe	87.75±2.2.78a	91.5±7.27a
Cu	2±0.57b	4.5±0.64a
Zn	22.25±2.21a	26±0.91a
B	34.75±3.32a	38.75±1.49a

*Means followed by same lowercase letters in the same row are not significantly different.

D. Minimum inhibitory concentration

Based on the MIC values, acetone extracts of aerial parts from leek plants that were exposed to fungus (treatment T3) performed significantly (df= 6, 62; P ≤0.05) better than the other treatments, including the positive controls, in inhibiting both *B. bassiana* and *F. oxysporum (*Table IV).

TABLE IV: MINIMUM INHIBITORY CONCENTRATION OF ACETONE EXTRACTS OBTAINED FROM AERIAL PARTS OF *A. PORRUM* GROWN IN ENTOMOPATHOGENIC FUNGUS-AMENDED MEDIA AGAINST *B. BASSIANA AND F. OXYSPORUM*

Treatments	MIC (mg/ml) at 12h	
	B. bassisana	*F. oxysopoum*
Control	0.07±0.07ab	0.13±0.03ab
T1(1×10^7 spores/ml)	0.09±1.11bc	0.16±0.02ab
T2 (1×10^6 spores/ml),	0.12±0.01c	0.25±0.07b
T3 (1×10^5 spores/ml)	0.04±1.11a	0.08±0.01a
Mancozeb	1.50±0.00d	1.50±0.00d
Odeon	1.50±0.00d	1.50±0.00d
Acetone	1.50±0.00d	1.50±0.00d

*Means followed by same lowercase letters in the same column are not significantly different following comparison using Tukey test. Mancozeb and Odeon are positive treatments while acetone was used as negative treatment.

E. Phytochemical Screening

Different phytochemical constituents were analysed from dry material of *A. porrum* aerial parts shown in Table V. There were no significant differences among treatments for all phytochemical constituents that were analysed (df= 3, 15; F=0.5; P≥0.05) and all their values showed similarities to each other.

TABLE V: PHYTOCHEMICAL SCREENING OF *A. PORRUM* DRY AERIAL PARTS EXPOSED TO DIFFERENT *B. BASSIANA* CONIDIA CONCENTRATIONS T1(1×10^7), T2(1×10^6), T3(1×10^5) AND CONTROL (DEIONIZED WATER).

Treatments	Polyphenol (mg GAE/g)	Flavonols (mg QE/g)	Alkaloids (mg AE/g)
Control	6.93±0.33a	3.5±0.27a	2.17±0.26a
T1	6.6±0.76a	4±0.39a	2.79±0.18a
T2	6.16±0.28a	3.6±0.27a	2.82±0.19a
T3	6.23±0.41a	3.9±0.48a	2.83±0.30a

*Means followed by same lowercase letters in the same column are not significantly different following comparison using Tukey test.

IV. DISCUSSION

Inoculating leek with the endophytic fungus *B. bassiana* did not lead to tissue colonization by the fungus. However, these results are not in agreement with previous findings from studies that inoculated plants using the soil drenching method [29-31]. Many previous studies have shown the ability of this fungus to colonize a wide array of plant species using different inoculation techniques, such as leaf spraying, root dipping, seed immersion, stem injection, mixing of dry conidia with growing potting medium, and vacuum infiltration [32-35]. The failure of the fungal endophyte to establish in the tissue of leek in the current study could be due to inherent characteristics of the fungal isolate used [31], the host species, the plant part evaluated, and the inoculation methods used — these factors could influence tissue colonization by an endophyte [30, 33, 35]. Literature on the influence of host plants' antifungal activities and bioactive constituents on fungal colonization of plants are scarce. Antifungal constituents of host plant could impair endophytic fungal colonization.

There was no distinct difference on the growth of *A. porrum* in terms of number of leaves, aerial part length and dry weight parameters. However, root fresh weights were significantly difference among the treatments. Many studies have reported that endophytic fungi *B. bassiana* have plant growth enhancing effects [36, 37]. In a paper by Lopez and Sword [38], *B. bassiana* significantly enhanced plant development in cotton plant. On the contrary, findings by Pai and Bushley [32] demonstrated that endophytic fungus can decreas plant growth and production in tomato plants. While there was no significant influence of fungal inoculation on secondary metabolite contents in this study, *B. bassiana* is known to produce a variety of bioactive secondary metabolites that can inhibit or promote growth [12, 36, 39].

Previous studies have demonstrated that inoculating plants with endophytic fungi *B. bassiana* promotes nutrient absorption [29, 40, 41]. In this study, inoculation of *B. bassiana* using soil drenching method showed a significant increase in the tissue contents of copper (Cu), manganese (Mn), and a decreased nitrogen (N) content in fresh aerial parts of plants exposed to *B. bassiana* conidia. Cartmill et al. [42] reported similar results. In their study, plants inoculated with mycorrhizal fungi had higher Cu and Mn content when compared to untreated plants.

Interestingly, our results showed that the lowest MIC value of acetone extracts of *A. porrum* was observed at T3 (0.04±1.11 mg/ml) and was the most bioactive against *B. bassiana*, but it was not statistically different from control (0.07±0.07 mg/ml) treatment at 12-hour post treatment (Table III). Overall, the acetone extracts of aerial parts of *A. porrum* was found to be bioactive against *B. bassiana* in the antifungal assay. The acetone extracts of plants exposed to the lowest fungal spore concentration and no spores were the most active against the fungi in the MIC bioassay. Moreover, acetone extracts of the aerial parts of *A. porrum* performed better than the tested positive controls (Mancozeb and Odeon) (Table III). The results suggested that aerial parts of *A. porrum* acetone extracts have fungistatic effects against *B. bassiana* and *F. oxysporum.* The high antifungal activity of leek plants might have prevented the *B. bassiana* conidia from successfully colonizing its tissue.

Further investigations are warranted to establish whether tissue colonization by fungal endophytes is influenced by secondary metabolite contents of plants.

In the present study, phytochemical investigation of *Allium porrum* (leeks) revealed the presence of polyphenols, flavonols and alkaloids in the plant extracts. Plants in the genus *Allium* are rich in phytochemicals and are exploited by agricultural, nutraceutical and pharmaceutical industries [6, 43-45].

V. Conclusion

Although successful leek tissue colonization by *B. bassiana* was not recorded in this study, we demonstrated that the presence of a fungus in the root region may still influence the plant physiologically in terms of nutrient availability. In nature, some of these entomopathogens are part of the rhizophere microbiome of the plant, and may influence nutrient availability to and absorption by plants; thus, influencing growth and secondary metabolite production in plants. More extensive studies on the influence of plant antifungal activities on endophytic fungal colonization of plants are warranted.

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