World Journal of Agricultural Sciences 5 (6): 720-728, 2009
ISSN 1817-3047

Trichoderma as a Seed Treatment to Control *Helminthosporium* Leaf Spot Disease of *Chrysalidocarpus lutescens*

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Abstract: *Helminthosporium* (Bipolaris) causes a leaf spot disease in *Chrysalidocarpus lutescens* (cane palm) in Sri Lanka. The losses could reach 90% of production during rainy weather conditions. Field experiments were carried out to test the efficacy of treatment of seeds of *C. lutescens* against *Helminthosporium* infection. In the fungal growth tests the isolates *T.harzianum* 1, *T.harzianum* 2, *T.viride* 1,*T.viride* 2 and *T.viride* 3 inhibited growth of the pathogen by 79.18, 69.03, 83.75, 82.99, 74.11% respectively. Isolates of *Trichoderma harzianum* and *Trichoderma viride* obtained from soil and having antagonistic activity against *Helminthosporium* were used in the field experiments. In the field trials, seed treatment with a spore suspension of *Trichoderma* completely eliminated the disease while *Trichoderma* and *Pseudomonas fluorescens* reduced the disease incidence significantly. The seed treatments also significantly increased seed germination, seedling growth and seedling vigor.

Key words: *Trichoderma* % Biological control % Leaf spot % *Helminthosporium* % Cane palm

INTRODUCTION

The soil and seed borne fungus *Helminthosporium* causes severe yield loss in *Chrysalidocarpus lutescens* (cane palm) in Sri Lanka. Although several measures including chemical treatments are used by growers to control the disease, the results are not satisfactory. Hence, there is an urgent requirement to develop alternative control measures and biological control is a promising alternative. *Trichoderma* is the most widely used biocontrol agent of plant diseases [1]. *Trichoderma harzianum* and *Trichoderma viride* are active rhizosphere colonizers and these fungi produce antibiotics such as gliotoxin, viridin, cell wall degrading enzymes and also biologically active heat-stable metabolites such as ethyl acetate [1]. These substances are involved in disease suppression and / or plant growth promotion. *Pseudomonas fluorescens* also has potential in the biological control of soil-borne plant pathogenic fungi [1]. When *P. fluorescens* is applied to planting material or to the soil, the bacteria colonize roots and prevent or limit the establishment of root pathogens and their secondary spread [2]. In addition to the antagonistic activity, *P. fluorescens* also produces numerous of growth substances in the soil which promotes plant growth [3].

The objective of this study was to examine the effect of *Trichoderma* spp. isolated from the soil as a seed treatment, either alone or in combination with *P. fluorescens*, for controlling the leaf spot disease of *C. lutescens* caused by *Helminthosporium* sp.

MATERIALS AND METHODS

Isolation of *Trichoderma* sp and *In-vitro* Studies: The *Trichoderma,* two species used in this study were isolated from soil samples obtained from Green farms Ltd, Marawila, Sri Lanka using the soil dilution technique [4]. Pure cultures of the *Trichoderma* isolates were maintained on PDA at 25± 2°C and the isolates were identified using morphological and reproductive characters [5-9]. The following isolates were Identified as.*viride1, T.viride2, T.viride3, T.harzianum1* and *T.harzianum2,*

Isolation of *Helminthosporium* From *Chrysalidocarpus lutescens* (Cane Palm): The pathogen was isolated from infected *C.lutescens* leaves showing typical leaf spot lesions by plating surface sterilized infected segments with 1%NaOCl for 2 min on PDA medium

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Helminthosporium species were identified using morphological and reproductive characteristics. Koch's postulates were followed to prove their pathogenicity [10].

Preparation of Conidia Suspensions: A conidia suspension of the test isolates of *Trichoderma* was prepared from a 7-day old culture of the isolate on PDA. The plate (9cm diameter) was flooded with 10ml of sterilized distilled water and shaken for a few minutes. The resulting suspension was filtered through muslin cloth. After filtering the suspensions through double layer of cheese towel, the conidial concentration was determined using a double ruled Nabuer's haemocytometer. The spore concentration of the filtrate was adjusted to10^4 conidia ml^{-1} using sterilized distilled water.

Effect of *Trichoderma* on *Helminthosporium:* "Poisoned food technique" [11] was followed to assesses the inhibitory effect of five *Trichoderma* isolates on one *Helminthosporium* isolate. Conidia suspensions (1ml) of *Trichoderma* isolates, prepared as described above, was poured into a sterilized Petri dish followed by 15ml of PDA. One ml of distilled water instead of the spore suspension was used in the control. Four mm diameter discs were obtained from an actively growing region of a 7-day old *Helminthosporium* culture on PDA and the disc was transferred aseptically to the centre of each *Trichoderma* amended PDA medium. The treatments were replicated five times in a completely randomized design and repeated three times. The Petri dishes were incubated at 28± 2°C and 72 ±4% RH. Growth of *Helminthosporium* was determined at 3 and 7 days after inoculations by measuring the diameter of the culture diametrically. The percentage growth inhibition (I) was calculated using the following formula [12].

Dual Culture Technique: Two isolates of *T. harzianum* and three isolates of *T. viride* were screened individually against *Helminthosporium* by employing dual culture technique [4, 13]. Complete randomized design was used and the treatments were replicated five times. The same set was repeated three times. *Helminthosporium* sp. was maintained as standard check. Plates were incubated at 26±2°C and seven days after inoculation the radial growth of *Helminthosporium* sp. was measured. Inhibition of mycelia growth of *Helminthosporium* sp. by different strains of *Trichoderma* sp. was recorded on the basis of radial growth in dual culture and in control using the following formula [12]. The best two isolates each in different species were selected based on percentage of growth inhibition and have been tested for confirmation.

$$[I\% = (C-T)/C] \times 100.$$

Where,
I = percentage inhibition of pathogen by antagonists
C= radial growth in control
T= radial growth in the treatment.

Seed Treatment: The seeds of *C. lutescens* were obtained from Green farms Ltd, Marawila, Sri Lanka. The treatments listed below were carried out to determine the efficacy of *Trichoderma* as a seed treatment.

- T1 seeds were soaked for 12 h in sterilized distilled water.
- T2 seeds were treated with a conidial suspensions of the test *Trichoderma* isolates (ratio 1:1) for 24 h (the conidia concentration (Mean value)of each isolates was 1x 10^{11}conidia /ml).
- T3 seeds were treated with a conidial suspension (1x 10^{11}conidia/ml) of *Trichoderma* isolate and *Pseudomonas fluorescens* (T.Stanes and Company Ltd, 'India'-[50ml/liter]) for 24 h
- T4 seeds were treated with 0.14mM NaOCl for 30min and washed in running water for one hour.

The treated seeds were air-dried for 7 days under ambient conditions before sowing as described below. The experiments were conducted for two consecutive growing seasons (2007-2008) under identical conditions-Average Annual Rain fall1620mm, Minimum and Maximum Relative humidity 60%-90%, Minimum and Maximum Day Temperature 25°C-34°C, Minimum and Maximum Night Temperature 20°C-27°C.

A field of 60 m^2 was prepared in which 12 micro plots were demarcated by raised margins. Seeds subjected to a treatment (T1, T2, T3 or T4) were sowed in three micro plots As replicates and 500 seeds were used for each. The distribution was in a complete randomized block design [1]. The field was irrigated at 3-day intervals after sowing. At 1, 2 and 3 months after sowing, twenty-five plants from each micro plot were randomly uprooted to determine dry-matter production and plant length (root /shoot). Observations were made on the occurrence of the disease at 2-week intervals for five months and the leaf spot incidence was calculated as follows.

$$\text{Leaf Spot Incidence (\%)} = \frac{\text{No. of infected plants in a micro plot}}{\text{Total no. of plants in a micro plot}} \times 100$$

Plant vigor index was determined as given below at monthly intervals for 3 months. Observations from the twenty-five plants of each micro plot were averaged and considered as one replicate [14].

Plant Vigor Index = Germination% x (shoot length+ root length).

RESULTS

Growth Inhibition *In-vitro*: All the *Trichoderma* isolates tested exhibited inhibition of growth of *Helminthosporium.* Percentage of growth inhibition of *Helminthosporium* by *T. harzianum* 1, *T. harzianum* 2, *T. viride*1, *T. viride*2, *T. viride*3 were 79.18%, 69.03%, 83.75%, 82.99%, 74.11% respectively (Fig. 1 and 2).

In dual culture technique the mycelium of both the cultures came in contact with each other on 3DAI. Seven days after inoculations the hyphal growth of *Helminthosporium* was found to be inhibited by the hyphae of *Trichoderma* sp (Figure 4). Further, *Trichoderma* almost inhibited the mycelia growth of the *Helminthosporium* at 10 DAI. The advancing hyphae of *Trichoderma* covered the entire Petri plates, suppressing the growth of *Helminthosporium* sp. When both the species of *Trichoderma* sp. were evaluated against *Helminthosporium,* Tv1 and Th1 were suppressed the growth of the mycelia of *Helminthosporium* (Fig. 3 and 4). Seven days after inoculation the radial growth of *Helminthosporium* sp.

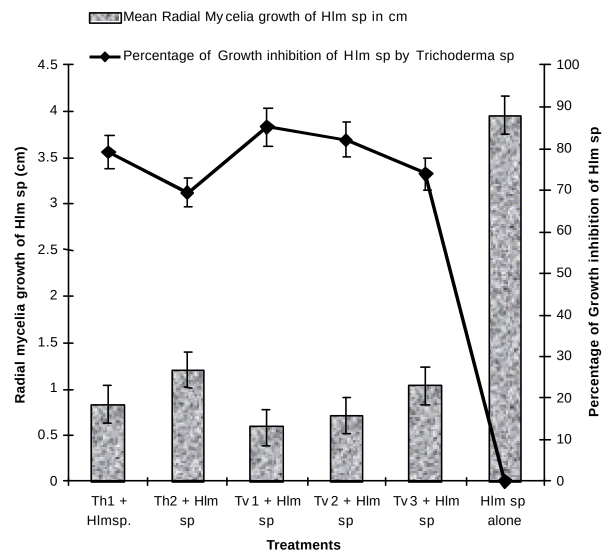


Fig. 1: Effect of *Trichoderma* isolates on the growth of *Helminthosporium*

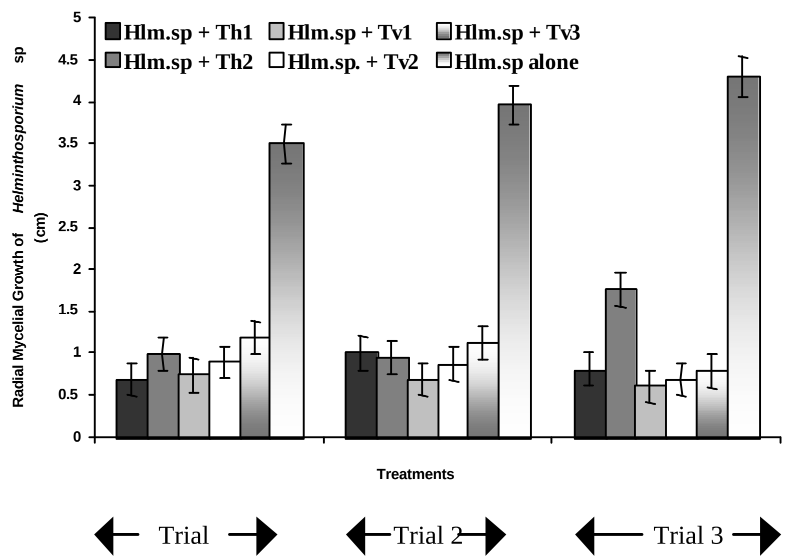


Fig. 2: Mycelia growth of *Helminthosporium-Bipolaris* in association with Antagonist Fungi *Trichoderma* sp

T1	*Helminthosporium* Pathogenic fungi with *T. harzianum* 1	Th1
T2	*Helminthosporium* Pathogenic fungi with *T. harzianum* 2	Th2
T3	*Helminthosporium* Pathogenic fungi with *T. viride* 1	Tv1
T4	*Helminthosporium* Pathogenic fungi with *T. viride* 2	Tv2
T5	*Helminthosporium* Pathogenic fungi with *T. viride* 3	Tv3
T6	*Helminthosporium* Pathogenic fungi alone	Hlm.sp

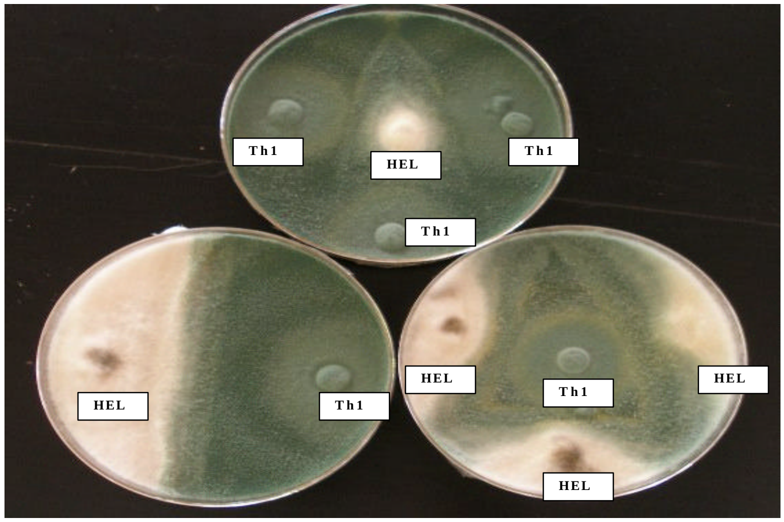
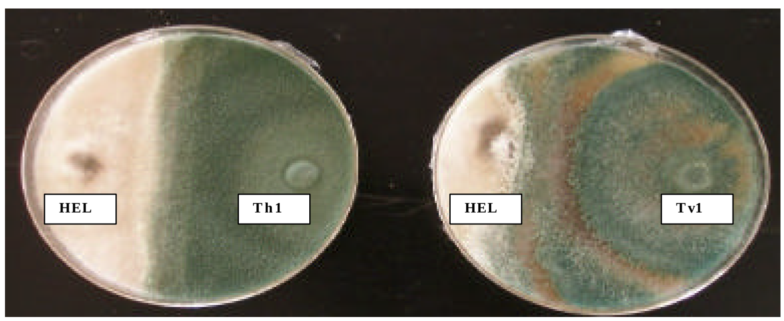
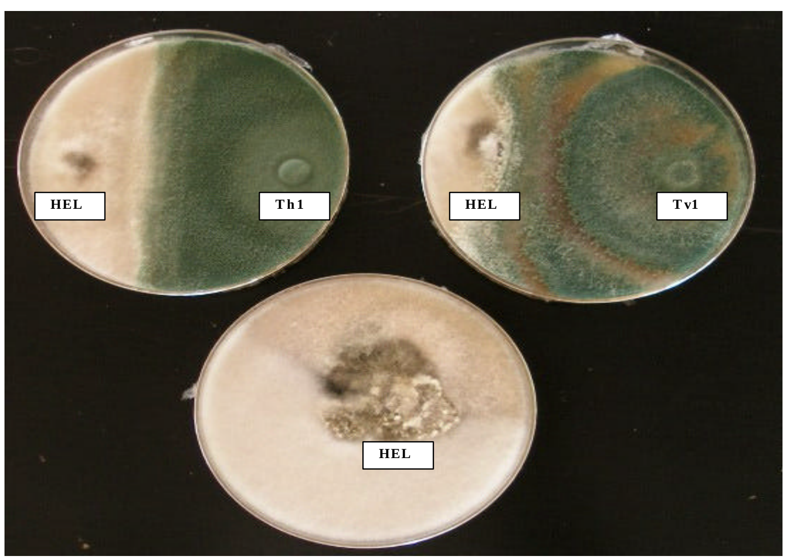


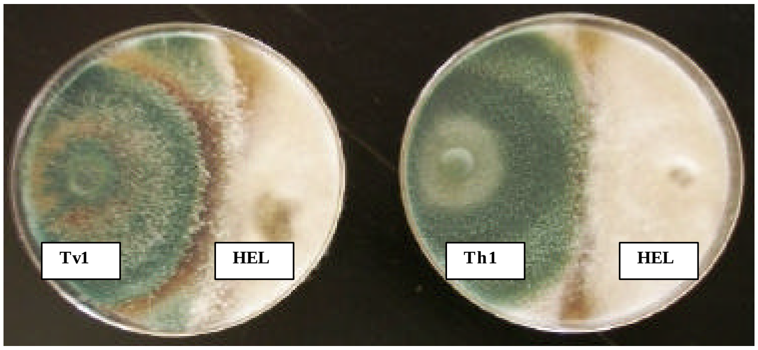
Fig. 3a: Seven days after inoculation fungal mycelia growth in dual culture technique.
HEL: - *Helminthosporium* Sp Th1: - *Trichoderma harzianum* Th1



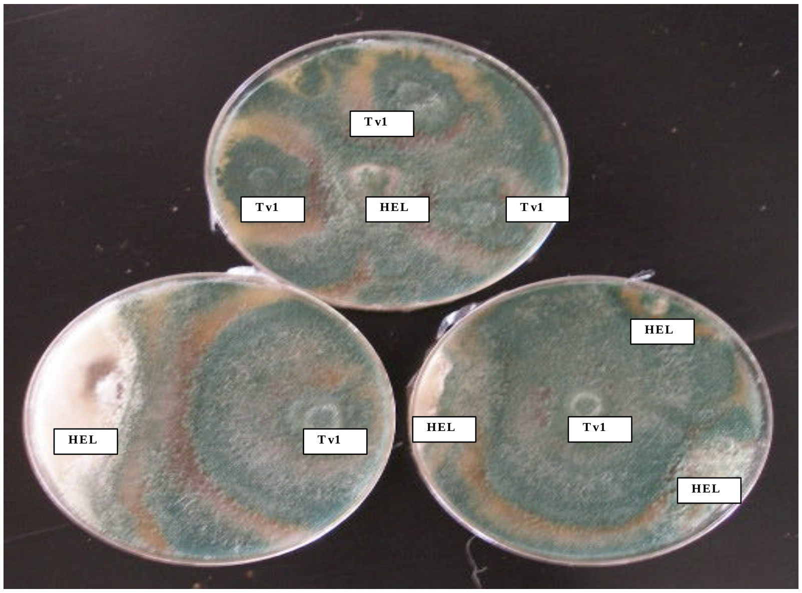
3(b): - Seven days after inoculation fungal mycelia growth in dual culture technique.
HEL: - *Helminthosporium* Sp Th1: - *Trichoderma harzianum* Th1 Tv1: - *Trichoderma viride* Tv1



3 c: - Seven days after inoculation fungal mycelia growth in dual culture technique.
HEL: - *Helminthosporium* Sp Th1: -- *Trichoderma harzianum* Th1 Tv1: - *Trichoderma viride* Tv1



3 d: - Five days after inoculation fungal mycelia growth in dual culture technique.
HEL: - *Helminthosporium* Sp Th1: - *Trichoderma harzianum* Th1 Tv1: - *Trichoderma viride* Tv1



3 (e): - Seven days after inoculation fungal mycelia growth in dual culture technique.
HEL: - *Helminthosporium* Sp Tv1: - *Trichoderma viride* Tv1

3 (f): - Seven days after inoculation fungal mycelia growth in dual culture technique.
HEL: - *Helminthosporium* Sp Th1: - *T. harzianum* Th1 Tv1: - *T.viride* Tv1

Fig. 3: Dual culture pictures

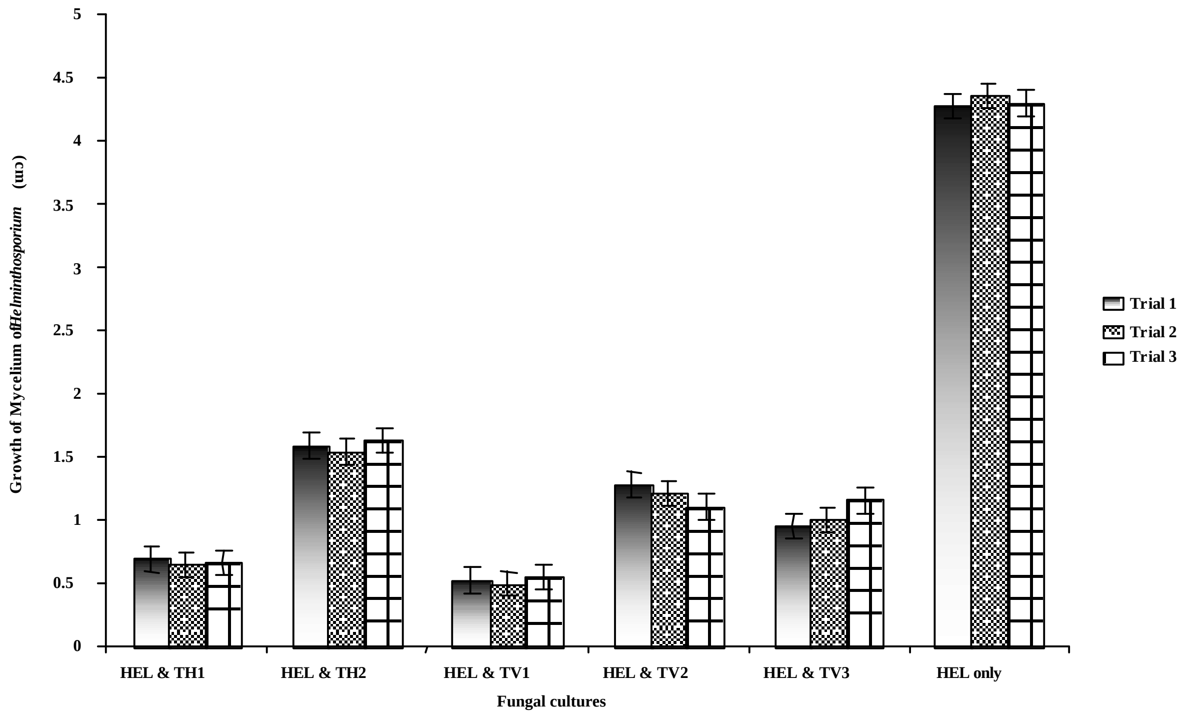


Fig. 4(a): Mycelia growth of Helminthosporium - Bipolaris in association with Antagonist Fungi Trichoderma sp in dual culture technique
Th1- *T.harzianum1*, Th2 -*T.harzianum* 2, Tv1 -*T.viride1*, Tv2 -*T.viride* 2 Tv3 -*T.viride* 3, *Hlm* - *Helminthosporium* sp

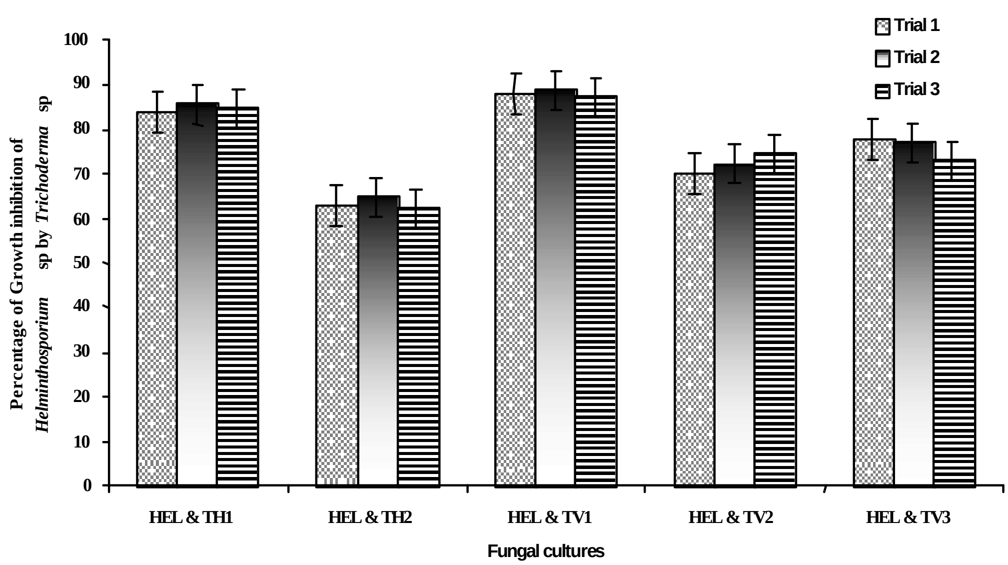


Fig. 4 b: Effect of Trichoderma isolates on the growth of Helminthosporium
Th1- *T.harzianum1*, Th2 -*T.harzianum* 2, Tv1 -*T.viride1*, Tv2 -*T.viride* 2
Tv3 -*T.viride* 3, *Hlm* - *Helminthosporium* sp

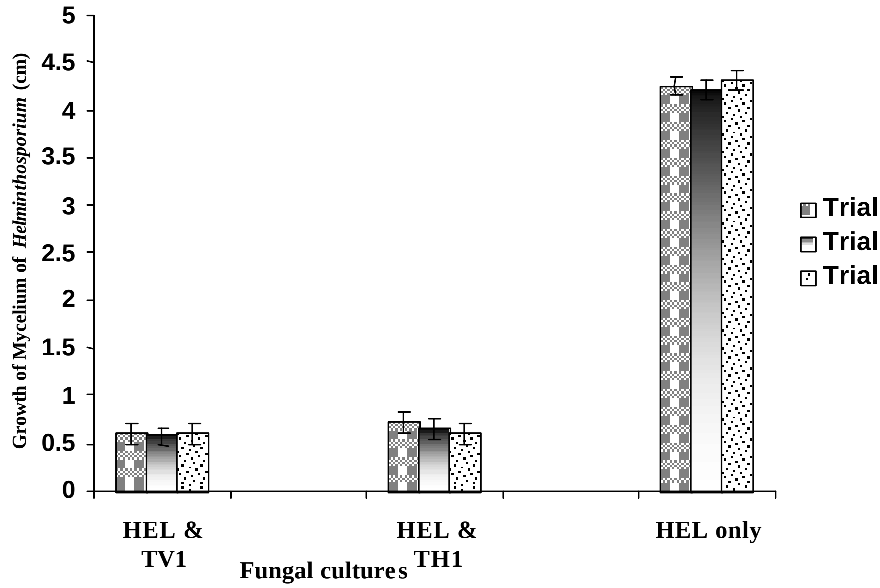


Fig. 4 (c): Mycelia growths of *Helminthosporium-Bipolaris* in association with Antagonist Fungi *Trichoderma* sp in dual culture technique
Th1-*T.harzianum1*, Tv1-*T.viride*1 and the pathogenic fungi Hlm-*Helminthosporium* sp

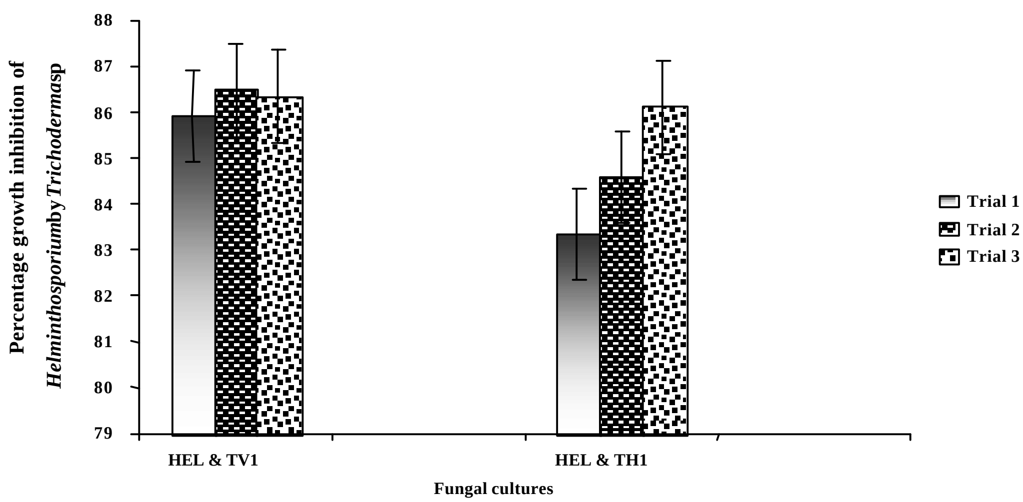


Fig. 4 (d): Effect of *Trichoderma* isolates on the growth of *Helminthosporium*
Th1-*T.harzianum1*, Tv1-*T.viride*1 and the pathogenic fungi Hlm-*Helminthosporium* sp

Table 1: Effect of seed treatment on germination, plant vigor and disease incidence

		Seedling vigor index			
Treatments	Germination %	1 month after germination	2 months after germination	3 months after germination	Disease Incidence%
Untreated Control	43.86[a]	893[a]	1277[a]	1555[a]	6%
T.viride and T.harzianum	54.40[b]	1391[b]	1651[b]	1918[b]	0%
T.viride+T.harzianum and *P fluorescens*	55.46[b]	1354[b]	1731[b]	2066[b]	1.8%
NaOcl	48.48[ab]	1282[b]	1493[ab]	1864[ab]	2%

- Means in a column for each treatment followed by the same letters are not significantly different according to LSD (p=0.05) test.
- Data are the means of five replicates at two weeks intervals in two consecutive trials.
- Disease incidence presented above is the average values of occurrence of the disease at two week intervals for five months obtained from all record combined.

had been measured and analyzed using SPSS. Significant differences ($p<0.05$) have been found in each treatment.

Seed Treatment and Field Experiments: Four seed treatments were evaluated. Seed treatment with spore suspension of *T.viride* + *T.harzianum* and *Trichoderma* species + *Pseudomonas fluorescens* reduced the disease incidence compared to untreated control. Such seed treatments also significantly increased seedling growth and seedling vigor index of *C lutescens.* The growth parameter and vigor index were high in *Trichoderma* Sp *+P. fluorescens* treatment. *Trichoderma* Sp with *Pseudomonas fluorescens* seed treatment enhanced seed germination by 26.44%, seedling vigor index by 32.87% and reduced leaf spot incidence by 78.57%. Seed treatment with *Trichoderma* alone enhanced seed germinations by 24.03%, seedling vigor index by 23.29% and reduced leaf spot incidence totally. Treatment with NaOcl also caused a reduction in disease incidence but its overall effect on seedling vigor was less the other treatment (Table 1).

DISCUSSION

Microscopic observations in dual culture revealed that coiling of antagonistic hyphae around *Helminthosporium* hyphae that ultimately resulted in a mycelial rope like appearance

This study reveals that seeds treatment with *Trichoderma* sp. have the potential to highly reduce the disease on Cane palm. *Trichoderma*, is reported to give systemic protection against many seed borne foliar diseases [15] and *Trichoderma* Sp.is also known to provide plants with useful molecules such as glucose oxidase and growth stimulating compounds that can increase their vigor and as a result resistance to pathogens [16, 17]. Moreover these fungi produce antibiotics such as gliotoxin, viridin and cell wall degrading enzymes and also biologically active heat-stable metabolites such as ethyl acetate. These substances are known to be involved in disease incidents suppression. [1]. Though higher seedling vigor and percentage of germination was obtained when *Trichoderma* was mixed with *P.fluorescens,* the differences were not significant. *Pseudomonas fluorescens* has been also reported to produce auxins, gibberellins and solubilises phosphorus in the soil which also promotes plant growth [3] and this could be the reason for the higher vigor and percentage germination.

The seed treatment with NaOCl also decreased disease incidence. Sodium hypochlorite is highly effective against many bacteria, fungi and viruses. The possible reason is that NaOcl kills microbes by oxidizing biological molecules such as proteins and nucleic acids and the chemical penetrates more easily through the seed coat. [18].

CONCLUSION

Trichoderma sp inhibits the growth of the pathogen *Helminthosporium* sp.and seed treatment with *Trichoderma* can be used to protect Cane palm seedlings from the leaf spot disease caused by *Helminthosporium*. The treatments also increase percentage of germination and seedling vigor.

ACKNOWLEDGEMENT

The authors are grateful to Mr.Arne Svinningen, Chairman, Managing Director, Green Farms Ltd, Marawila, Sri Lanka for his kind assistance through-out the research period. The research work was fully supported by Green Farms, Ltd, Marawila. The authors also appreciate Mr.M.D.S.D Karunaratne, Technical Manager, Ms.K.P.Rashani and Staff members, Laboratory unit, Green Farms Ltd, for their support through-out the research period.

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