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Small Scale Production of *Trichoderma viride* on Locally Available Liquid Waste and Other Substrates

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Abstract: In eco friendly agriculture, *Trichoderma viride* is a promising bio control agent for various plant pathogens. It can be multiplied in solid and liquid media but liquid fermentation yields high reproductive capacity and is more convenient than solid state fermentation system. High cost of substrates and storage methods are major problems to accelerate the production. Therefore experiments were carried out to screen out suitable liquid wastes and other liquid media as suitable substrates for small scale production of *T. viride*. Locally available household and industrial liquid wastes such as Black gram soaked water, Coconut water, Rice mill effluent from the red pericarp variety, 5% Distillery spent wash and other liquid substrates such as 1% Palmyrah jaggery solution, 5% Palmyrah toddy and 1% Palmyrah fruit pulp extract, 10% Cow urine, 10% *Gliricidia sepium* and 10% *Thespesia populnea* leaves extracts were individually investigated. Among these substrates higher growth and sporulation of *T. viride* was recorded in black gram soaked water ($35.9x10^7$spores/ml), followed by 1% Jaggery solution ($30.0x10^7$spores/ml), Coconut water ($28.8x10^7$spores /ml), Rice Mill Effluent ($28.7 x10^7$spores/ml) and 1% Palmyrah fruit pulp extract ($27.1x10^7$spores/ml) after 14 days of incubation in dark room at 30°C. *T. viride* grown on black gram soaked water resulted highest fungal growth inhibition of 83.72% against *Sclerotium rolfsii* in 7 days. The present study revealed that locally available liquid substrates are potential source for liquid fermentation of *T.viride*.

Key words: *Trichoderma viride* · Black gram soaked water · Coconut water · Liquid fermentation · Growth inhibition

INTRODUCTION

Extensive applications of agrochemicals lead to numerous health and environmental problems. Very recently Chronic Kidney Disease of unknown etiology (CKDU) is reported in North Central province and some other parts of Sri Lanka [1, 2]. Therefore government banned most of the commonly used synthetic pesticides. In addition to that, chemical pesticides are not a long term and only remedy for sustainable agriculture, because development of resistance to certain toxic chemicals are reported and also have less selectivity and their effect is temporary as well [3]. This has increased the interests on bio pesticides and the biocontrol agents are also used effectively to control the plant pathogens.

Trichoderma species are the most effective fungi against many soil borne and foliar pathogens [4, 5]. It has been known since 1930 to exhibit the antagonistic activity and commonly used them to control most of the plant diseases [6,7]. In addition, *Trichoderma* based bio control agents have the ability to promote plant growth and soil remediation activity [8].

Trichoderma sp is multiplied by solid and liquid fermentation methods [9]. Enormous literatures are available for the mass multiplication of *T. viride, T. harzianum, T. reesei* and T. *koningii*. Glucose, cellulose, soluble starch and molasses are used as substrates to conventional synthetic solid media but the availability and high cost of these media are major limitations [10]. Therefore many researchers have successfully used low cost solid materials like rice husk, saw dust [3] rotten wheat grains and vegetable waste As a substitute to the substrates [11]. However solid fermentation has its own limitations such as requirement of high substrate volume, contamination and prolong fermentation.

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Table 1: Collection sites of locally available substrates

Locally available substrates	Collection sites
Palmyrah fruit pulp extract and jaggery	Palmyrah development board, Jaffna
Gliricidia sepium leaves extract	Farmer field, Jaffna
Thespesia populnea leaves extract	Farmer field, Jaffna
Black gram soaked water and coconut water	A Restaurant at Jaffna
Rice mill effluent	KRS rice mill, Thavadi, Jaffna
Cow urine	Cattle Farm, Jaffna
Distillery spent wash and Palmyrah toddy	Distillery unit, Navaly, Jaffna

T. viride produces three kinds of propagules: hyphae, chlamydospores and conidia [12]. Among these, hyphae are the major propagule. However hyphae cannot withstand during drying and dehydration process [12]. Chlamydospores and conidia have been used as active propagules in most of the *Trichoderma* sp. formulation [13, 14]. More over liquid medium has more advantages such as easy application through irrigation system, less time consumption for fermentation and application. In addition, mass multiplication of bio agent through liquid fermentation helps to minimize the liquid waste generation. Therefore present investigation is carried out to evaluate the locally available liquid wastes and other materials for the mass multiplication of *T. viride* by small scale farmers to strengthen sustainable agriculture.

Materials and Methods

Preparation of Initial Inoculation: *T. viride* and *Sclerotium rolfsii* were obtained from the collection of department of Agricultural biology, Faculty of Agriculture, University of Jaffna and cultured on potato dextrose agar (PDA) medium. Initial inocula were prepared by scraping seven days old fully sporulated cultures grown on the surface of PDA plate using with sterile scalpel in distilled water. Initial spore count ($3.02x10^6$ spores/ ml) was determined by haemocytometer.

Collection and Preparation of Liquid Media: The locally available substrates and the collection sites were given in Table 1. 10% Distillery spent wash, 10% *Gliricidia sepium* leaves extract,10% *Thespesia populnea* leaves extract, Black gram soaked, Rice mill effluent, Coconut water, 5%Palmyrah Toddy, 1% Palmyrah fruit pulp extract, 1% Palmyrah Jaggery solution were prepared and Chemical parameters like pH, total soluble solids (TSS), electrical conductivity (EC) were measured using multi- meter (HACA).

Evaluation of Growth and Sporulation of *T. viride* in Locally Available Substrate: An experiment was conducted using complete randomized design with 3 replicates of each substrate. Glass containers (180ml) were cleaned with disinfectant. 50ml of substrates were poured in to glass containers and plugged with non-absorbent cotton. pH of the substrate solutions were adjusted from 6 to7 using 1% HCl and 1% NaOH solutions. Total soluble solid content (TSS) and Electrical conductivity (EC) were measured by using HACA multi- meter. Substrates were sterilized at 121°C, 15PSI for 15 to 20 minutes [15]. *T. viride* ($3.02x10^6$spores/ml) was inoculated into the sterilized substrate under aseptic condition and inoculated samples were incubated at dark room for two weeks at ambient temperature (30±2°C). The final spore counts of each samples were taken by using haemocytometer twice at 7^{th} and 14^{th} days after inoculation.

Antagonistic Effect of *T. viride* Grown on Locally Available Substrates Against *Sclerotium rolfsii*: Antagonistic effect of *T. viride* was evaluated against *Sclerotium rolfsii* using dual culture technique described by Rangeswaran and Prasad [16]. A mycelial disc of 9 mm diameter was obtained from 7 days old culture. *T. viride* and selected fungal pathogen were dually placed on freshly prepared PDA medium with equal distance of 3cm from the periphery [17]. Following treatments were carried out under *in-vitro* conditions.

T1- Control
T2- *T. viride* in Rice mill effluent
T3 -*T. viride* in Blackgram soaked water
T4 -*T. viride* in 1%Palmyrah Jaggery solution
T5 -*T. viride* in Coconut water

Then inoculated plates were incubated at 30±3°C for 5 days. At the end of incubation period, the radial growth and the antagonistic zones were measured. Percentage of Inhibition of mycelial growth for the tested pathogen was calculated using the formula I = [100 (C-T)]/C as described by Kulkarni and Lingappa [18].

where
I = Inhibition of mycelial growth.
C = Mycelial growth in control
T = Mycelial growth in treated with *T.viride*.
Each treatment was replicated four times
Each treatment was replicated four times.

Statistical Analysis: The *In-Vitro* experiment was designed according to the complete randomized design (CRD) and the derived data were statistically analyzed using SAS package significance among the treatments were determined according to the least significant difference (LSD) test at 95% of the confidence interval. [19].

RESULT AND DISCUSSION

Chemical Characteristics of Locally Available Substrates: Chemical characteristics of locally available substrates were presented in Table (2). The pH, Electrical conductivity (EC) and Total soluble solid (TSS) of tested substrates are given in Table 2. The recorded pH was high in cow urine (8.33) and low in palmyrah toddy 5% (3.96). All other substrates contain low acidic to near neutral pH. Among the tested substrates highest EC 9.29mS/cm was observed in coconut water where as lowest was in palmyrah jaggery solution (1.178mS/cm). Total soluble solid content (TSS) was recorded as high as in coconut water (4.5 g/L) and low in 1% Jaggery solution (0.523g/L) among the tested substrates.

Growth and Sporulation of *T. vir*ide in Locally Available Substrates: The growth and sporulation of *T.viride* in locally available substrates are shown in Table 3. The highest spores count was recorded after 7th day of incubation in black gram soaked water 16.4×10^7/ml. Next highest spores count was observed in coconut water (14.8x10^7/ml), then Rice mill effluent (14.4 x10^7/ml), 1% Palmyrah jaggery solution (12.6 x10^7/ml) and 1% Palmyrah fruit pulp extract (9.22x10^7/ml). Lowest spore count was recorded in 0.26x10^7 /ml in 5% Distillery spent wash, 5%Toddy (0.3 x10^7 /ml) and 10% *Gliricidia* leaf extract (0.5x10^7/ml).

After 14th day of incubation the highest spore count was recorded in black gram soaked water (35.9 x10^7/ml), 1% Jaggery solution (30.4 x10^7/ml), Coconut water (28.8x10^7/ml), Rice mill effluent(28.7 x10^7/ml) and 1% Palmyrah fruit pulp extract (27.1 x10^7/ml), respectively. Low spore count was recorded in 5%Toddy, 5% Distillery spent wash and 10% *Gliricidia* leaf extract 0.6 x10^7/ml, 0.84 x10^7 /ml and 1.6 x10^7/ml, respectively.

*T. vir*ide growth on black gram soaked water (A), rice mill effluent (B), *T. vir*ide coconut water (C).

The results of the present study revealed that locally available liquid substrates such as Black gram soaked water, Coconut water; rice mill effluent, Palmyrah jaggery solution and Palmyrah fruit pulp extract can be used for the mass multiplication of *T. viride* by small scale farmers. Another study reveals that pulses are the potential sources for maximum biomass production of *Trichoderma* spp. [4]. Black gram soaked water and Coconut water are locally available liquid waste from domestic kitchen and south Indian restaurants. Palmyrah is one of the widely distributed natural resources in northern and eastern part of Sri Lanka [20]. Some previous studies also proved the mass multiplication of *T. viride* in liquid medium such as molasses soy flour broth and potato dextrose broth [3]. A study revealed that sporulation of *T. viride* is high in liquid media compared to solid media of the same substrate as vegetable waste, fruit juice and rotten wheat [21]. Small scale multiplication of *T. viride* through liquid fermentation helps to produce microbial starter for the compost tea production. Cow urine [22] and *Thespesia* leaves extract [23] have antimicrobial activity. Palmyrah Toddy (pH 3.96) and Distillery spent wash (pH 4.28) contain low pH, it may affect the growth and sporulation of *Trichoderma viride*, because optimum pH ranges between 5.5and 7.5 for growth and sporulation of *Trichoderma* spp. [24].

Antagonistic Effect of *T. viride* Against *Sclerotium rolfsii*: Antagonistic effect of *T.viride* grown on locally available substrate against *Sclerotium rolfsii* is shown in Table 4. Highest percentage of fungal inhibition was observed in T3- *T.viride* grown on black gram soaked water treatment as 83.72%(14.65 mm MCD) followed by 62.77% (33.75 mm MCD) inhibition from T5 -*T. viride* grown on Coconut water, 62.05% (34.15mm MCD) from T4 -*T. viride* grown on Palmyrah Jaggery solution 1%, 53.66% (41.70mmMCD) from T2- *T. viride* grown on Rice mill effluent. Therefore *T. viride* grown on black gram soaked water has shown highly significant antagonism than other treatments and control (Table 4).

Sclerotium rolfsii is one of the virulent soil born pathogen causing root rot, stem rot and wilt on more than 500 plant species including almost all the agricultural and horticultural crops [25]. *T. viride* shows antagonistic effect on *Sclerotium rolfsii* [26] and other several plant pathogens such as banana stem rot fungus *Marasmiellus* spp [27], onion basal rot causing fungus, *Fusarium oxysporum f. sp. cepae* and onion leaf twisting fungal pathogen *Colletotrichum gleosporioides* [19].

Table 2: Chemical characteristics of locally available substrates

No	Substrate	Chemical characteristics		
		pH	EC(mS/cm)	TSS(g/L)
1	10%Distillery spent wash	4.28	1.305	0.582
2	Cattle urine	8.33	1.432	0.697
3	10%*Gliricidia* leaves extract	5.43	4.970	2.330
4	10%*Thespesia* leaves extract	6.75	2.710	1.210
5	Black gram soaked water	5.85	2.100	1.120
6	Rice mill effluent	4.92	1.850	0.849
7	5%Palmyrah Toddy	3.96	2.136	0.940
8	Coconut water	4.30	9.290	4.500
9	1%Palmyrah fruit pulp extract	4.29	4.340	2.096
10	1%Palmyrah Jaggery solution	6.95	1.178	0.523

Plate 1: Growth and sporulation of *T. viri*de in locally available substrates

Table 3: Spores count of *Trichoderma viride* after 7th and 14th day of in

Locally available substrates	Mean spores count per ml	
	After 7 days ($x10^7$)	After 14 days ($x10^7$)
Black gram soaked water	16.4	35.9
Coconut water	14.8	28.8
Rice mill Effluent	14.4	28.7
1% Palmyrah jaggery solution	12.6	30.4
1% Palmyrah fruit pulp extract	9.2	27.1
Cow urine	0.9	0.2
Thespesia leaves extract	0.6	1.2
Gliricidia leaf extract 10%	0.5	1.6
5% Palmyrah Toddy	0.3	0.6
5% Distillery spent wash	0.2	0.8

Table 4: Antagonistic effect of *T.viride* in locally available substrate against *Sclerotium rolfsii*

Treatment	Mean colony diameter (mm)*	Growth inhibition (%)
T1- Control	90.00^a	0.00
T2- *T. viride* grown on Rice mill effluent	41.70^b	53.66
T3 -*T. viride* grown on Blackgram soaked water	14.65^c	83.72
T4 -*T. viride* grown on 1%Palmyrah Jaggery solution	34.15^b	62.05
T5 -*T. viride* grown on Coconut water	33.50^b	62.77

* Figures having same alphabet in a column indicate the values are not significantly different according the LSD at 0.05 á and 95% confidence interval All the values are the means of four replicates

CONCLUSION

Use of *T.viride* as a potential bio control agent reduces the health, social and environmental issues caused by the synthetic pesticides. Locally available household and industrial liquid waste and other liquid substrates are potential cost effective substrates for the small scale production of *Trichoderma* spp. Among this the black gram soaked water ($35.93x10^7$spores/ml), Palmyrah jaggery solution ($30x10^7$spores/ml), Coconut water ($28.8x10^7$spores /ml) rice mill effluent ($28.7 x10^7$spores/ml) and Palmyrah fruit pulp extracts produced highest proliferation of mycelia and spores. Locally available substrates do not affect the antagonistic ability of T.*viride.* Therefore these locally available liquid substrates can be used as the potential sources of substrates for the multiplication of *T. viride* in small scale level.

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