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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Biochemical factors and enzymes governing resistance in Indian mustard (*Brassica juncea*) against Sclerotinia stem rot (*Sclerotinia sclerotiorum*)

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Four mustard cultivars possessing different degrees of resistance to Sclerotinia stem rot *(Sclerotinia sclerotiorum)* were used to study the biochemical and enzymatic basis of resistance. These include two moderately resistant cultivars (RGN-13 and RRN-505) and two susceptible cultivars (Bio-902 and T-59). Phenol content in all the mustard cultivars tested was found to increase at all three stages of inoculation, that is, 60, 75 and 90 days after sowing in diseased plants as compared to their respective healthy plants. Reduction in the contents of total sugars and reducing sugars were observed in diseased plants of all four cultivars irrespective of their susceptibility towards *S. sclerotiorum*. Peroxidase (PO) activity was observed to be high in moderately resistant cultivars as compared to susceptible ones. Maximum increase in PO activity was measured in diseased tissue of moderately resistant cultivar RRN-505 followed by RGN-13 and minimum PO activity in susceptible Bio-902. Significant increase in polyphenol oxidase (PPO) activity was recorded in moderately resistant cultivars as compared to susceptible cultivars. The phenylalanine ammonia lyase (PAL) activity was maximum in moderately resistant cultivar RGN-13. The PAL activity increased with days of inoculation in healthy as well as in diseased plants. Significant decrease in catalase activity was observed in diseased plants as compared to healthy ones and this decrease was more pronounced in susceptible cultivar T-59. The pathogen related enzymes polygalacturonase trans-eliminase (PGTE), pectin trans-eliminase (PME), polygalacturonase (PG) and cellulolytic (Cx) activities were found to be lower at initial stages of enzymes activity.

Key words: Enzymes, phenol, peroxidase, phenylalanine ammonia lyase and polyphenol oxidase.

INTRODUCTION

Oilseed crops in India account for almost 5% of Gross National Product (GNP) and 10% of value of agricultural products. Rapeseed mustard (*Brassica*) contributes 32% of the total oilseed production in India, and it is the second largest indigenous oilseed crop (Moza, 2006). Indian mustard [*Brassica juncea* (L.) Czern & Coss] is a major oilseed crop grown in *rabi* (post rainy) season in different regions of India. Sclerotinia stem rot or stem

blight or white rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary has become a serious problem in mustard in North India. *S. sclerotium* is a necrotrophic plant pathogen that is able to infect more than 400 host plant species (Boland and Hall, 1994) and causes yield losses in a broad range of agricultural crops, including oilseeds, vegetables and pulses (Bolton et al., 2006). Diseases caused by *S. sclerotiorum* are often sporadic in occurrence and severity, with losses in individual crops ranging from 0-100% (Krishnia et al., 2000; McDonald and Boland, 2004; Ghasolia et al., 2004; Shukla, 2005). The explosive pathogenicity of this fungus under favorable conditions and the ability of its sclerotia to withstand adverse conditions allow it to be a successful pathogen. Peroxidase (EC 1.11.1.7) and phenylalanine ammonia-lyase (EC 4.3.1.5; PAL) are two enzymes frequently associated with infection by phytopathogens (Hammerschmidt et al., 1982; Shirashi et al., 1989; Southerton and Deverall, 1990). The stimulation of the activities of these enzymes has been correlated with resistance to infection in many of the published reports, but conclusive evidence of their role in the defense mechanism(s) of plants is not yet available. The biochemical processes involved in the expression of resistance in *B. napus* are rarely known. However, the accumulation of a phytoalexin in *Brassica* sp. in relation to a hypersensitive reaction towards *L. maculans* has been reported (Rouxel et al., 1989). *S. sclerotiorum* is known to produce pectinolytic and cellulolytic enzymes (Lumdsen, 1969; Favaron et al., 1988; Marciano et al., 1982). The level of these enzyme activities correlates with the development of disease symptoms (Favaron et al., 1988; Lumdsen, 1976). Aside from pectic and cellulolytic enzymes, the diversity of polysaccharidases produced by *S. sclerotiorum* and the mechanisms controlling expression of cell wall-degrading enzymes are poorly understood. Pectic enzymes are one of a complex of factors involved in pathogenesis, in certain diseases, they appear to be principal factor, in another, they apparently are of relatively little or of no importance. However, information on the biochemical and enzymatic basis of resistance of mustard to Sclerotinia stem rot is limited. The objective of this study was to examine in detail the changes in biochemical factors and enzymes in different cultivars of mustard governing resistance or susceptibility against *S. sclerotiorum*.

MATERIALS AND METHODS

Biochemical changes in infected host plants

To study the biochemical basis of Sclerotinia stem rot in healthy and infected plants of Indian mustard, host related enzymes, that is, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and catalase, pathogen related enzymes, polygalacturonasetranseliminase (PGTE), pectin transeliminase (PTE), polygalacturonase (PG) and biochemical constituents, soluble phenolic contents and total sugars were estimated in two moderately resistant (RGN-13 and RRN-505) and two susceptible cultivars (T-59 and Bio-902) of healthy and diseased plants (stem samples), during *rabi* 2008-09. Mustard plants were raised in research farm at College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner following recommended agronomic practices. The plants were artificially inoculated at 60, 75 and 90 days after sowing.

Isolation, purification and artificially inoculation of *Sclerotinia sclerotiorum*

Small pieces of diseased tissues together with adjoining healthy area and sclerotia found in diseased stem were surface sterilized by dipping in mercuric chloride solution (1:1000) for two minutes followed by three washings with sterile water and blot dried then plated aseptically on Potato Dextrose Agar (PDA) in Petri dishes. These were incubated in BOD incubator for growth of the fungus at 27 ± 2°C. Sub cultures from pure peripheral growth were made on PDA slants and Petri dishes. Plant materials were inoculated following the method of Reglinski et al. (1997) with some modifications. Young, healthy plants aged 60, 75 and 90 days, were selected from mustard field raised in research farm. Using a sharp needle, small injuries were made in the middle of half the stem of each cultivar. Mycelial bit (4 mm in diameter) taken from the growing edge of 3-day-old cultures on potato dextrose agar were used for inoculation. Inoculum bits were placed with the mycelium side down in the middle of each stem injury. Control stem were treated with uninfected PDA bits. The stems were then immediately sealed with two layers of parafilm to maintain high humidity. All the biochemical constituents were estimated in fresh samples of healthy and diseased plants of Indian mustard after 7 days of inoculation with the pathogen. The stem samples were collected and stored in deep freeze at -30°C till further use for biochemical analysis of phenol, total sugars and enzyme activity of the sample.

Estimation of soluble sugar content

The soluble sugar content of the leaf samples was determined by using the method of Hedge and Hofreiter (1962). The soluble sugar content of the leaf samples was determined by using the method of Hedge and Hofreiter (1962). A 100 mg of the sample was hydrolyzed by keeping it in a boiling water bath for 3 h with 5 ml of 2.5 N HCl and cooled at room temperature. After that the sample was neutralized with solid sodium carbonate until the effervescences ceased and centrifuged. The supernatant was collected and 0.5 and 1 ml aliquots was took for analysis. The standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard in which '0' served as blank. The volume was made to 1 ml in all the tubes including the sample tubes by adding distilled water followed by addition of 4 ml anthrone reagent. The green to dark green colour was read at 630 nm. Standard graph was drawn by plotted concentration of the standard on the x-axis versus absorbance on the Y-axis and calculations were made to observe the carbohydrate in samples.

Reducing sugar content was measured by following "Nelson's modification of "Somogyi's method" (Somogyi, 1952) using arseno-

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molybdate colour forming reagent and two copper reagent "A" (sodium carbonate anhydrous 2.5 g, potassium sodium tartrate 2.5 g, sodium bi carbonate 2.0 g, sodium sulphate 20.0 g and distilled water 80.0 ml) and "B" (copper sulphate, 15 g concentrated sulphuric acid, 1 drop and distilled water, 80 ml). 1 ml of leaf sample (100 times diluted) was added with a mixture of 1 ml copper reagent prepared from 24 part of copper "B" solutions. This mixture in test tubes was heated in boiling water bath, cooled, added with the colour-forming reagent (arseno-molybdate) and absorbance was measured at 620 nm on Spectronic-20. The value was plotted against a standard curve prepared from glucose.

Estimation of soluble phenol content

The soluble phenol content was estimated by the method described by Thimmaiah (1999). One gram of fresh mustard leaf sample was macerated in pestle and mortar with 10 ml ethanol 80%. The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was pooled and evaporated to dryness in water bath. The residue was dissolved in 5 ml distilled water. An aliquot of 0.2 ml was transferred in test tube and the volume was made to 3 ml with distilled water. Folin ciocalteau reagent (0.5 ml) was added to each test tube. After three minutes, 2 ml of 20% Na_2CO_3 was added in each tube and mixed thoroughly. The tubes were then kept in boiling water for one minute and the absorbance was measured at 650 nm against reagent blank in spectrophotometer. The standard curve was prepared using different concentrations of catechol. The phenol content was expressed as mg phenols/g fresh tissue.

Estimation of soluble protein content

The soluble protein content of the leaf samples was determined by using the method of Lowry et al. (1951). One gram of fresh leaf was macerated in mortar with 5 ml 0.1 M sodium phosphate buffer (pH 7.0). The homogenate obtained was centrifuged at 16,000 g for 20 min. The supernatant was used for estimation of soluble protein content. For this purpose, 0.2% sodium carbonate (anhydrous) in 0.1 N NaOH (Solution A) was prepared. Similarly, 0.5% copper sulphate ($CuSO_4$ $5H_2O$) in 1% sodium potassium tartarate (freshly made) was prepared and was regarded as solution B. From these two reagents, solution C (alkaline copper solution) was prepared by mixing 50 ml of solution A with 1 ml of solution B just before use. An aliquot of 0.1 ml of supernatant was taken in the test tube and the volume was made to 1 ml with distilled water followed by addition of 5 ml solution C, mixed well and incubated at room temperature for ten minutes. 0.5 ml of Folin ciocalteau reagent was diluted, mixed well and incubated at room temperature in dark for 30 min. The absorbance was recorded at 660 nm against blank. The amount of protein in sample was determined from the standard curve prepared by using different concentrations of bovine serum albumin.

Host related enzymes

Estimation of peroxidase (EC 1.11.1.17) activity: Peroxidase activity was assayed by the method described by Thimmaiah (1999). One gram leaf sample was macerated in previously chilled mortar in 10 ml ice cold 0.1 M phosphate buffer at pH 6.0. The homogenate was strained through two fold of muslin cloth and centrifuged at 16,000 g for 20 min at 4°C. The supernatant was used as enzyme source. In order to assay the enzyme activity, 1 ml 0- dianisidine, 0.5 ml H_2O_2, 1 ml of phosphate buffer and 2.4 ml of distilled water were pipetted in test tubes. The blank was prepared by excluding H_2O_2 and adding additional volume of water in the place of H_2O_2. The reaction was initiated by adding 0.2 ml of enzyme extract (supernatant) and incubating at 30°C for five minutes. The reaction was stopped by adding 1 ml of 2 N H_2SO_4. The absorbance was measured at 430 nm against reagent blank. The unit of enzyme was defined as absorbance/min/mg protein.

Estimation of polyphenol oxidase (EC1.14.18.1) activity: The polyphenol oxidase activity was determined by the method of Mayer et al. (1965). One gram leaf sample was homogenized in 2 ml 0.1 M sodium phosphate buffer at pH 6.5 and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was used as enzyme source. The reaction mixture consisted of 0.2 ml enzyme extract (supernatant) and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 0.2 ml of 0.1 M catechol was added and the change in absorbance was recorded at 30 s intervals for up to 3 min at 495 nm. The activity of enzyme was expressed as absorbance/min/mg protein.

Estimation of phenylalanine ammonia lyase (EC 4.3.1.5) activity: Phenylalanine ammonia lyase activity was assayed by the method described by Thimmaiah (1999). Three gram leaf sample was macerated in a mortar in presence of 2.6 ml of 0.2 M sodium borate buffer (pH 8.7) containing 2-mercaptoethanol (0.8 ml/L). The homogenate was filtered through cheese cloth. Acetic acid (0.1M) was added drop by drop to bring the pH of filtrate to 5.5. Protamine sulphate solution (0.002 g + 0.008 ml of 1 M sodium acetale buffer pH 5.5 diluted 0.1 ml) was added to filtrate and stirred for 10 min followed by centrifuging at 7,000 g for 10 min. The supernatant was used as enzyme source. In order to determine the enzyme activity, an assay mixture consisting of 1 ml of 0.05 M Tris-HCl buffer (pH 8.8), 0.5 ml of 0.01 M L-phenylalanine and 0.4 ml of distilled water was incubated at 30°C for 5 min. The reaction was initiated by adding 0.1 ml enzyme extract (supernatant) and again incubated at 30°C for 60 min. The blank without L-phenylalanine was run. The reaction was stopped by adding 0.5 ml of 1 N HCl. The residue was dissolved in 3 ml of 0.05 N NaOH. The absorbance was recorded at 290 nm. The standard curve was prepared using different concentrations of cinnamic acid. The unit of enzyme was expressed as µmoles cinnamic acid produced/min/mg protein.

Estimation of catalase (EC 1.11.1.6) activity: Catalase activity was measured by adopting the procedure of Sinha (1972). The reaction mixture containing 0.4 ml of 0.2 M H_2O_2, 0.1 ml of enzyme extract and 0.5 ml of 0.01 M phosphate buffer (pH 7.0) was incubated at 37°C for 1 min along with continuous shaking and then 3 ml of dichromate reagent (5% potassium dichromate; glacial acetic acid, 1:3) was added. The mixture was heated for 10 min in a boiling water bath. After cooling, the intensity of colour was measured at 570 nm. The enzyme activity was expressed as µ moles of H_2O_2 utilized min^{-1} mg^{-1} protein.

RESULTS AND DISCUSSION

In the present studies, an attempt was made to analyze the activity of host related enzymes, pathogen related enzymes, soluble phenolic compounds and total sugars in healthy and diseased plants of moderately resistant and susceptible cultivars of Indian mustard inoculated with *S. clerotiorum* at three stages: 60, 75 and 90 days after sowing.

Changes in biochemical constituents in healthy and infected host plants

Effect on phenolic contents

There was a significant increase in phenolic content of

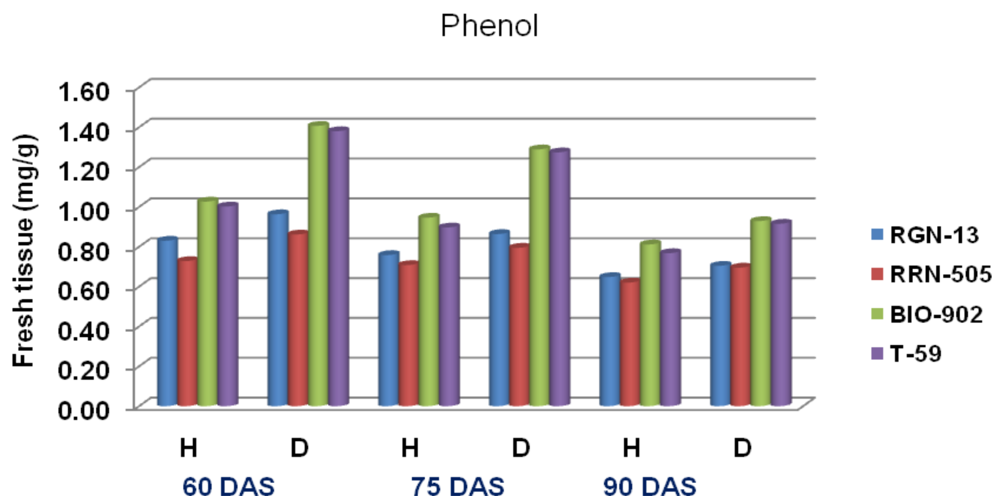

Figure 1. Comparisons of soluble phenol content in four Indian mustards RGN-13, RRN-505, BIO-902 and T-59 at different days after infection with *Sclerotinia sclerotiorum*. DAS- Inoculated days after sowing

mustard stem due to *S. sclerotiorum* infection as compared to healthy plants (Table 1 and Figure 1). Phenol content in all the mustard cultivars tested *viz*., RGN-13, RRN-505, Bio-902 and T-59 was found to be increased at all three stages of inoculation: 60, 75 and 90 days after sowing in diseased plants as compared to their respective healthy plants (Table 1). Data revealed that the total phenolic contents were highest in diseased plants of moderate cultivar, RRN-505 increase of total phenolic compounds was observed in moderately resistant cultivar RGN-13 and RRN-505 as compared to susceptible cultivars Bio-902 and T-59 at 60,75 and 90 days after sowing. Gupta et al. (1990) also found similar trend for phenolic compounds in mustard and reported that resistant cultivar RC-781 possessed higher amount of phenols than susceptible genotypes. The higher level of total phenols in diseased leaves with *A. brassicae* as compared to healthy leaves in the four cultivars of Indian mustard was also reported by Gupta and Kaushik (2002). In contrast, decrease in total phenol of the leaves of *Mentha arvensis* with increase in initial inoculum of *S. sclerotiorum* was also reported by Perveen et al. (2010). Beniwal et al. (2008) also reported higher quantity of phenolic contents in resistant cultivars of wheat in response to flag smut than susceptible and highly susceptible cultivars. The level of total phenol contents indicated the involvement of phenolics in resistant mechanism through the phenomenon of oxidation to quinones which are more toxic to the pathogens (Arora and Bajaj, 1981; Bajaj et al., 1983).

Sugars

Significant decrease in total sugars was observed in *S. sclerotiorum* infected plant tissues of both moderately resistant cultivars as well as susceptible cultivars at all three stages (Table 2 and Figure 2). The total sugars also decreased with the days of sowing, it was higher when mustard cultivars were inoculated at 60 days of sowing as compared to 90 days of sowing in all four cultivars (Table 2). Similar trend was also found in reducing sugars (Table 3 and Figure 3). In the present studies, total, reducing and non-reducing sugars were observed to be low in Sclerotinia infected stem tissues of moderately resistant and susceptible cultivars of mustard. However, in similar study, Jobic et al. (2007) observed a decline in sugars and amino acids in the plant and fungus total content during infection. Sucrose and fructose, initially present almost exclusively in plant, were reduced by 85%. Kiran et al. (2003) reported increased amount of reducing and total sugars in calli of *Brassica* species subcultured on medium having different concentrations of culture filtrate of *Alternaria brassicae.* Guleria and Kumar (2006) studied biochemical basis of host-pathogen interaction between white stem rot and rapeseed-mustard in field experiment and found that total sugars content in the plant tissue of *S. sclerotiorum* resistant *Brassica* cultivars *viz*., JTC-1 and PCC-5 was found to be lower than susceptible cultivars *viz*., Neelam and Sheetal. Kumar et al. (1998) also reported reduction in total sugars in sunflower seeds due to infection of *S. sclerotiorum*.

Host related enzymes

Effect on peroxidase (PO) and polyphenol oxidase (PPO)

The findings of the present study revealed that the

Table 1. Comparisons of phenol content in four Indian mustard cultivars at different days after infection with *Sclerotinia sclerotiorum*.

Variety	Phenol content (mg/g fresh tissue)								
	60 DAS*			75 DAS			90 DAS		
	H	D	Mean	H	D	Mean	H	D	Mean
RGN-13 (MR)	1.027	1.407 (36.97)**	1.217	0.946	1.289(36.26)	1.118	0.812	0.929(14.46)	0.870
RRN-505 (MR)	1.002	1.380(37.76)	1.191	0.897	1.274(42.03)	1.086	0.768	0.916(19.23)	0.842
Bio-902 (S)	0.830	0.963(16.02)	0.897	0.758	0.864(13.93)	0.811	0.648	0.704(8.69)	0.676
T-59 (S)	0.728	0.862(18.31)	0.795	0.708	0.795(12.24)	0.752	0.620	0.695(12.09)	0.658
Mean	0.897	1.153		0.827	1.056		0.712	0.811	
	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV
Variety (V)	0.010	0.031	2.44	0.015	0.046	3.98	0.008	0.026	2.71
H/D	0.007	0.022		0.011	0.033		0.006	0.018	
V x H/D	0.014	0.044		0.022	0.066		0.012	0.036	

MR- Moderately resistant, S- susceptible, H– healthy, D- disease. *DAS- Inoculated days after sowing. **Values in parentheses indicate percent deviation in diseased leaves over healthy leaves of corresponding variety.

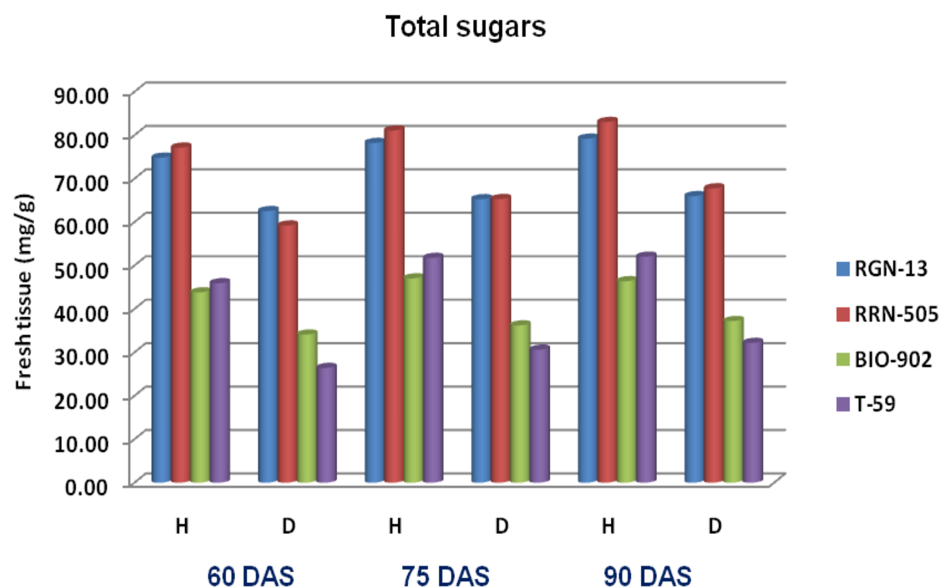

Figure 2. Comparisons of total sugars in four Indian mustards RGN-13, RRN-505, BIO-902, and T-59 at different days after infection with *Sclerotinia sclerotiorum*. DAS- Inoculated days after sowing

Table 2. Comparisons of total sugars in four Indian mustard cultivars at different days after infection with *Sclerotinia sclerotiorum.*

Variety	Total sugar content (mg/g fresh tissue)								
	60 DAS*			75 DAS			90 DAS		
	H	D	Mean	H	D	Mean	H	D	Mean
RGN-13 (MR)	74.83	62.53(-16.43)**	68.68	78.23	65.27(-16.47)	71.75	79.21	65.97(-16.72)	72.59
RRN-505 (MR)	77.16	59.21(-23.26)	68.19	81.10	65.32(-19.45)	73.21	83.05	67.77(-18.40)	75.41
Bio-902 (S)	43.83	34.11(-22.17)	38.97	47.04	36.22(-23.01)	41.63	46.39	37.21(-19.78)	41.80
T-59 (S)	45.93	26.42(-42.47)	36.18	51.76	30.62(-40.84)	41.19	52.03	32.19(-38.13)	42.11
Mean	60.44	45.57		64.53	49.36		65.17	50.79	
	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV
Variety (V)	0.32	0.98	1.49	0.40	1.22	1.73	0.37	1.11	1.55
H/D	0.23	0.69		0.28	0.86		0.26	0.78	
V x H/D	0.45	1.38		0.57	1.72		0.52	1.57	

MR-Moderately resistant, S- susceptible, H – healthy, D- disease. *DAS- Inoculated days after sowing. **Values in parentheses indicate per cent deviation in diseased leaves over healthy leaves of corresponding variety.

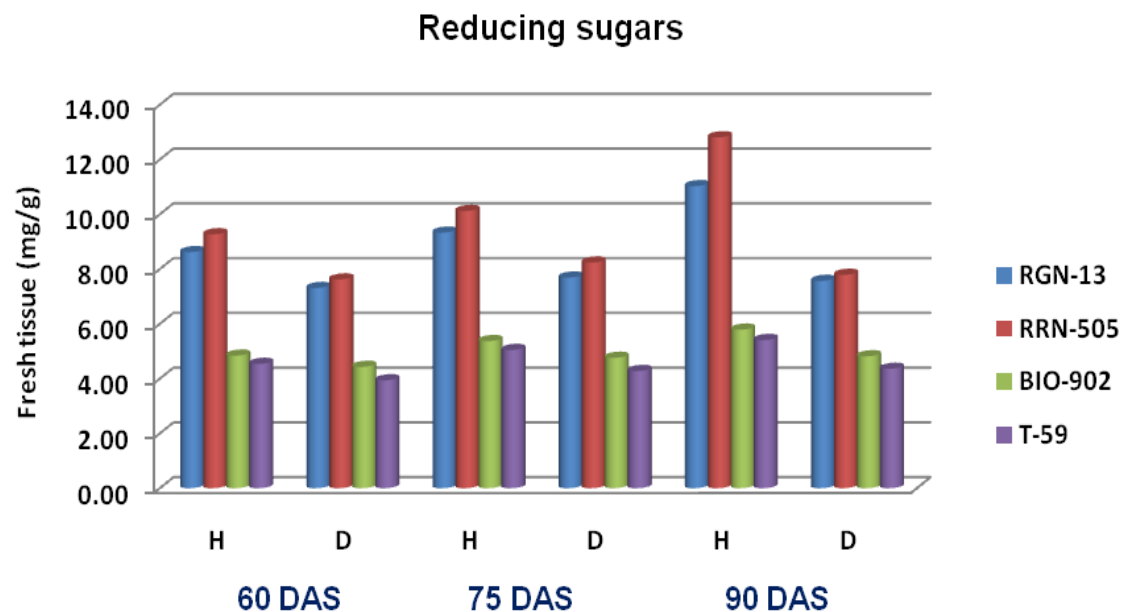

Figure 3. Comparisons of reducing sugars in four Indian mustards RGN-13, RRN-505, BIO-902 and T-59 at different days after infection with *Sclerotinia sclerotiorum.* DAS- Inoculated days after sowing

Table 3. Comparisons of reducing sugars in four Indian mustard cultivars at different days after infection with *Sclerotinia sclerotiorum.*

Variety	Reducing sugar content (mg/g fresh tissue)								
	60 DAS*			75 DAS			90 DAS		
	H	D	Mean	H	D	Mean	H	D	Mean
RGN-13 (MR)	8.62	7.31(-15.20)**	7.97	9.33	7.69(-17.58)	8.51	11.03	7.57(-31.37)	9.30
RRN-505 (MR)	9.27	7.62(-17.80)	8.45	10.12	8.24(-18.57)	9.18	12.81	7.79(-39.19)	10.30
Bio-902 (S)	4.84	4.43(-8.47)	4.64	5.37	4.76(-11.36)	5.07	5.79	4.82(-16.81)	5.30
T-59 (S)	4.54	3.94(-13.22)	4.24	5.05	4.28(-15.19)	4.66	5.41	4.36(-19.41)	4.89
Mean	6.82	5.83		7.47	6.24		8.76	6.13	
	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV
Variety (V)	0.07	0.22	2.83	0.08	0.24	2.83	0.10	0.29	3.15
H/D	0.05	0.16		0.06	0.17		0.07	0.21	
V x H/D	0.10	0.31		0.11	0.34		0.14	0.41	

MR-Moderately resistant, S- susceptible, H– healthy, D- disease. *DAS- Inoculated days after sowing. **Values in parentheses indicate percent deviation in diseased leaves over healthy leaves of corresponding variety.

Table 4. Comparisons of peroxidase activity in four Indian mustard cultivars at different days after infection with *Sclerotinia sclerotiorum.*

Variety	Peroxidase activity (mg/g fresh tissue)								
	60 DAS*			75 DAS			90 DAS		
	H	D	Mean	H	D	Mean	H	D	Mean
RGN-13 (MR)	196.47	221.58(12.78)**	209.03	187.42	214.25(14.22)	200.84	137.91	152.09(10.28)	145.00
RRN-505 (MR)	204.73	247.55(20.96)	226.14	196.82	236.61(20.22)	216.72	143.77	162.29(12.88)	153.03
Bio-902 (S)	134.14	173.51(29.35)	153.83	127.23	162.33(27.59)	144.78	105.01	132.83(26.49)	118.92
T-59 (S)	125.18	166.31(32.80)	145.75	118.64	157.39(32.66)	138.02	106.86	129.56(21.24)	118.21
Mean	165.13	202.24		157.53	192.65		122.19	145.86	
	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV
Variety (V)	2.33	7.05	3.10	2.26	6.87	3.17	1.32	3.99	2.41
Enzyme activity (EA)	1.64	4.99		1.60	4.86		0.93	2.82	
V x EA	3.29	9.98		3.20	9.71		1.86	5.65	

MR-Moderately resistant, S-susceptible, H – healthy, D- disease. *DAS - Inoculated days after sowing. **Values in parentheses indicate percent deviation in diseased leaves over healthy leaves of corresponding variety.

peroxidase (PO) and polyphenol oxidase (PPO) activities were higher in diseased plants as compared to healthy plants of mustard (Tables 4 and 5). The enzyme activity was also higher in moderately resistant genotypes in comparison with susceptible mustard genotypes. The enzyme PO and PPO activity were decreased with age of plant in all four cultivars of mustard. A sharp increase in PPO and PO activities following infection was

Table 5. Comparisons of polyphenol oxidase activity in four Indian mustard cultivars at different days after infection with *Sclerotinia sclerotiorum*.

Variety	Polyphenol oxidase activity (Absorbance/mg protein)								
	60 DAS*			75 DAS			90 DAS		
	H	D	Mean	H	D	Mean	H	D	Mean
RGN-13 (MR)	1.132	1.429(26.24)**	1.281	1.215	1.685 (38.68)	1.450	1.117	1.438(28.74)	1.278
RRN-505 (MR)	1.076	1.417(37.69)	1.247	1.152	1.598(38.72)	1.375	1.014	1.341(32.25)	1.178
Bio-902 (S)	0.432	0.538(24.54)	0.485	0.452	0.558(23.45)	0.505	0.389	0.487(25.28)	0.438
T-59 (S)	0.392	0.486(23.17)	0.439	0.434	0.546(25.90)	0.490	0.383	0.465(21.32)	0.424
Mean	0.758	0.968		0.813	1.097		0.726	0.933	
	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV
Variety (V)	0.012	0.038	3.51	0.016	0.047	4.01	0.009	0.028	2.74
Enzyme activity (EA)	0.009	0.027		0.011	0.034		0.007	0.020	
V x EA	0.017	0.053		0.022	0.067		0.013	0.040	

MR- Moderately resistant, S- susceptible, H- healthy, D- disease. *DAS- Inoculated days after sowing. **Values in parentheses indicate per cent deviation in diseased leaves over healthy leaves of corresponding variety.

observed in all the cultivars which appeared to be more associated with susceptibility (Table 4-5 and Figure 4-5). Similar increases in both enzymes in many other host-parasite combina-tions have been documented (Gangopadhyay and Lal, 1986; Gowda et al., 1989; Gupta et al., 1990). Watpade and Mehta (2012) reported activity of oxidative enzymes *viz.*, polyphenol oxidase and peroxidase increase in tolerant calli with the increase in concentration of culture filtrate of *S. sclerotiorum*, whereas catalase activity increased in susceptible calli in response to increase in concentration of culture filtrate. In contrast, the lower PPO activity was observed in susceptible genotypes at both stages (pre and post infection) of determination (Mahatma et al., 2008).

Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) activity was increased in diseased plants when compared with healthy plants tissue in all the mustard cultivars used, at all three stages of inoculation (Table 6). The PAL activity was significantly higher in mode-rately resistant cultivars as compared to susceptible cultivars. The enzyme activity increased uniformly with age of plant (Table 6 and Figure 6). Chakraborty et al. (1993) determined activities of peroxidase and phenylalanine ammonia lyase in leaves of healthy and inoculated with *Leptosphaeria maculans* in *Brassica napus* cultivars and observed increased activity of both enzymes in moderately resistant cultivars. These findings corroborate with observa-tions made by Joshi et al. (2004) and Muthukumar (2007) in Alternaria leaf spot of cluster-bean and tuberose plants, respectively. Chakraborty et al. (2004) reported the association of defense enzymes peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in resistance mechanism in tea leaves against *Exobasidium vexans*.

Catalase

The healthy plants of moderately resistant cultivar RGN-13 and RRN-505 had higher catalase activity in comparison with susceptible Bio-902 and T-59. When the diseased plants were analyzed for catalase activity, it was observed that there was significant decrease in catalase activity in diseased plants as compared to healthy plants in all four cultivars and this decrease was more pronounced in susceptible cultivar (Table 7 and Figure 7). Contrary to PPO and PO, the activity of catalase remained lower in diseased plants as compared to healthy ones. The present findings are in agreement with the earlier reports made by the Saharan et al. (2001) that the healthy leaves of resistant cultivars of cluster bean to *Alternaria* blight had higher catalase activity than susceptible cultivars both at 65 and 80 days after sowing. Gupta et al. (1995) reported that catalase activity was maximum at 40 DAS in healthy leaves, which declined after this stage in all the mustard species. The presence of this enzyme in substantial amount at initial stages of plant growth indicates its possible role in tissue. Changes in peroxidase and catalase activity in

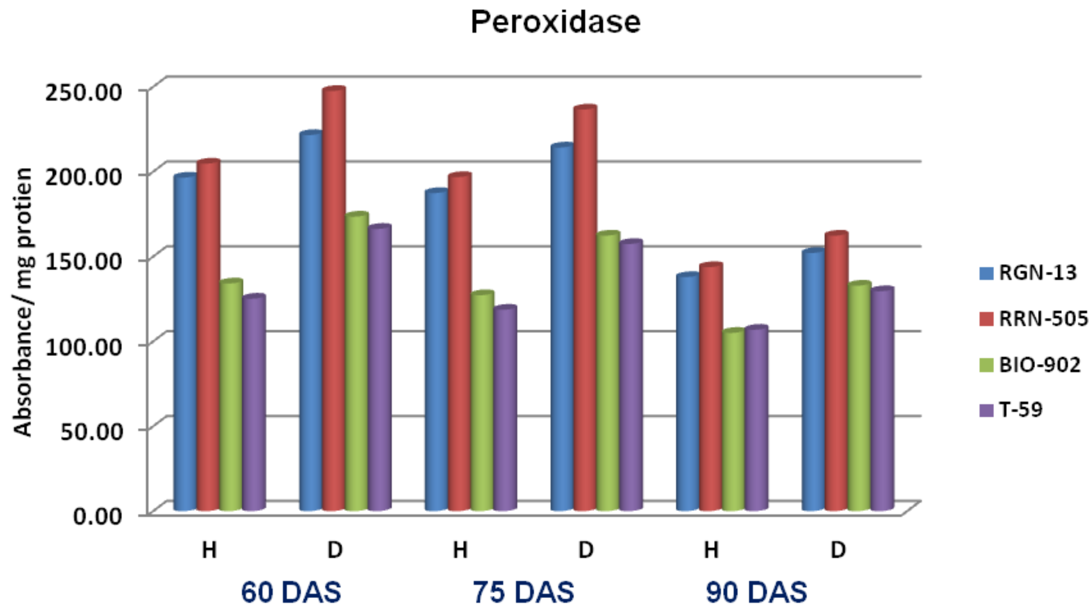

Figure 4. Comparisons of peroxidase in four Indian mustards RGN-13, RRN-505, BIO-902, and T-59 at different days after infection with *Sclerotinia sclerotiorum.* DAS- Inoculated days after sowing

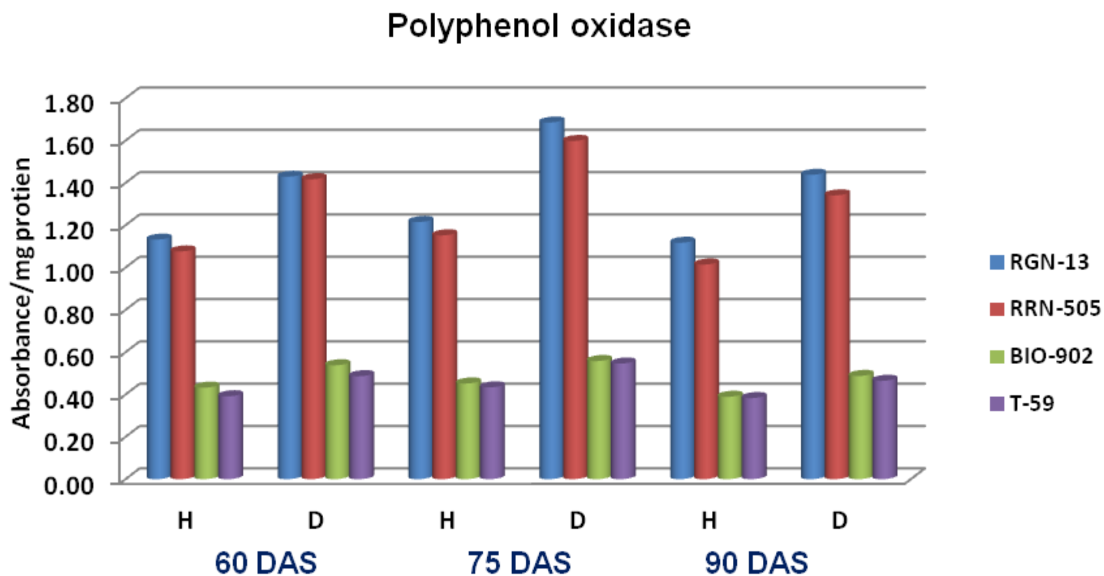

Figure 5. Comparisons of polyphenol oxidase in four Indian mustards RGN-13, RRN-505, BIO-902, and T-59 at different days after infection with *Sclerotinia sclerotiorum.* DAS- Inoculated days after sowing

healthy and diseased fruits of mango due to black tip were also reported by Agrawala et al. (1960).

Conflict of Interests

The authors have not declared any conflict of interests.

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Table 6. Comparisons of phenylalanine ammonia lyase in four Indian mustard cultivars at different days after infection with *Sclerotinia sclerotiorum*.

Variety	Phenylalanine ammonia lyase activity (μ mole trans-cinnamic acid/min/mg protein)								
	60 DAS*			75 DAS			90 DAS		
	H	D	Mean	H	D	Mean	H	D	Mean
RGN-13 (MR)	232.69	255.57(9.83)**	244.13	318.70	363.67(14.12)	341.19	326.62	368.67(12.86)	347.65
RRN-505 (MR)	212.37	245.53(15.61)	228.95	298.38	336.61(12.81)	317.50	305.38	342.61(12.19)	324.00
Bio-902 (S)	102.73	114.54(11.50)	108.64	116.17	127.59(9.92)	121.88	117.84	129.59(9.97)	123.71
T-59 (S)	95.33	110.97(16.41)	103.15	113.67	128.62(13.15)	117.81	118.67	131.28(10.63)	124.98
Mean	160.78	181.65		211.72	237.46		217.14	243.04	
	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV
Variety (V)	2.03	6.15	2.90	2.83	8.58	3.07	1.32	4.00	1.41
Enzyme activity (EA)	1.43	4.35		2.00	6.07		0.93	2.83	
V x EA	2.87	8.69		4.00	12.13		1.87	5.66	

MR- Moderately resistant, S- susceptible, H- healthy, D- disease. *DAS- Inoculated days after sowing. **Values in parentheses indicate per cent deviation in diseased leaves over healthy leaves of corresponding variety.

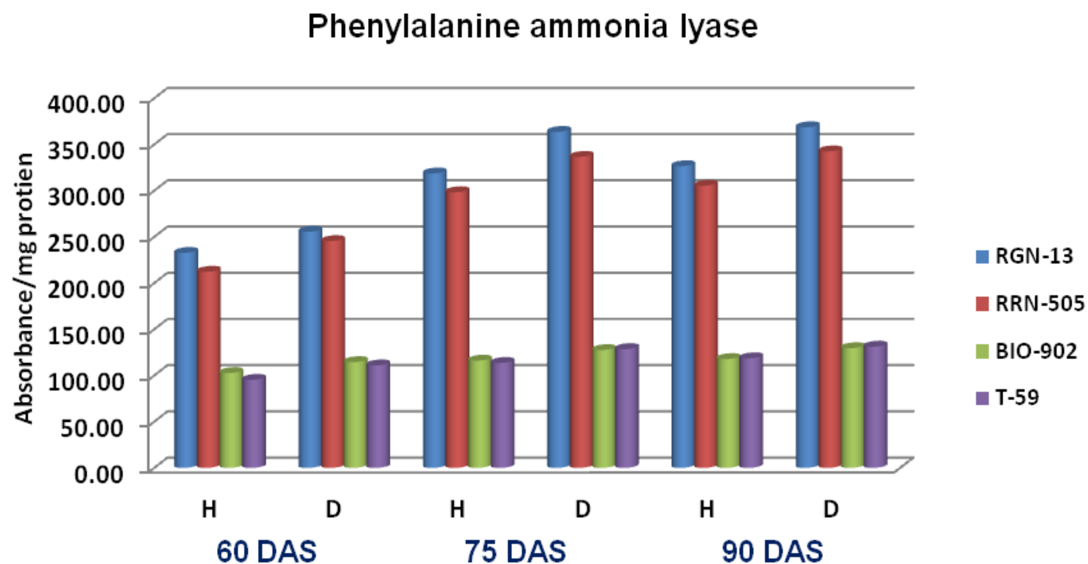

Figure 6. Comparisons of phenylalanine ammonia lyase in four Indian mustards RGN-13, RRN-505, BIO-902 and T-59 at different days after infection with *Sclerotinia sclerotiorum*. DAS- Inoculated days after sowing

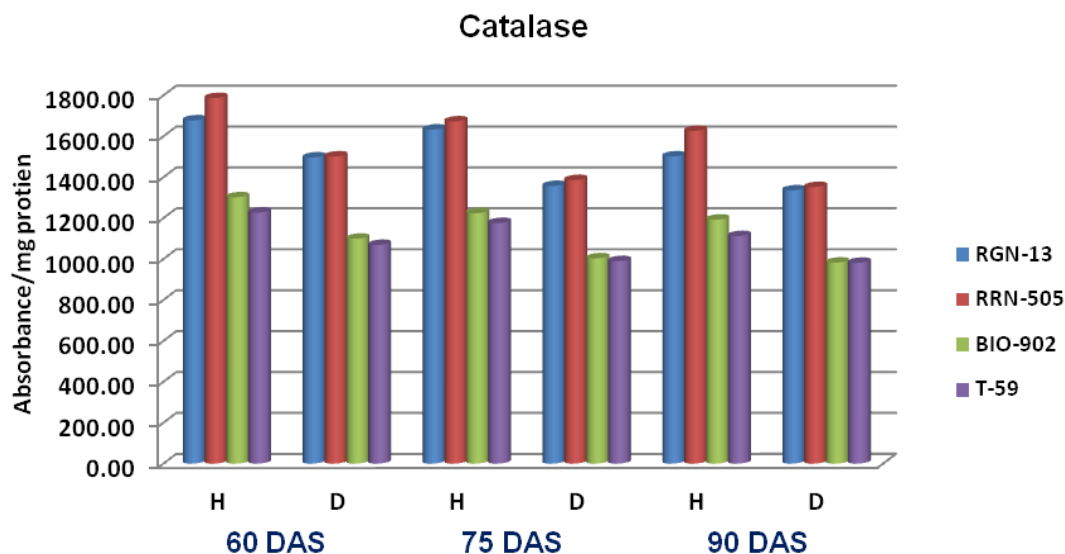

Figure 7. Comparisons of catalase in four Indian mustards RGN-13, RRN-505, BIO-902, and T-59 at different days after infection with *Sclerotinia sclerotiorum.* DAS- Inoculated days after sowing

Table 7. Comparisons of catalase activity in four Indian mustard cultivars at different days after infection with *Sclerotinia sclerotiorum.*

Variety	Catalase activity (Absorbance/mg protein)								
	60 DAS*			75 DAS			90 DAS		
	H	D	Mean	H	D	Mean	H	D	Mean
RGN-13 (MR)	1677.89	1496.59 (-10.81)**	1587.24	1633.04	1357.23(-16.89)	1495.13	1501.59	1335.83(-11.04)	1418.71
RRN-505 (MR)	1787.02	1501.56(-15.97)	1644.29	1672.96	1386.47(-17.12)	1529.72	1626.66	1352.22(-16.87)	1489.44
Bio-902 (S)	1302.05	1099.67(-15.54)	1200.86	1224.25	1003.49(-18.03)	1113.87	1192.67	982.57(-17.62)	1087.62
T-59 (S)	1227.09	1069.09(-12.88)	1148.09	1176.27	990.08(-15.83)	1083.18	1112.33	981.71(-11.74)	1047.02
Mean	1498.51	1291.73		1426.63	1184.32		1358.31	1163.08	
	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV	SEm±	CD (P=0.05)	CV
Variety (V)	27.91	84.64	4.90	18.53	56.21	3.48	23.56	71.47	4.58
Enzyme activity (EA)	19.73	59.85		13.10	39.75		16.66	50.53	
V x EA	39.46	119.70		26.21	79.50		33.32	101.07	

MR- Moderately resistant, S- susceptible, H- healthy, D- disease.
*DAS- Inoculated days after sowing.
**Values in parentheses indicate percent deviation in diseased leaves over healthy leaves of corresponding variety.

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Full Length Research Paper

Cloning and expression of *Bacillus thuringiensis cry1Ia* in *Escherichia coli* and its insecticidal activity

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The crystal (Cry) proteins produced by *Bacillus thuringiensis* determine a particular strain's toxicity profile. This study was focused on cloning a *cry1Ia* gene based on amplicon restriction fragment length polymorphism (ARFLP) profile which would be helpful in developing new biopesticides with broader and higher spectrum of toxicity against Lepidoptera and Coleoptera insect pests. The present paper describes *cry1Ia* gene from a local isolate of *Bacillus thuringiensis* (*B.t*) CFE20(3). A PCR-restriction fragment length polymorphism method for identification of *cry1I*-type genes from *Bacillus thuringiensis* was established by designing a pair of primers based on the conserved regions of the genes to amplify 2,169 bp *cry1I*-type gene fragments. Amplification products were digested with the *Kpn*I and *Xba*I, and new kind of *cry1I*-type genes was successfully identified. *Escherichia coli* DH5α was transformed with recombinant DNA comprising pTZ57R/T and *Bt cry1I* (2169 bp) amplified from a native isolate CFE20(3) for cloning. The cloned 2169 bp was sequenced and then ligated in the expression vector pQE30 for transformation of *E. coli* M15 and SG13009 for expression analysis. The sequence obtained shows 99% homology with known *cry1Ia* from *B. thuringiensis subsp. Kurstaki.* An expected band size of 81 KDa was observed after sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicating the expression of *cry*1I. The toxicity of crude recombinant Cry1I proteins was determined against third instar larvae of Diamond back moth, *Plutella xylostella* and *Spodoptera litura.* Cry1I protein was found to be effective against *Plutella xylostella.*

Key words: *Bacillus thuringiensis*, *cry*1I, SDS-PAGE, *Plutella xylostella*, *Spodoptera litura*.

INTRODUCTION

Crystal proteins from the Gram-positive spore-forming bacterium, *Bacillus thuringiensis* are toxic to a wide variety of insects that are economically important as pests. Many different genes encoding the *B. thuringiensis* endotoxin have been isolated and characterized. The genes have been classified as *cry1* to *cry72*, *cyt1*, *cyt2* and *cyt3* and are ranked according to their homology. The 72 groups of *cry* genes are divided into class and subclasses according to their amino acid similarities (Crickmore et al., 1998; Song et al., 2003; Pooja et al., 2013; *B. thuringiensis* toxin nomenclature website at http://www.biols.susx.ac.uk/home/Neil_Crickmore /Bt/). Cry1 proteins that are active against lepidopteran insects are produced as crystalline parasporal inclusions during

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sporulation. Generally, the crystals are composed of pro-toxins of approximately 130 kDa, but *cry1I*-type genes are usually silent genes capable of encoding a protein of about 81 kDa in *B. thuringiensis* strains (Choi et al., 2000; Gleave et al., 1993). We decided to screen *B. thuringiensis* isolates for *cry1I* genes with the aim of finding novel *cry1I* genes, which could encode insecticidal proteins toxic to insensitive or resistant insect pests (Song et al., 2003).

The sporulating cells of *Bt* release crystal proteins into the surrounding medium and after exposure to alkaline environment the crystal protein is activated. The C terminal end of the protein recognizes the receptor site in the gut membrane and then its N terminal end undergoes conformational changes and loop like structure is formed which ultimately insert in the membrane and make pores. The lysis of the cells due to the formation of non specific pores causes the paralysis of the gut and the larvae stop feeding. This brings about larval death (Schwartz et al., 1993; Lorence et al., 1995; Pietrantonio and Gill, 1996).

The Cry proteins are classified based on similarities among amino acid sequences, and are grouped into 72 classes. There are more than 500 different *cry* genes that encode these proteins that may be done using the polymerase chain reaction technique. This technique has been applied in several studies, including to find new isolates with entomopathogenic potential. The type of *cry* gene present in an isolate correlates with the specificity of the produced Cry protein (Lamptey et al., 1991).

Some of these proteins have toxicities to more than one insect order; for example, Cry1I is toxic to the Lepidoptera and Coleoptera orders (Tailor et al., 1992). Other *cry1I* genes have been characterized and published (Tounsi et al., 2003; Selvapandiyan et al., 1998; Gleave et al., 1993; Choi et al., 2000; Bergamasco et al., 2011; Pooja et al., 2013). The *cry1I* genes encode proteins of about 70 to 80 kDa that are segregated during the vegetative phase of *B. thuringiensis* and do not accumulate as crystals (Grossi-de-Sa et al., 2007).

With advances in biotechnology, genes that encode proteins with insecticidal characteristics may be isolated from *B. thuringiensis,* cloned into *Escherichia coli* or *B. thuringiensis* mutants for *cry* genes (cry-B) and even modified to express adequately in plants. *Bt* cotton plants have already been adopted by some farmers, which has decreased insecticidal application by 50 to 65% (Bergamasco et al., 2011; Santos et al., 2003). The objec-tives of the present work were to clone the *cry1Ia* gene of a native isolate into an expression vector, express the Cry1Ia protein in *E. coli* and evaluate its insecticidal activity against *Plutella xylostella* and *Spodoptera litura.*

MATERIALS AND METHODS

Bacillus thuringiensis strains, plasmids

Native *Bacillus thuringiensis* isolate CFE20(3) and CFE25(2) which are available in Institute of Agri Biotechnology, University of Agricultural Sciences, Dharwad was used to amplify *cry*1Ia gene. HD1 was used as a reference strain. The T/A cloning vector pTZ57R/T was obtained from InsTA clone PCR cloning kit #K1213, Fermentas and pQE30 from Qiagen (Cat. No. 32915), the host *E. coli* JM109 for maintenance and *E. coli* M15 and SG13009 for expression analysis from Qiagen.

Amplification of *cry*1I gene

Gene specific primer which was synthesized at Sigma Aldrich Pvt. Ltd., Bangalore, was used for amplification of *cry*1I gene. The forward and reverse primers used were 5'GGATCCATGAAACTAAAGAATCAAGATAAGC3' and 3'CTGCAGCATGTTACGCTCAATATGGAGT 5', respectively. PCR was performed with 3U Taq DNA polymerase, 1 mM dNTP, 5 pM primer each, 25 mM $MgCl_2$ in a final volume of 100 µl. Amplification was done in an Eppendorf thermal cycler under the following conditions: 5 min of denaturation at 94°C followed by 35 cycles of amplification with a 1 min denaturation at 94°C, 1 min of annealing at 50.4°C, 2 min of extension at 72°C, final extension step of 45 min at 72°C.

Amplicon restriction fragment length polymorphism (ARFLP)

The amplified product of *cry* genes from the potent isolates was used for checking the variants by restriction analysis on the basis of ARFLP of the PCR amplified DNA as outlined by Kuo and Chak (1996). The purified DNA of the isolates *viz.*, CFE20(3) and CFE25(2) were digested with different restriction endonucleases *viz.*, *Hind*III, *BamHI*, *Pst*I, *Xma*I and *Kpn*I separately for 3 h by incubating the reaction mixture at 37°C. The enzyme was heat inactivated by incubating the preparation at 65°C for 20 min. 20 µl of the digested sample was loaded on 0.7% agarose gel along with uncut total DNA to check the digestion. The *cry1I* gene of the corresponding native *B. thuringiensis* isolates were identified by their ARFLP patterns. Isolate CFE20(3) which shown differences in their amplicon restriction fragment length polymorphism was selected for further cloning and expression in *E. coli.*

Molecular cloning and nucleotide sequencing

PCR amplified products were ligated to the T/A cloning vector pTZ57R/T (Sambrook and Russell, 2001) using the Fermentas DNA ligation kit. The transformed cells were spread on LB agar plates containing X-gal (20 mg/ml), Isopropyl-β-D-thiogalactopyranoside (IPTG) (24 mg/ml) and ampicillin (100 µg/ml). The plates were then incubated at 37°C for 12 to 16 h and the transformed colonies were further streaked on Luria agar with ampicillin (100 µg/ml). The confirmation for the presence of desired DNA fragment in cloning vector was done by PCR using gene specific primers and by restriction analysis, and subsequently, gel electrophoresis carried was out. Nucleotide sequencing was done by using M13 forward and reverse primer at Chromous Biotech Pvt. Ltd., Bangalore. In order to express the *cry*1I gene, the construct containing *cry*1I was inserted into the multiple cloning site of an expression vector pQE30 to generate the recombinant expression construct. The complete amplified gene was gel purified using the Mini Elute PCR purification kit (Qiagen) according to the manufacturer's instruct-tions. The insert sequence and its reading frame were confirmed by *Bam*HI and *Pst*I digestion. The ligated product was first transferred into *E. coli* JM109 cells for maintenance and then into *E. coli* M15 (pREP4) (Qiagen) and *E. coli* SG13009 (pREP) (Qiagen) for expression analysis. For confirmation of the clones, the plasmid was isolated by using alkaline lysis protocol of Birmboim and Doly

(1979) and restriction analysis was done for the plasmids of selected clones by using *Bam*HI and *Pst*I restriction endonucleases.

Protein analysis and expression studies

For protein analysis, about 5 ml of Luria broth with kanamycin (50 mg/ml) and ampicillin (50 mg/ml), the protein was extracted and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis inoculated with a colony of *E. coli* containing the recombinant construct and incubated at 37°C overnight under shaking conditions. Overnight grown culture was diluted in fresh Luria broth in 1:100 ratio without selection pressure and incubated at 37°C until the culture reached the log phase of growth (A550-0.5 to 1.0) under shaking conditions which took approximately 3 h. The expression of target protein was induced based on the optimal values of IPTG (1 mM) concentration and it was again incubated for 5 h at 37°C in a shaker. For extraction of proteins, the cell culture was centrifuged at 13,000 rpm for 1 min at room temperature. The pellet was resuspended in 100 µl of $T_{10}E_1$ and 100 µl of 1X SDS gel loading buffer was added to it. The mixture was heated at 90°C on a thermo mixer and centrifuged for 10 min at 4°C. The supernatant was collected in micro centrifuge tubes and protein was quantified by using NanoDrop. The protein preparations were analyzed by SDS-PAGE as described by Sambrook and Russell (2001).

Mass multiplication of *Plutella xylostella* and *Spodoptera litura*

P. xylostella

Diamondback moth was mass multiplied in the laboratory by following the method described by Liu and Sun (1984) with minor modifications. The larvae were collected from the infested cabbage field and were reared separately on cabbage leaves which were raised in the green house under insecticide free conditions. Pupae obtained were kept in Petri plate and placed in a cage of 25 cm^3 for adult emergence. When the moths started to emerge, mustard seedlings were provided for oviposition. Plastic cups of 6 cm height and 4.5 cm diameter were filled with sterilized vermiculite to a depth of 4 cm and pre-soaked mustard seeds (24 h) treated with Bavistin (2 g/kg) were sown in cups and allowed to germinate under natural conditions. Four to five days after germination, the cups were placed in the oviposition cage and replenished at 24 h interval. The moths laid eggs on both sides of cotyledons. The cups with eggs were transferred to plastic tubs (45 x 30 x 15 cm) for mass rearing. Ten percent honey solution containing the multivitamin/multimineral capsule, Becadexamin from GlaxoSmithkline Pharmaceuticals Limited was provided for the adults as food through cotton swab kept in a sterilized petriplate. For raising the mustard seedlings and rearing of DBM larvae, approximately 12:12 (L:D) photoperiod and 27±2°C temperature was maintained under laboratory conditions.

Eggs hatched in 2-3 days and neonates mined the mustard cotyledons and fed on them. When the seedlings were completely consumed, larvae were transferred to fully expanded cabbage leaves with petiole covered in wet cotton swab to maintain leaf turgidity. The third instar larvae (0.5 ± 0.15 cm length; 1.65 ± 0.20 mg weight) were used for the bioassay.

S. litura

The larvae collected from the infested fields of cabbage were reared separately on cabbage leaves raised in green house under insecticidal free condition. Pupae thus obtained were kept in a sterilized Petri plate and placed in the cage of 25 cm^3 for adult emergence. When the moth started emerging, 25-30 days old small cabbage heads were provided for oviposition. The moth laid eggs both on ventral and dorsal surface of leaves, leaves with eggs were transferred to plastic tubs (45 x 30 x 15 cm) for mass rearing. Ten percent of honey solution containing the multivitamin/multimineral capsule, Becadexamin from GlaxoSmithkline Pharmaceuticals Limited was provide as food for adults in sterilized vial with cotton plug. The 3rd instar F_1 generation larvae were used for bioassay.

Bioassay of *cry*1I clones

10 ml of Luria Bertani broth was inoculated with *E. coli* containing *cry*1I construct and kept in shaker overnight at 37°C. 1:100 dilution was made for *cry*1I construct , M15 host and plain pQE30 vector and keep in shaker till it reaches log phase. IPTG was induced (1 mM) of *cry*1I construct, M15 host and plain pQE30 vector. Cells were pelleted at 5000 rpm for 15 min at 4°C and supernatant was discarded. Cells were resuspended in binding buffer (20 mM sodium phosphate + 500 mM NaCl). Lysozyme was added at the final concentration of 1 mg/ml and incubated on ice for 30 min. Triton X100 was added to the above suspension (1%) and incubated on 4°C for 10 min on a rocking platform. Cell suspension was centrifuged at 5000 rpm for 30 min at 4°C and supernatant was collected in fresh tube. A constant concentration of protein was used for bioassay of *P. xylostella* and *S. litura.*

Insecticidal activity against third instar larvae of *P. xylostella* and *S. litura* was measured which was conducted on fresh leaf disks by leaf dip bioassays. Disks cut from leaves of cabbages incorporate a suspension of purified inclusions of *cry1I* clones. Toxicity studies on larvae of the *P. xylostella* and *S.* grown in the greenhouse were used for *P. xylostella* and *S. litura*. Ten larvae were placed on a leaf disk, and their fates were monitored after 2 days for *P. xylostella* and *S. litura*. Bioassays were repeated at least twice.

RESULTS

Amplicon restriction fragment length polymorphism was done in order to identify the presence of *cry* gene variants among the native isolates. The annealing temperature of *cry1I* full length primer was standardized by using reference strain *B. thuringiensis* subsp. *kurstaki* (HD1). CFE20(3) and CFE25(2) were selected for amplicons restriction length polymorphism analysis in order to find out the variants. When the PCR amplicon of CFE20(3) and HD1 were restricted with *Kpn*I and *Xma*I, there were differences observed in restriction fragments in relation to the reference strain HD1 (Figure 1).

There was one restriction site of *Xma*I in CFE20(3) giving rise to 1869 and 300 bp as compared to the reference strain HD1 which give an unrestricted band of 2169 bp. CFE20(3) showing varied restriction pattern were selected for further cloning and expression studies.

Based on the ARFLP pattern of the *cry1I*, variant from the native *B. thuringiensis* isolates viz., CFE20(3) was cloned into T/A cloning vector pTZ57R/T. The large scale amplification of full length gene encoding *cry1I*(2169 bp) from CFE20(3) was obtained using *cry1I* specific primers. A sharp amplicon of the expected size (2169 bp) was eluted from the preparative gel, which was then cloned into a cloning vector, pTZ57R/T. These constructs was transformed into *E. coli* DH5α and transformants were

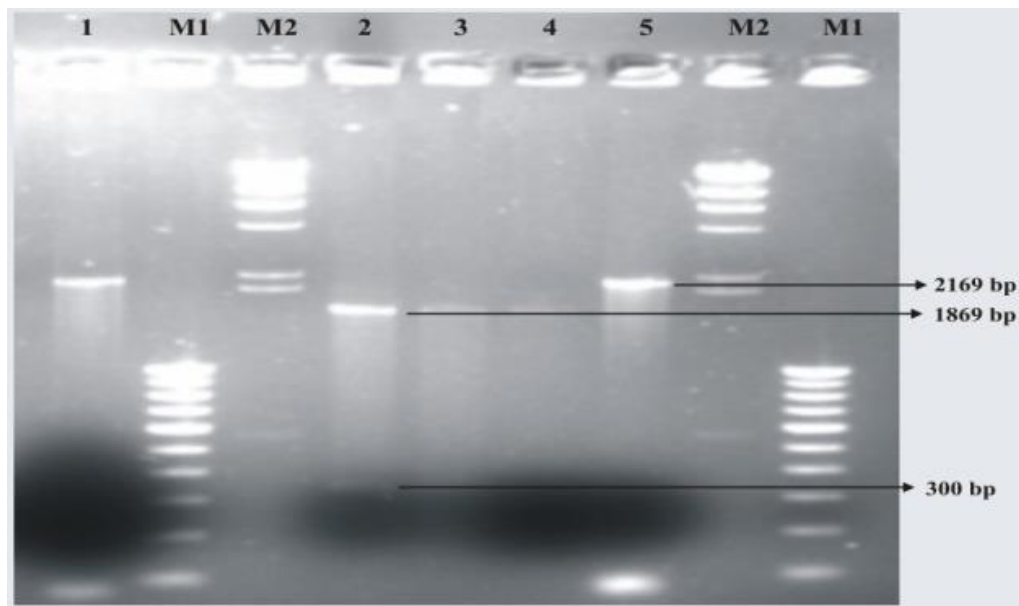

Figure 1. Amplicon restriction fragment length polymorphism of full length *cry1I* genes from native *B. thuringiensis* isolates. M1 represents 100 bp marker. M2 represents double digest marker. Lanes 2, 3 and 4 represents *cry1I* amplicon from CFE20(3) restricted with *Xma*I. Lane 5 represents *cry1I* amplicon from HD1 restricted with *Xma*I.

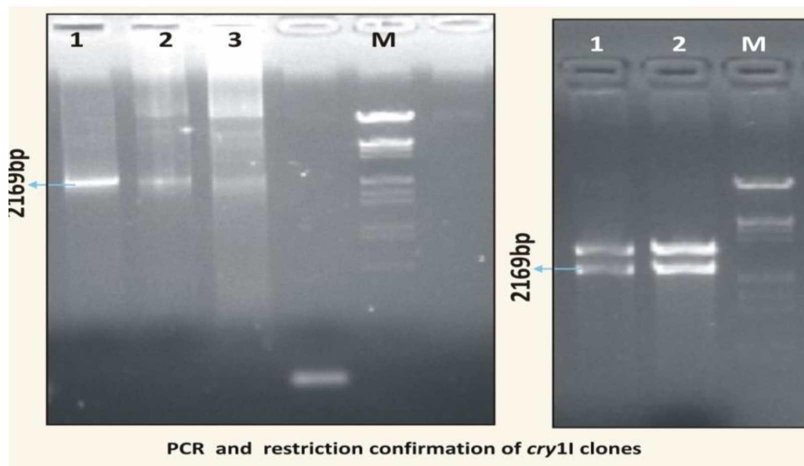

Figure 2. PCR and restriction confirmation of *cry*1I clones in pQE30 expression vector. Lane 1, 2, 3: PCR confirmation of clones. Lane 1, 2: Restriction confirmation of clones.

confirmed by PCR and restriction analysis using *Bam*HI and *Pst*I endonucleases separately giving rise to linear fragment of 4.9 kb including both vector and insert.

The construct containing *cry1I* was sequenced through primer walking employing M13 primers. The available sequence information from cloned fragments was analyzed using BLAST algorithm available at http:/www.ncbi.nlm.nih.gov. Multiple alignment of amino acid sequences showed 99 percent homology to that of the published *cry1IAa* sequence.

Following restriction, the 2169bp *cry1I* fragment from the plasmid DNA were individually cloned into *Bam*HI and *Pst*I sites of the expression vector pQE30 and transformed into *E. coli* JM109 cells which is a maintenance host. The transformants with *cry1I* were picked and streaked on Luria agar plates containing Amp100 and Nal10 selection pressure. The recombinants obtained from clones were confirmed by digestion with *Bam*HI and *Pst*I containing both insert (2169bp) and vector (3400 bp) following electrophoresis (Figure 2).

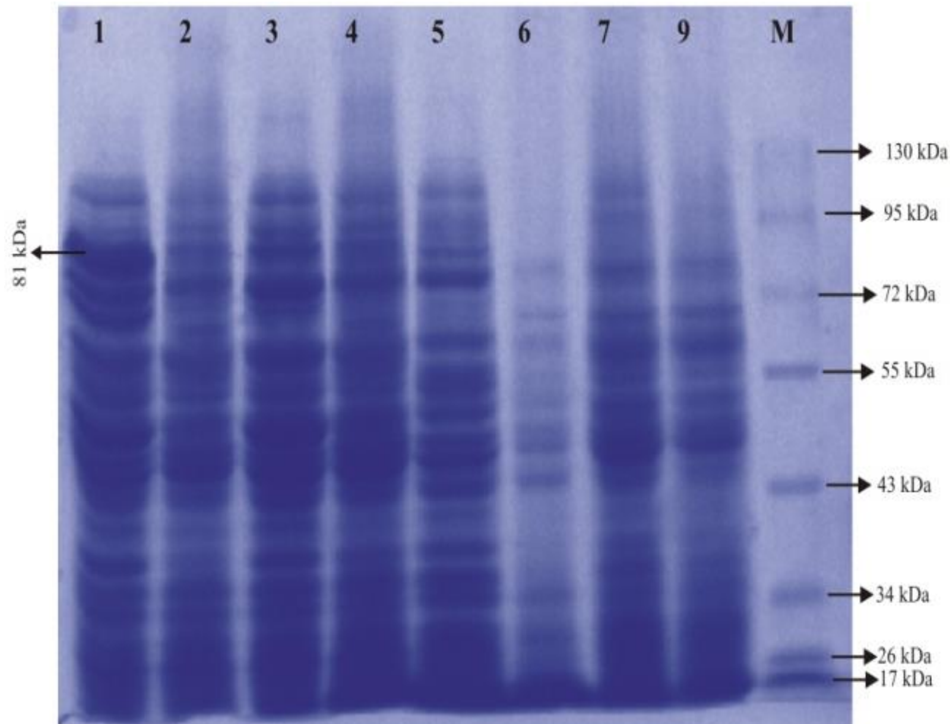

Figure 3. Detection of the recombinant protein with a 10% SDS PAGE gel. M- Page ruler plus prestained protein ladder marker in kDa; Lane 1- Protein from induced *cry*1I clone; Lane 2- Protein from uninduced *cry*1I clone; Lane 3- Protein from induced *cry*1I clone; Lane 4- Protein from uninduced *cry*1I clone; Lane 5- Protein from induced pQE 30 vector; Lane 6- Protein from uninduced pQE 30 vector; Lane 7- Protein from induced M15 host; Lane 8- Protein from uninduced M15 host.

Later, the plasmid from the confirmed recombinant clones in *E. coli* JM109 cells were transformed into two expression hosts viz. *E. coli* M15(pREP4) and *E. coli* SG13009(pREP4). The recombinants were again confirmed by digestion with *Bam*HI and *Pst*I containing both insert (2169 bp) and vector (3400 bp) fragments of the plasmid pREP4 which is present in the host strains *E. coli* M15 (pREP4) and *E. coli* SG13009(pREP4) following electrophoresis.

To check the expression of cloned *cry1I* gene in pQE30, the total protein from IPTG induced *cry1I* clones in *E. coli* M15 (pREP4) and *E. coli* SG13009 (pREP4), plain pQE30 (negative control) and the plain host strain viz. *E. coli* M15 (pREP4) and *E. coli* SG13009(pREP4) (negative control) were subjected to SDS PAGE. The protein band of approximately 81kDa in clones and absence of such bands in negative control when loaded at equal amount of protein (500 µg) were observed (Figure 3).

For further confirmation of *cry1I* clones which expressed 81kDa proteins, bioassay was done against third instar larvae of *P. xylostella* and *S. litura* by feeding the total protein of 500 mg/ml. Plain vector pQE30 in both *E. coli* M15 (pREP4) and the host strains viz. *E. coli* M15(pREP4) without insert and leaf discs dipped in distilled water served as negative controls. The percent mortality at 24, 48 and 72 h interval was recorded. At 72 h interval, the percent mortality of clones ranged from 60 to 90%. The clone containing *cry1I* recorded 86.67% mortality (Figure 3). In negative controls, which included the plain *E. coli*, *E. coli* with vector and plain water, no mortality was recorded.

When compared with an *E. coli* extract containing the empty vector, SDS-PAGE analysis demonstrated the presence of a band with a molecular weight of approximately 81 kDa, corresponding to the Cry1Ia protein and several other *E. coli* proteins (Figure 3).

In the *P. xylostella* bioassay, protein lysate of cry1Ia construct under induced conditions shows the mortality of 90% under uninduced conditions 40%, where as protein lysate of PQE30 plain vector shows mortality of 30% (Abbott's formulae, 1925) (Figure 4). Protein lysate of *cry1Ia* construct was found to be ineffective against *S. litura.*

DISCUSSION

The use of *E. coli* as a power house to express genes of various microorganisms has been broadly used successfully. With this technique, a *cry1Ia* gene from a

Figure 4. Bioassay of *cry*1I construct against diamond back moth (*Plutella xylostella*). IN- induced; UN- uninduced.

local isolate of *B. thuringiensis* CFE20(3) was cloned, expressed in *E. coli* and the toxicity of the recombinant protein subsequently tested against third instar larvae of Diamond back moth, *P. xylostella* and In a similar study conducted by Bergamasco et al. (2011), the expression of Cry1Ia protein was confirmed with molecular weight of approximately 81KDa. In a study conducted by Grossi-de-Sa et al. (2007), a new *cry*1I gene, designed as *cry*1Ia7, was isolated and protein expressed in *E. coli*. This protein was demonstrated to be soluble in water and toxic to various insect species. Song et al. (2003) reported that Cry1I protein was shown to be toxic to the diamond back moth (*Plutella xylostella*), Asian corn borer (*Ostrinia furnacalis*) and soyabean pod borer (*Leguminivora glycinivorella*). It was not toxic to the cotton boll worm (*Helicoverpa armigera*), beet armyworm (*Spodoptera exigua*), or elm leaf beetle (*Pyrrhalto aenescens*) in bioassays. Similarly, in the present study, it was observed that expressed product was toxic to the *P. xylostella* and showed 86.67% mortality but it was not effective against *S. litura*. Cry1Aa, Cry1Ab, Cry1C and Cry1Da were found to cause more than 93% mortality against third instar larvae of *P. xylostella* (Cheng et al., 2005). Bergamasco et al. (2011) observed that Cry1I was found to be toxic against *Spodoptera frugiperda* and *Anthonomous grandis.* Although many toxins have been found in *B. thuringiensis* strains, only a few of them have been used to effectively control some determined insect pests. Moreover, some insect pests have developed resistance against some *B. thuringiensis* toxins. In order to solve these problems, isolation of new strains and toxins is crucial. This study provided a identification of *cry*1I type gene with new insecticidal properties. However, its insecticidal characteristics may be explored if the Cry1I proteins were successfully expressed in transgenic plants. The present work demonstrated the efficiency of the bacterial system for the expression of the *B. thuringiensis* Cry1Ia protein, and the high toxicity of the protein to *P xylostella*.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Efficacy of fungicides, botanicals and bioagents against *Rhizoctonia solani* inciting leaf blight on turmeric (*Curcuma longa* L.)

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Leaf blight caused by *Rhizoctonia solani* is one of the most serious diseases that cause damage to turmeric crop. Fifty percent disease incidence was observed in turmeric growing areas of Andhiyur (Erode district) and Mettur (Salem district) of Tamil Nadu, India. The antifungal efficacy of botanicals, bioagents and fungicides were tested against *R. solani in vitro.* It was observed that seed extract and oil cake of *Madhuca longifolia* suppressed the mycelia growth (34.81; 49.63%), sclerotial formation (100%) and production of biomass (2.18; 2.41 mg) of *R. solani* followed by leaf extracts of *Azadirachta indica* and *Littorina littorea.* Among the bioagents native isolate of BSoya1 of *Bacillus* spp., Cg1 of *Chaetomium* spp., Pf1 of *Pseudomonas* spp. and Tv1 of *Trichoderma* spp. shown more than 50% antagonistic effect on mycelia growth and 100% on sclerotial formation. The complete fungal growth inhibition was observed in Nativo and Bavistin fungicides amended medium. Then, the basal soil application of mahua cake and *T. viride* in the ratio of 1:2 (1 g of mahua cake mixed with 2 g *T. viride* per kg of pot soil) followed by foliar spray of Nativo @ 0.5% significantly mitigated the leaf blight (8.6%) over the inoculated control (50.51%) in pot culture experiments.

Key words: Turmeric, efficacy, fungicides, bioagents, botanicals, *Rhizoctonia solani*.

INTRODUCTION

Turmeric (*Curcuma longa* L.) known as the "golden spice", is one of the most important herbs in tropical and subtropical countries. It is a rhizomatous perennial plant of the ginger family, Zingiberaceae (Dixit et al., 2002). Turmeric is used as condiment, dye, drug and cosmetic in addition in religious ceremonies (Gescher et al., 2005). India is the leading producer, consumer and exporter of turmeric in the world and contributes about 80% of the world production and 60% to the total trade (Parthasarthy et al., 2007).

Several fungal, bacterial and viral diseases are reported in turmeric (Hudge and Ghugul, 2010). Among the diseases, leaf blight caused by *Rhizoctonia solani* is an emerging disease to the turmeric crop (Roy, 1992). The pathogen is known to be soil borne and sclerotia are often found in

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Table 1. Standard evaluation system for screening rice sheath blight.

Grade	Description
0	No incidence
1	Less than 1% sheath area affected
3	1-5% sheath area affected
5	6-25% sheath area affected
7	26-50% sheath area affected
9	51-100% sheath area affected

the soil. Limited information is available on sustainable management and the disease control is generally with chemical applications. The increasing awareness of fungicide related hazards has emphasized the need for adopting biological methods as an alternative disease control method, which is also ecofriendly (Khare et al., 2010).

Biological control appears to be the best solution for long term sustainability and effective management of soil borne disease which can considerably minimize the disease. Successful management of *R. solani* on various crops by bioagents has been previously reported (Lahlali and Hijri, 2010; Seema and Devaki, 2012; Srinivas et al., 2014).

Hence, considering economic importance of the crop and the disease, the present investigation was undertaken to conduct the disease survey, to evaluate the efficacy of fungicide, botanicals and bioagents against *R. solani* and to find out the suitable management practice to mitigate the disease.

MATERIALS AND METHODS

Survey

Turmeric growing villages of Tamil Nadu, India were surveyed to assess the leaf blight disease incidence and severity on turmeric. The disease scoring was done in 50 plants at three different locations in a single village. The disease rating scale (0 to 9) was followed for the assessment of incidence and severity of leaf blight disease. The blight disease incidence was assessed and expressed in percent disease index (PDI) and also relative lesion height (RLH) was assessed based on the lesion height (Sharma et al., 1990). The following formula was used to calculate PDI.

$$\text{PDI} = \frac{\text{Sum of individual ratings}}{\text{Total number of plants observed}} \times \frac{100}{\text{Maximum grade}}$$

Standard evaluation system for screening rice sheath blight (0-9 scale) is shown in Table 1

The following formula was used to calculate relative lesion height (RLH):

$$\text{RLH} = \frac{\text{Lesion height}}{\text{Plant height}} \times 100$$

Isolation of pathogen

R. solani was isolated from the naturally infected leaf samples through tissue segment method collected from different places of Tamil Nadu. Small portions of sheath showing typical lesions were cut into small bits of 0.5 - 1 cm, surface sterilized with 0.1% mercuric chloride and rinsed three times with sterile distilled water. Then, they were transferred to Petri dish containing potato dextrose agar (PDA) medium aseptically (Riker and Riker, 1936). The pure culture of the pathogen was obtained by single hyphal tip technique and the culture was maintained on PDA slants to carry out further studies (Taheri et al., 2007).

Pathogenicity tests

Diseases free (healthy) turmeric rhizomes (Erode local 8) were planted in 30 cm diameter earthen pots containing pot mixture at two rhizomes per pot. Actively growing plants at 30 days after planting (DAP) were inoculated with *R. solani* by inserting young immature sclerotia (two sclerotia per sheath) and incubated for seven days for the development of typical blight symptoms on the plants (Sriram et al., 1997).

In vitro effect of botanicals on radial mycelial growth of *R. solani*

Leaves of nine plants viz., *Lawsonia inermis* L. (Maruthani), *Ocimum tenuiflorum* L. (Thulasi), *Azadirachta indica* L. (Neem), *Morinda citrifolia* L. (Noni), *Vinca major* (Periwinkle), *Gloriosa suberba* (Glory lily), *Justicia adhatoda* (Adathoda), *Vitex nigundo* (Nochi) and *Madhuca longifolia* (Mahuva seed extract) and seven oilcakes *viz.,* mahuva (*Mahua longifolia L.*), pungam (*Pongamia glabra L.*), sesamum (*Sesamum indicum L.*), groundnut (*Arachis hypogaea L.*), castor (*Ricinus communis L.*), neem (*Azadirachta indica L.*) and sunflower (*Helianthus annuus L.*) were collected and tested for their efficacy against *R. solani* by poisoned food technique. Fresh plant leaves and seed extracts were prepared by cold water extraction method (Shekhawat and Prasad, 1971). The leaves were first washed with distilled water and finally with sterile water and the oilcakes were first soaked in sterile distilled water at the rate of one g in 1 ml of water and kept overnight. Then, ground in pestle and mortar by adding sterile water at the ratio of 1:1 (w/v). The macerate was squeezed using cotton to get the extract. The extract was strained through two layers of muslin cloth and finally through Whatman No.1 filter paper and this formed the standard plant extract solution (100%). This was further diluted with sterilized distilled water (v/v) to have the required concentrations (10, 15, and 20%). The PDA medium was mixed with different concentrations *viz.,* 10, 15 and 20% of plant extracts and oil cakes. Mycelial plug of the pathogens (7 mm) was placed at the centre of each Petri plate and incubated at 28 ± 1°C and three replications were maintained for each treatment. The experiment was arranged in a completely randomized block design. The fungus grown on PDA without any plant extracts and oil cakes served as control. The growth diameter was recorded and the percent inhibition was calculated.

In vitro efficacy of botanicals on biomass production of *R. solani*

The effect of plant products on mycelia growth of the pathogen in liquid medium was done by the method suggested by Neslihan et al. (2008). Different concentrations (10, 15, and 20%) of plant extracts and oil cakes were amended with the 100 ml of potato dextrose broth. A mycelial plug (7 mm) was transferred to the flasks which contain different concentrations of plant extracts and incubated at

28±2°C for one week. Three replications were maintained for each treatment. The experiment was arranged in a completely randomized block design.The fungal mycelial mat was collected, dried and mycelium weight recorded.

In vitro efficacy of bioagents on mycelial growth and sclerotial formation of *R. solani*

The native isolates of bio-agents *viz., Bacillus* isolates (EPCO8, EPCO44, EPCO26, BG1, Bsoya1, Bsoya2, BG2, BC3, EPCO81, EPCO PF, BC1, EPCO78, BC9, BC8, BC7, BC6, EPCO P6); *Pseudomonas* isolates (Pf1, KAU46, APF6, Pf3, PC3, 18P, 6P, PC4 and Pf26); *Chaetomium* isolates (Cg1, Cg2, Cg3, Cg4, Cg5, Cg6 and Cg249) and *Trichoderma viride* isolates (Tv1, Tv2, Tv3, Tv4, Tv5, Tv6 and Tv7) were used for testing their efficacy against *R. solani* by dual plate technique (Dennis and Webster, 1971). The bioagents as well as the pathogen were inoculated equidistant on PDA medium aseptically and incubated at 28 ± 1°C. In each case, three replications were maintained for each isolates along with control. The experiment was arranged in a completely randomized block design. After obtaining the maximum growth in the control, the observation on radial growth of pathogen was taken and percent inhibition was worked out using Vincent's formula. The sclerotial formation was recorded on 7th and 14th days after inocu-lation.

In vitro efficacy of bioagents on biomass production of *R. solani*

The effect of culture filtrates of the native isolates of bioagents *viz., Bacillus* isolates (EPCO8, EPCO44, EPCO26, BG1, Bsoya1, Bsoya2, BG2, BC3, EPCO81, EPCO PF, BC1, EPCO78, BC9, BC8, BC7, BC6, EPCO P6) ; *Pseudomonas* isolates (Pf1, KAU46, APF6, Pf3, PC3, 18P, 6P, PC4 and Pf26); *Chaetomium* isolates (Cg1, Cg2, Cg3, Cg4, Cg5, Cg6 and Cg249) and *Trichoderma viride* isolates (Tv1, Tv2, Tv3, Tv4, Tv5, Tv6 and Tv7) on the biomass production of *R. solani* was studied as per method given by Dennis and Webster (1971). Sterilized potato dextrose (PD) broth (100ml) was taken in 250 ml flask and inoculated with mycelial plugs (7 mm) of the biocontrol native isolates taken from the edge of four day old culture. Inoculated flasks were incubated at 28 ± 2°C for one week and the cultural filtrate was extracted by centrifuging the content at 10000 rpm for 30 min and the culture filtrate was collected. The culture filtrate of bioagents were mixed with PD broth at a rate of 1:4 (v/v) and inoculated with mycelial plugs of pathogen (7 mm), then incubated at 28±2°C for seven days. Three replications were maintained for each isolates. The experiment was arranged in a completely randomized block design. After the incubation period, fungal mycelial mat was collected and dried. The dried mycelial weight was recorded.

In vitro efficacy of fungicides on mycelial growth of *R. solani*

Evaluation of fungicides was carried out for their efficacy to inhibit the mycelial growth of *R. solani* isolate by "poisoned food technique" as described by Sharvelle (1961). The fungicides *viz.,* azoxystrobin (Mirador 250 SC), tebuconazole (Orius 25.9% EC), tridemorph (Calixin 80% EC), fosetyl-Aluminium (Aliette WP 80), trifloxystrobin (Flint 50 WG), fenamidone 10% + mancozeb 50% (Sectin 60 WG), Carbendazim (Bavistin 50WP), tebuconazole 50% + trifloxystorbin 25% (Nativo WG 75) were used at 10, 50, 100, 250, 500, 750 and 1000 ppm concentration. Mycelial plugs of the pathogens (7 mm) taken from a seven day old culture was placed at the centre of each Petri plate and incubated at 28 ± 1°C. Three replications were maintained for each treatment. The experiment was arranged in a completely randomized block design. The fungus grown on PDA without any fungicides served as control. The radial growth of the colony was recorded when maximum growth was observed in the control and it was calculated by using the following formula.

$$I = \frac{C - T}{C} \times 100$$

Where, I = Percent inhibition; C = radial growth in control; T = radial growth in treatment.

The sclerotial formation was recorded on 7th and 14th days after inoculation.

Management of leaf blight of turmeric incited by *R. solani* – Greenhouse studies

Studies were conducted to test the efficacy of effective treatment against leaf blight disease of turmeric under greenhouse condition with nine treatments with fungicide, bioagents and botanical. The Erode local 8 was sown in the pots containing 5 kg/pot of sterilized soil. The pathogen was inoculated 30 DAP. Three replications were maintained for each treatment. The experiment was arranged in a completely randomized block design. The blight incidence was recorded on 40 days after sowing and the effectiveness of the treatments on the intensity of leaf blight disease was observed seven days after inoculation, with a 0-9 scale of the Standard Evaluation System for rice, IRRI (2002) and expressed as percent disease index (PDI).

RESULTS AND PLUGUSSION

Survey and pathogenicity studies of *R. solani* in turmeric

Among the fifteen villages surveyed, the turmeric plants at Andhiyur, Erode district recorded the maximum of 54.5% disease index (PDI) of leaf blight which was followed by Mettur in Salem district which recorded 44.3 PDI. The disease incidence was prevalent in only four villages *viz.,* Annur (Rs1), Andhiyur (Rs2), Mettur (Rs3) and Parama-thivellur (Rs4) and the incidence ranged from 6.4 to 54.5 PDI (Table 2). The results revealed that placing of sclerotia in the injured sheath region exhibited the maximum lesion length of 16.89 cm and leaf blight lesion length. The sym-ptom observed under field condition was very typical when compared with the artificially inoculated plants. Though the symptoms produced by all the isolate were similar, the most virulent Andhiyur, Erode isolate was highly virulent as compared to other isolates and produced larger lesion length.

Efficacy of fungicides on turmeric leaf blight pathogen (*R. solani)*

Nativo and Bavistin were individually effective against the pathogen even at the lowest concentration of 10 ppm by completely inhibiting the mycelia growth and sclerotia

Table 2. Survey of leaf blight of turmeric incited by *R. solani*.

Isolates	Village	District	*PDI	*Relative Lesion Height (RLH)
Rs1	Annur	Coimbatore	6.4	7.14
Rs2	Andhiyur	Erode	54.5	16.67
Rs3	Mettur	Salem	44.3	11.63
Rs4	Paramathivellur	Namakkal	28.6	8.8
	SEd		0.5279	0.2606
	CD(.05)		1.2173	0.6009

* Mean of three replications.

Table 3. Efficacy fungicides on the mycelia growth of *Rhizoctonia solani*.

Fungicides	Mycelia growth inhibition over control (%)* (ppm)						
	10	50	100	250	500	750	1000
Mirador 250 SC	4.21	100.00	100.00	100.00	100.00	100.00	100.00
Orius 25.9% EC	27.4	100.00	100.00	100.00	100.00	100.00	100.00
Calixin 80 % EC	27.44	100.00	100.00	100.00	100.00	100.00	100.00
Aliette WP 80	0.00	0.00	0.74	3.70	19.26	19.83	48.15
Flint 50 WG	59.63	87.04	94.44	100.00	100.00	100.00	100.00
Sectin 60 WG	0.00	41.85	72.59	72.89	87.41	100.00	100.00
Bavistin 50WP	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Nativo WG 75	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	T	**ppm**					
SEd	0.04292	0.03591					
CD (0.05)	0.08487	0.07101					

* Mean of three replications.

production. It was significantly superior over other fungicides and on par with each other. It was followed by Mirador, Orius and Calixin which exhibited total inhibition at 50 ppm (Table 3). The least effective fungicides were Aliette, Flint and Sectin (48.15% inhibition at 1000 ppm). The same trend was observed in the liquid culture technique in which the two fungicides *viz.,* Nativo and Bavistin completely inhibited the mycelia growth at the lowest concentration of 10 ppm (Table 3). Aliette had recorded sclerotial formation of 86 numbers on 14 DAI at 100 ppm. As the concentration of fungicides increased, the sclerotial formation was arrested (Table 4).

Fungicides *viz.,* trifloxystrobin + tebuconazole, tebuconazole and propiconazole showed higher level of efficacy against *R. solani* of rice in laboratory conditions (Hunjan et al., 2011). Among the new formulations, Filia and Nativo were equally effective in controlling sheath blight (Swamy et al., 2009). In the present study, among different fungicides screened for, *R. solani* Nativo and Bavistin were individually effective against the pathogen in inhibiting the mycelia growth and sclerotial production even at the lowest concentration of 10 ppm.

In vitro antagonistic effect of *bioagents* against *R. solani*

Bacillus sp.

Bacillus isolates, BSoya1 and BC3 respectively recorded 32 and 33 mm colony diameter which accounted for 58 and 57% mycelia growth inhibition over the control. BSoya1 and BC3 *Bacillus* isolates were significantly superior and on par with each other in controlling the mycelia growth of *R. solani.* It was interesting to note that the sclerotial formation was completely inhibited in eleven isolates. The remaining six isolates of *Bacillus viz.,* EPCO26, BG1, BSoya2, BG2, BC7 and BC8 had sclerotial formation. Among the seventeen isolates a maximum number of 84 sclerotia were observed in the BG2 as against the control which recorded 228.67 sclerotia (Table 5). All the *Bacillus* isolates reduced the mycelial weight of the pathogen over the control. The least biomass production was seen with the *Bacillus* isolate EPCO26 (0.52 mg) followed by Bsoya1 (0.65 mg)

Table 4. Effect of fungicides on the *Rhizoctonia solani* sclerotial formation.

Fungicides	*Sclerotial formation (DAI)	10 ppm	50 ppm	100 ppm	250 ppm	500 ppm	750 ppm	1000 ppm
Mirador 250 SC	7	2.67	-	-	-	-	-	-
	14	8	-	-	-	-	-	-
Orius 25.9% EC	7	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-
Calixin 80 % EC	7	6.33	-	-	-	-	-	-
	14	13.67	-	-	-	-	-	-
Aliette WP 80	7	20	40	78.67	-	-	-	-
	14	25	50	86.00	-	-	-	-
Flint 50 WG	7	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-
Sectin 60 WG	7	96.00	-	-	-	-	-	-
	14	101.67	-	-	-	-	-	-
Bavistin 50WP	7	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-
Nativo WG 75	7	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-
Control	7	164	154.9	134	158	191	145	169
	14	189	162	173	169	204	163	182

- : Sclerotia not formed. DAI: Days after inoculation.

and BC3 (0.78 mg) (Table 6). Similar antagonistic effect of *Bacillus* spp. against *R. solani* infecting many other crops was reported by several workers. Calvo et al. (2010) reported that, *Bacillus* spp. from rhizosphere region of potato showed high antagonistic effect against *R. solani* causing various diseases in crops plants. Padaria and Kapoor (2011) reported that, *Bacillus pumillus* obtained from rice field exhibited antagonistic activity against *R. solani* infecting various crops. *Bacillus* sp. strain 916, isolated from the soil showed strong activity against *R. solani* causing rice sheath blight (Wang et al., 2012). *Bacillus* sp. shows inhibition against the growth of a wide range of plant fungal pathogens like *R. solani* and *Fusarium verticillioides* by producing morphological modifications on the pathogenic fungi hyphae like total collapse of the cell. In the present study, among the 17 *Bacillus* isolates, BSoya1 and BC3 were significantly superior over other antagonists and it also reduced the mycelial weight and sclerotial production of the pathogen.

Pseudomonas spp.

Among nine isolates of *Pseudomonas* spp., Pf1 strain was significantly superior and recorded 59.22% mycelial growth inhibition and 1.18 mg biomass production. This was followed by KAU46 which recorded 56.7 mm colony diameter which accounted for 37% mycelial growth inhibition over control (Table 6). After 14 days of inoculation, the sclerotial count was recorded and it was observed that except three *Pseudomonas* strains *viz.,* KAU46, Pf3 and Pf26, all the other strains supported sclerotia formation of *R. solani* but the production was drastically reduced. Among the various strains of *Pseudomonas*, the maximum number of 142 sclerotia was recorded in PC3 strain as against the control which recorded 198.67 numbers of sclerotia. Though the *Pseudomonas* isolate KAU 46 exhibited 56.7 mm mycelial growth, it had completely inhibited the sclerotial formation in *R. solani.* Pf 26 has recorded full growth of the test pathogen but it had also inhibited the sclerotial formation (Table 7). Hence the mycelial growth could not be correlated with the sclerotial formation or inhibition. Similarly, Zachow et al. (2010) found that *P. fluorescens* L13-6-12 applied to the *R. solani* infected soil in sugar beet, formed large microcolonies consisting of hundreds of cells.

Chaetomium sp.

On studying the interaction between the *Chaetomium* and the pathogen, the isolate Cg1 showed the maximum percent inhibition (61.89%) which was significantly higher than other isolates (Table 6). It was followed by Cg249 (46.67%) and Cg2 (45.22%) and they were on par with each other. The reduction in mycelial biomass was also

Table 5. Antagonistic effect of *Bacillus* isolates against *Rhizoctonia solani.*

***Bacillus* isolates**	***Colony diameter (mm)**	***Mycelial growth inhibition over control (%)**	***Biomass production (mg)**	***Sclerotial formation (nos.)**	
				7 DAI	**14 DAI**
EPCO8	43.00	47.00	1.02	-	-
EPCO44	50.70	39.30	1.19	-	-
EPCO26	43.30	46.70	0.52	-	2
BG1	45.00	45.00	1.08	-	3
BSoya1	32.00	58.00^{a}	0.65	-	-
BSoya2	41.70	48.30	0.88	13	19
BG2	41.70	48.30	0.97	38	84
BC3	33.00	57.00	0.78	-	-
EPCO81	48.70	41.30	1.19	-	-
EPCO Pf	73.00	17.00	2.52	-	-
BC1	74.30	15.70	2.31	-	-
EPCO78	55.00	35.00	1.28	-	-
BC9	43.00	47.00	1.02	-	-
BC8	90.00	0.00	2.79	-	3
BC7	90.00	0.00	2.56	36	73
BC6	63.30	26.70	1.29	-	-
EPCO P6	52.70	37.30	1.25	-	-
Control	90.00	0.00	3.65	175.67	228.67
SEd	1.7825	0.0978	0.0648		
CD(.05)	3.6155	0.1985	0.1314		

*Mean of three replications. Means in a column followed by same superscript letters are not significantly different and the means are compared with LSD. Sclerotia not formed. DAI- Days after inoculation.

Table 6. Antagonistic effect of *Pseudomonas* isolates against *Rhizoctonia solani.*

***Pseudomonas* isolates**	***Colony diameter (mm)**	***Mycelial growth inhibition over control (%)**	***Biomass production (mg)**	***Sclerotial formation (nos.)**	
				7 DAI	**14 DAI**
Pf1	36.7	59.22	1.18	1	2
KAU46	56.7	37.00	1.26	-	-
APF6	62.0	31.11	2.12	3	3
Pf3	76.7	14.78	2.52	-	-
PC3	78.7	12.56	2.49	45	142
18P	90.0	0.00^{f}	3.59	43	50
6P	90.0	0.00	3.65	3	5
PC4	90.0	0.00	3.52	10	14
Pf26	90.0	0.00	3.63	-	-
Control	90.0	0.00	3.65	122.67	198.67
SEd	1.9772	0.0843	0.2708		
CD (0.5)	4.1244	0.1759	0.5649		

*Mean of three replications. Means in a column followed by same superscript letters are not significantly different and the means are compared with LSD. - Sclerotia not formed. DAI- Days After Inoculation.

recorded with Cg1 (1.39 mg) and the isolate was individually significantly superior when compared with other isolates. It was followed by Cg249 (1.99 mg) and Cg2 (2.01 mg). All the isolates drastically reduced the number of sclerotial formation over control. The isolates Cg2 completely inhibited the sclerotial formation. The

Table 7. Antagonistic effect *Chaetomium* isolates against *Rhizoctonia solani.*

Chaetomium isolates	*Colony diameter (mm)	*Mycelial growth inhibition over control (%)	*Biomass production (mg)	*Sclerotial formation (nos.)	
				7DAI	14 DAI
Cg1	34.30	61.89	1.39	28	54
Cg2	49.30	45.22	2.01	-	-
Cg3	51.70	42.56	2.10	3	4
Cg4	56.70	37.00	2.30	3	6
Cg5	65.30	27.44	2.58	5	12
Cg6	52.70	41.44	2.12	6	10
Cg249	48.00	46.67	1.99	36	74
Control	90.00	0.00	3.65	113.02	132
SEd	1.4269	0.0979	0.0624		
CD(.05)	3.0250	0.2075	0.1322		

*Mean of three replications. Means in a column followed by same superscript letters are not significantly different and the means are compared with LSD. Sclerotia not formed. DAI- Days after inoculation.

Table 8. Antagonistic effect *Trichoderma viride* isolates against *Rhizoctonia solani.*

Trichoderma isolates	*Colony diameter (mm)	*Mycelial growth inhibition over control (%)	*Biomass production (mg)	*Sclerotial formation (nos.)	
				7 DAI	14 DAI
Tv1	19.7	78.11	0.40	-	-
Tv2	43.3	51.89	1.42	-	-
Tv3	38.7	57.00	1.10	-	-
Tv4	41.0	54.44	1.74	-	-
Tv5	35.0	61.11	1.41	-	-
Tv6	39.7	55.89	1.61	-	-
Th	49.7	44.78	2.01	-	-
Control	90.0	0.00	3.65	97	121
SEd	1.0212	0.1080	0.0577		
CD(.05)	2.1648	0.2290	0.1224		

*Mean of three replications. Means in a column followed by same superscript letters are not significantly different and the means are compared with LSD. Sclerotia not formed. DAI- Days after inoculation.

maximum sclerotial production was recorded in the control (132 numbers), where as the minimum sclerotial production was seen in the isolate Cg 3 (4 numbers) (Table 7). Kaushik et al. (2010) reported that *Chaetomium globosum* showed good activity against *Sclerotinia sclerotiorum*, *Fusarium oxysporum* and *R. solani*.

Trichoderma viride

Among the seven isolates of *Trichoderma* spp. tested, maximum percentage of inhibition was recorded with the isolate Tv1. All the seven isolates inhibited the growth of *R. solani* in dual culture. The isolate Tv1 showed maximum percent inhibition of mycelial growth (78.11%) and it was individually significantly superior, followed by Tv5 (61.11%) and Tv3 (57.00%). The least percent inhibition was observed with the isolate *T. harzianum* (44.78%) (Table 8). The Tv1 culture filtrate significantly reduced the mycelial biomass of the pathogen by recording 0.40 mg biomass production. It was followed by Tv3 (1.10 mg) and Tv5 (1.41 mg). All the isolates of *Trichoderma* inhibited the formation of sclerotia over control which recorded 121 sclerotia after 14 days of inoculation (Table 8).

Similarly, Khan and Sinha (2007) reported that, *T. harzianum* (rice leaf sheath isolate) was found most effective against *R. solani* in *in vitro* and glasshouse conditions. *T. harzianum* is well known biocontrol agents against several soil borne pathogens. Osman et al. (2011) showed that, *T. harzianum* was found effective in inhibiting the *R. solani* causing soya beans root rot. In the

present study, *T. viride* was found to be effective among the other antagonists. Among the seven isolates of *Trichoderma* spp tested, maximum percentage of inhibition was recorded with the isolate Tv1. All the seven isolates inhibited the growth, mycelial biomass and sclerotial production of *R. solani* in dual culture. Alamri et al. (2012) proved that the mechanism of *T. harzianum* JF419706 to suppress the pathogenic fungi *viz., Alternaria alternate*, *Fusarium oxysporum*, *Exserohilum rostratum*, *Macrophomina phaseolina*, *Pythium ultimum*, and *R. solani* was though competition for nutrients, mycoparasitizing and lysis of pathogen's cell walls. Harman et al. (2012) mentioned that *Trichoderma* spp. parasitize a range of phytopathogenic fungi.

In vitro effect of botanicals against *R. solani*

Madhuca longifolia (mahua) seed extract showed maximum inhibition of 34.81% at 20% concentration and it was found significantly superior to other extracts (Plate 1). This was followed by *A. indica* leaf extract which showed an inhibition of 20% at 20% concentration and *O. tenuiflorum* (thulasi) leaf extract, 18.15%. All the nine aqueous plant extracts were ineffective at 10% concentration (Table 9). The botanicals reduced the mycelial biomass of the pathogen over control. Extract from the *M. longifolia* seed significantly reduced the mycelial biomass (2.18 mg) over other treatments. It was followed by *O. tenuiflorum* (2.73 mg) and *A. indica* (2.95 mg) and they were on par with each other. The leaf extracts of *A. indica*, *L. littorea* and *M. longifolia* seed extract completely inhibited the sclerotial formation at all the three concentrations tested *viz.*, 10, 15 and 20% (Table 8).

Plant extract are not only easy to prepare but also non-polluting and low priced as compared to commercial fungicides. This is supported by the work of Alabi and Olorunju (2004). In their studies, plants sprayed with neem seed extract gave yields higher than the plants sprayed with black soap and cow dung extract (Alabi and Olorunju, 2004). Gujar et al. (2012) reported that *A. indica* and *A. vera* showed inhibition of mycelial growth of the pathogen and can be utilized for the management of fungal diseases caused by the *Aspergillus niger*, *Aspergillus flavus*, *R. solani*, *Rhizoctonia bataticola*. In this present study, *M. longifolia* seed extract showed maximum inhibition and it was significantly superior to other plant extracts. All these plant extracts inhibited the mycelial weight and sclerotial formation completely.

All the oil cake extracts tested were not inhibitory at the lowest concentration of 10%. Mahua oil cake was the only extract which was inhibitory at 15% concentration recording 14.81% mycelial growth inhibition over the control. Among the seven extracts, mahua oil cake extract exhibited the maximum mycelial growth inhibition of 49.63% against the pathogen *R. solani* at 20% concentration (Plate 2). It was followed by neem oil cake (14.07%) and castor oil cake (8.15 %) (Table 11). Mahua oil cake and neem oil cake extract completely inhibited the formation of sclerotia at all concentrations. Among the treatments, maximum sclerotial formation was seen in the Petri plate poisoned with gingelly oil cake extract (117 numbers) at 20% concentration after 14 days of inoculation. The control recorded 199 numbers of sclerotia. It was interesting to note that the lower concentration of the seven oil cake extracts *viz.,* 10 and 15% had inhibited sclerotial formation in solid medium (Table 10). Alice et al. (1998) reported that the presence of antifungal principles present in the mahua cake extract (10%) was effective in combating the jasmine wilt incidence caused by *Sclerotium rolfsii*.

Management of leaf blight of turmeric under greenhouse studies

The effective treatments were culled out from the present research findings and used to perform the pot culture experiments to find out its efficacy against *R. solani* causing leaf blight of turmeric. Among the seven treatments, the basal soil application of mahua cake and *T. viride* in the ratio of 1:2 (1 g of mahua cake mixed with 2 g *T. viride* per kg of pot soil) followed by foliar spray of Nativo @ 0.5% significantly reduced the percent disease incidence over other treatments. This effective treatment recorded 8.6 PDI and a plant height of 160.8 cm on 180 DAP as against the inoculated control which recorded 50.51 PDI with a plant height of 138.6 cm. The disease reduction over control was 83.84% and the increase in plant height accounted for 11.00% (Table 11). The reduction in the turmeric sheath blight incidence and increased plant height in the treated plants in the present study is due to the biocontrol nature of all the treatments imposed.

Similarly, application of *T. harzianum* amended with organic fertilizer was more efficient alone in managing damping off disease (*R. solani*) in cucumber (Huang et al., 2011). Clove extract at a concentration of 4% as well as the chemical fungicide (Rizolex-T) significantly reduced the incidence of *R. solani* in pea (Al-Askar and Rashad, 2010). Addition of mustard oil cake in French bean (*Phaseolus vulgaris*) along with the inoculation of arbuscular mycorrhizal fungi (AMF) and *P. fluorescens* was found highly effective in reducing the root rot caused by *R. solani* (Neeraj and Kanchan, 2011).

Conclusion

Research presented here has expanded knowledge of the association of *R. solani* with turmeric crops in Tamil Nadu, India and about disease symptomology. Fungicide, botanical and bioagents were shown to vary in effectiveness at controlling mycelia growth, sclerotial

Table 9. *In vitro* effect of plants extracts on growth and sclerotial production of *Rhizoctonia solani*.

Plant products/ concentration	*Mycelial growth inhibition over control (%)			*Biomass production (mg)			*Sclerotial production			
	10%	15%	20%	10%	15%	20%	DAI	10%	15%	20%
Lawsonia inermis	0.00	0.00^{j}	2.59	3.65	3.65	3.54	7	3.00	6.33	97.33
							14	5.33	9.00	100.33
Ocimum tenuiflorum	0.00	0.00^{j}	18.15	3.62	3.61	2.73	7	-	194.33	-
							14	-	205.00	-
Azadirachta indica	0.00	6.30^{f}	20.00	3.62	3.56	2.95	7	-	-	-
							14	-	-	-
Morinda citrifolia	0.00	0.00	12.96	3.60	3.43	3.20	7	189.00	-	-
							14	195.00	-	-
Vinca major	0.00	0.00	3.70	3.63	3.60	3.58	7	-	-	-
							14	-	-	-
Gloriosa superba	0.00	0.00	0.00	3.65	3.65	3.65	7	93	95	84
							14	98	100	89
Justicia adhatoda	0.00	0.00	0.00	3.65	3.65	3.60	7	-	103	37
							14	-	117	43
Vitex nigundo	0.00	0.00	1.85	3.65	3.61	3.58	7	-	-	98.67
							14	2	5	104.33
Mahuva seed extract	0.00	14.81	34.81	3.62	3.50	2.18	7	-	-	-
							14	-	-	-
Control	0.00	0.00	0.00^{j}	3.65	3.65	3.65	7	192	186	163
							14	232	227	263
	T	**Concentration**	**T**	**Concentration**						
SEd	0.0284	0.01559	0.06410	0.03511						
CD(0.05)	0.0569	0.03118	0.12823	0.07023						

* Mean of three replications. Means in a column followed by same superscript letters are not significantly different and the means are compared with LSD. - Sclerotia not formed. DAI- Days after inoculation.

Table 10. *In vitro* effect of oil cake extracts on growth and sclerotial production of *Rhizoctonia solani.*

Oil cake/concentration	*Mycelial growth inhibition over control (%)			*Biomass production (mg)			*Sclerotial production			
	10%	15%	20%	10%	15%	20%	DAI	10%	15%	20%
Mahua	0.00	14.81^{a}	49.63^{a}	3.50^{a}	3.45^{a}	2.41^{a}	7	-	-	-
							14	-	-	-
Neem	0.00	0.00^{e}	14.07^{c}	3.58^{ab}	3.53^{ab}	3.50^{b}	7	-	-	-
							14	-	-	-
Groundnut	0.00	0.00^{e}	0.00^{e}	3.68^{c}	3.68^{c}	3.68^{c}	7	-	-	21
							14	-	-	26
Gingelly	0.00	0.00^{e}	0.00^{e}	3.68^{c}	3.68^{c}	3.68^{c}	7	-	-	108
							14	-	-	117
Sunflower	0.00	0.00^{e}	0.00^{e}	3.68^{c}	3.68^{c}	3.68^{c}	7	-	-	50
							14	-	-	100
Castor	0.00	0.00^{e}	8.15^{d}	3.68^{bc}	3.58^{bc}	3.53^{bc}	7	-	-	15.67
							14	-	-	24.6
Coconut	0.00	0.00^{e}	0.00^{e}	3.68^{c}	3.68^{c}	3.68^{c}	7	-	-	45.1

Table 10. Contd

							14	-	-	50.1
control	0.00	0.00^{e}	0.00^{e}	3.68^{c}	3.68^{c}	3.68^{c}	7	167	154	176
							14	184	179	199
	T	**Concentration**	**T**	**Concentration**						
SEd	0.02219	0.01359	0.06211	0.03803						
CD(0.05)	0.04461	0.02732	0.12488	0.07647						

* Mean of three replications. Means in a column followed by same superscript letters are not significantly different and the means are compared with LSD. - Sclerotia not formed; DAI- Days after inoculation.

Table 11. Management of leaf blight of turmeric under greenhouse conditions.

Treatment	Plant height* (cm)				Leaf blight*	
	45 DAP	**90 DAP**	**135 DAP**	**180 DAP**	**(PDI)**	**Reduction over control (%)**
Mahua cake	50.9^{b}	92.3^{b}	141.2^{b}	153.8^{b}	35.49^{b}	29.74^{ef}
T. viride	47.6^{de}	87.3^{de}	138.1^{de}	142.0^{de}	33.52^{de}	33.64^{e}
Mahua cake + *T. viride*	52.3^{bc}	89.6^{bc}	136.5^{bc}	148.6^{bc}	20.15^{bc}	60.11^{d}
Nativo WG 75 @ 0.5 %	48.02^{e}	84.12^{e}	135.6^{e}	138.6^{e}	18.39^{e}	63.59^{cd}
Mahua cake + Nativo WG 75 @ 0.5 %	49.9^{bc}	88.3^{bc}	141.2^{bc}	147.8^{bc}	13.04^{bc}	74.18^{bc}
T. viride + Nativo WG 75 @ 0.5 %	48.6^{cd}	87.88^{cd}	138.98^{cd}	143.34^{cd}	15.02^{cd}	70.26^{c}
T. viride :Mahua cake (2:1) + Nativo WG 75 @ 0.5%	55.9^{a}	98.3^{a}	145.2^{a}	160.8^{a}	8.16^{a}	83.84^{a}
Control (uninoculated)	49.02^{de}	85.2^{de}	137.6^{de}	140.6^{de}	—	—
Control (Inoculated)	38.3^{f}	79.92^{f}	132.3^{f}	138.6^{f}	50.51^{de}	0.00^{g}

*Mean of three replications.

formation and biomass production of *R. solani*. Basal application of *T. viride* + mahua cake and also foliar application of Nativo 0.5% were explored as a means for integrated disease management of leaf blight disease in turmeric crops.

Conflict of Interests

The authors have not declared any conflict of interests.

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Soil microbial properties, growth and productivity of pearl millet (*Pennisetum glaucum* L.) as influenced by moisture management and zinc fortification under rainfed conditions

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Pearl millet (*Pennisetum glaucum* L.) is an important crop in rainfed conditions and marginal land areas; it is grown under improper crop establishment and imbalanced fertilization. Proper moisture management with zinc fortification has potential to improve productivity, solve zinc malnutrition problem, maintain soil health and economic sustainability. The present study was conducted during 2012 and 2013 at IARI, New Delhi to find out the effect of moisture management and zinc fortification on soil microbial properties, growth and productivity of pearl millet under rainfed conditions. During both years, moisture management and zinc fortification treatments resulted in considerable improvement in soil microbial properties, growth and productivity of pearl millet. Flat bed with 5.0 t/ha crop residue recorded significantly higher dehydrogenase activity, microbial biomass carbon, plant height, dry matter accumulation and grain weight per ear head as compared to flat bed and flat bed with 2.5 t/ha crop residue. In terms of total number of tillers, number of ear heads and length of ear head flat bed with 2.5 and 5.0 t/ha crop residue and narrow bed and furrow with 2.5 t/ha crop residue remained statistically similar with each other. Significantly higher grain (2.52 and 2.72 t/ha), stover (8.21 and 8.65 t/ha) and biological yield (10.72 and 11.37 t/ha) were observed under flat bed with 5.0 t/ha crop residue during both years. Under zinc fortification treatments, application of 5.0 kg Zn/ha to pearl millet recorded significantly higher value of soil microbial properties over control and 2.5 kg Zn/ha. Application of 5.0 and 2.5 kg Zn/ha is at par with each other and proved significantly better over control in terms of growth parameters, yield attributes and yield of pearl millet. Residual effect of zinc fortification was also found to be significant in pearl millet. Soil microbial properties were improved significantly up to 5.0 kg Zn/ha. However, growth parameters and yield attributes were increased significantly only up to 2.5 kg Zn/ha. Application of 5.0 kg Zn/ha produced significantly higher grain (2.57 t/ha), stover (8.22 t/ha) and biological yield (10.78 t/ha) as compared to control. Final results revealed that pearl millet planting under flat bed with 5.0 t/ha crop residue or narrow bed and furrow with 2.5 t/ha crop residue and application of 2.5 kg Zn/ha to pearl millet or chickpea proved to be better.

Key words: Flat bed, crop residue, narrow bed and furrow, dehydrogenase, microbial biomass carbon, moisture management,root length, root volume, grain yield, pearl millet, Zn.

INTRODUCTION

Pearl millet (*Pennisetum glaucum* L.) is the fifth most important cereal grain crop next to rice, wheat, maize and

sorghum. The crop is cultivated for grain as well as fodder in the semi-arid tropical regions of Africa and Asia including India. In India, annual planting area is 8.69 million hectares producing nearly 10.05 million tonnes of grains (Anonymous, 2012). Today, it is getting more attention due to increasing evidence of less seasonal rainfall, terminal heat, frequent occurrence of extreme weather events coupled with scanty water resources (Singh et al., 2010). Pearl millet traditionally is an indis-pensable component of dry-farming system and it is consi-dered more efficient in utilization of soil moisture, and has a higher level of heat tolerance than even sorghum and maize. It is the food for millions of people in the poor regions of semi-arid tropics. From quality point of view, it is nutritionally better than many cereals as it is a good source of minerals (2.0-3.5%) parti-cularly iron (284ppm) and fat (4.0-8.0%). Pearl millet grains possess higher protein content (10.5-14.5%) with higher levels of essential amino acids. The grains of pearl millet possess a biological value similar to wheat and rice and impart substantial energy to the body. It occupies a distinct position in the agricultural economy of the country. With the advent of pearl millet hybrids in mid-sixties, the pearl millet cultivation received a fillip. As a result, the productivity almost tripled from about 350 kg in mid-sixties to about 1156 kg in 2012. The crop is mostly confined to low fertile water deficit soils. Because of its remarkable ability to withstand and grow in harsh environment, reason-able and nearly assured harvests are obtained.

Dryland agriculture has a distinct place in Indian agri-culture, occupying around 80 m ha area (58%) out of 141 m ha net cultivated area. This implies that the country will continue to grapple with the problems of rainfed agri-culture. The main problem of rainfed areas is uncertainty and uneven distribution of rainfall and loss of water through runoff which leads to low and unstable productivity due to moisture stress at critical stages of crop growth. It is a well known fact that about 85% of annual rainfall is received during south-west monsoon season. In this period, know-ledge of crop growth phases and moisture availability is more essential because the deficiency of rain water at any critical growth stage may affect the plant growth and yield. Moisture stress further affect the nutrient availability to the crop since nutrient mobility depends on optimum soil moisture. The risk factor can be minimized through *in situ* moisture conservation, adoption of suitable crops and their varieties (Munish Kumar et al., 2008). Residue application helps in maintaining proper growth and development of crop by conserving the moisture in soil profile and ultimately enhancing the productivity of crops (Singh et al., 2012; Tetarwal et al., 2012). Moisture conser-vation through organic residue application is a viable approach to retain soil moisture and nutrient under water scarcity situations (Tetarwal and Rana, 2006; Sharma et al., 2010).

Another problem of the present scenario is zinc deficiency in soils. It is well a known fact that zinc is now considered as fourth most important yield-limiting nutrient after nitrogen, phosphorus and potassium (Maclean et al., 2002). In-creasing zinc concentration in food crops, resulting to better crop production and improved human health is an important global challenge. Among the micronutrients, Zn deficiency is occurring in both crops and human (White and Zasoski, 1999). Zn deficiency reduces not only the grain yield, but also the nutritional quality of grain and ultimately nutritional quality of human diet. Zn is essential for both plants and animals because it is a structural constituent and regulatory co-factor in enzymes and proteins involved in many biochemical pathways. Besides improving photo-synthesis and regulation of auxin concentration, Zn plays an important role in nitrogen metabolism and protein synthesis. It also involved in formation of chlorophyll and carbohydrate. Under dryland conditions reduced soil moi-sture in surface soil layer reduce zinc adsorption and may cause zinc deficiency. Cereal crops are generally the most susceptible to zinc deficiency and show a high response to zinc fertilization. Agronomic approaches such as appli-cation of Zn-containing fertilizers appear to be a rapid and simple solution to address the Zn deficiency in crop and human health. Biofortification of cereal grains through use of Zn fertilizers is required for keeping sufficient amount of available Zn in soil solution, maintaining adequate Zn transport to the seeds during reproductive growth stage and optimizing the success of biofortification of staple food crops with Zn through use of different approaches. Chaube et al. (2007) and Badiyala and Chopra (2011) reported that use of Zn increase the productivity as well as improve the fertility status of soil. Thus, keeping these facts in view, a research was undertaken to find out the effect of zinc fortification under different moisture management practices on soil microbial properties and performance of pearl millet in rainfed conditions.

MATERIALS AND METHODS

Climate and soil

A field experiment was conducted at the research farm of Indian Agricultural Research Institute, New Delhi during *kharif* 2012 and 2013 under rainfed conditions. The experimental farm is situated at 28°37' N latitude, 77°09'E longitude and 224 m above mean sea level. The maximum and minimum temperature during the growing season (July-September) was 44.2 and 20.2°C during 2012 and 39.0 and 21.0°C during 2013, respectively. The total rainfall received during the cropping season was 416 and 928.6 mm, respectively, out of which 316.8 (76.1%) and 401.9 mm (43.3%) was effective (Figures 1 and 2). The region has typical semi-arid and sub-tropical climate with extremes of cold and hot situations (Sehgal et al., 1992). The experimental soil was sandy loam in texture

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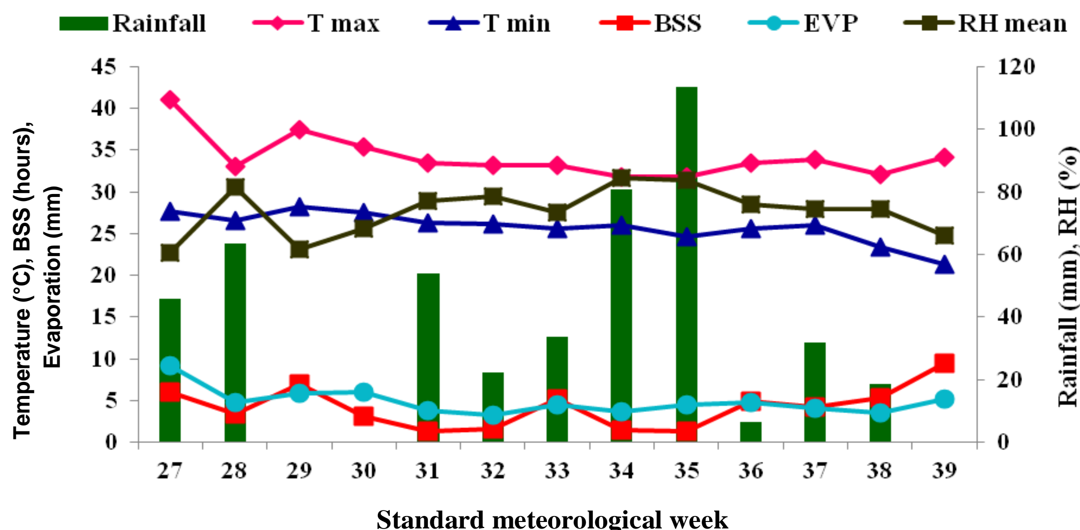

Figure 1. Weather parameters during *kharif* 2012.

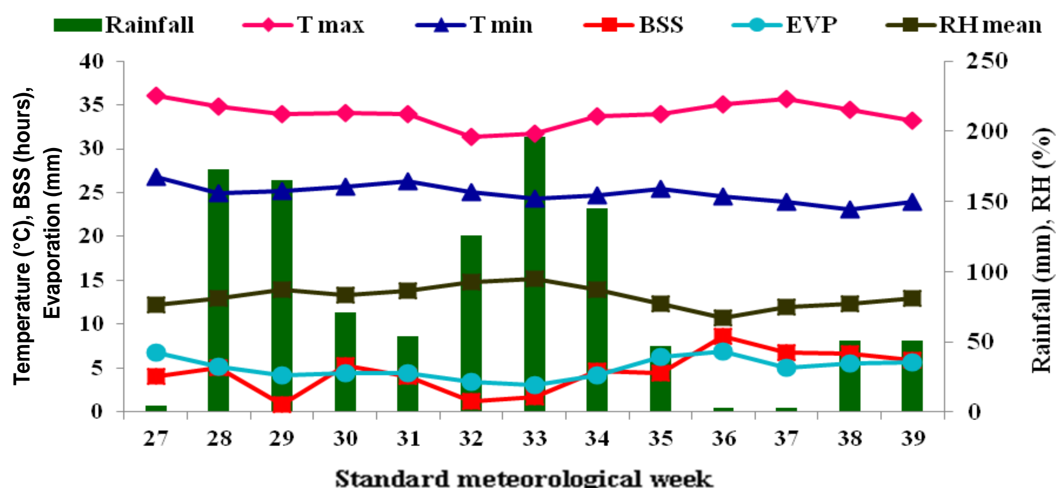

Figure 2. Weather parameters during *kharif* 2013.

having 61.48% sand, 12.66% silt and 25.86% clay contents. Chemical analysis of nutrients for the experimental soil were carried out by using the Modified Kjeldahl method (Jackson, 1958) for determination of available N, Olsen's method for available P (Olsen et al., 1954), Flame photometer method (Jackson, 1958) for available K, DTPA extraction method (Lindsay and Norvell, 1978) for available Zn and Walkley and Black's rapid titration method (Jackson, 1973) for organic carbon. The experimental soil was low in available nitrogen (135.4 kg N/ha), medium in available phosphorus (12.8 kg P/ha), potassium (178.8 kg K/ha) and Zn (0.63 mg/kg of soil) and low in organic carbon content (0.40%). The pH of the soil was 7.7 and determined in soil water suspension in the ratio of 1:2.5 with glass electrode pH meter.

Experimental set-up and management

The experiment comprised of four treatments of moisture management (Flat Bed, Flat Bed with 2.5 t residue/ha, Flat Bed with 5.0 t residue/ha and Narrow Bed and Furrow with 2.5 t residue/ha) in main plot and three treatments of zinc fortification (control, 2.5 kg Zn/ha and 5.0 kg Zn/ha) in sub plot to pearl millet and in sub-sub plot to chickpea. The experiment was laid out in split plot design during *kharif* 2012 and in split-split plot design from subsequent season and replicated thrice. The pearl millet variety 'Pusa composite 443' was taken for experiment and planted at 50 x 15 cm spacing. Recommended dose of fertilizers (60 kg N, 40 kg P_2O_5 and 40 kg K_2O/ha) were applied through urea, single super phosphate (SSP) or diammonium phosphate (DAP) and muriate of potash (MOP). Half dose of nitrogen and full dose of phosphorus and potassium was applied as basal dose at the time of sowing and remaining half dose of nitrogen was as top dressing at 40 DAS. Chickpea residue was applied in main plots as per treatment just after sowing of crop during both the years. Zinc fortification treatments were applied as per treatment through zinc sulphate ($ZnSO_4.7H_2O$) containing 21% zinc and 10% S at the time of sowing as basal dose. The amount of sulphur was adjusted through SSP in all the plots. The crop was grown with recommended

package of practices. Need based application of pesticide was also followed to protect the crops from termites. The crop toke 81 and 77 days for completion of life cycle during 2012 and 2013, respectively.

Soil samples from surface depth (0-15 cm) and near plant roots were taken in small polythene bags from each plot by core sampler at 50% flowering stage. The soil samples were air-dried, ground and passed through 2 mm mesh-sieve, and analysed for microbial parameters *viz.*, microbial biomass carbon and dehydrogenase activity. Microbial biomass carbon was estimated by chloroform fumigation method (Nunan et al., 1998) and dehydrogenase activity was estimated as described by Casida et al. (1964). Five plants were selected randomly from each plot, tagged permanently and used for measurement of plant height. For dry matter accumulation, five plants from each plot were uprooted randomly from sample rows and after removal of root portion, the samples were first air dried for some days followed by drying in an electric oven at 65°C till constant weight. The weight was recorded and expressed as g/plant. The total number of tillers and number of earheads per metre row length were counted at harvest from three different spots from each plot and the average was worked out. Root samples were taken from the sample row at flowering stage 50 DAS. A root auger of 4.8 cm diameter and 10 cm height (core volume = 180.86 cm3) was used to take root samples up to 0-15 cm depth of soil profile. The root samples taken from each plot were thoroughly washed in running water to remove the dust particles. Then, root samples were put into polythene bag and used to measure root length and volume by scanning. Scanning and image analysis using RHIZO system was operated in a computer mounted with the scanner of RHIZO system. After taking root length and volume, root samples were put first and air dried for some days followed by drying in an electric oven at 65°C till constant weight. The weight was recorded and expressed as g/plant. Five earheads were randomly selected from each plot and the length of earhead was measured from the basal whorl of spikelet to the tip of earhead. The length of earhead was measured in centimetre and mean length was calculated. Same five earheads of pearl millet which were used to measure length were also used for recording grain weight. The weight of the thoroughly sun dried harvested produce from net area of each plot was recorded separately before threshing and expressed as biological yield in t/ha. After proper drying of harvested product, they were threshed separately. Grain yield from each net plot was recorded and computed as grain yield t/ha. The stover yield for each plot was worked out by subtracting grain yield from total biomass of each net plot and stover yield was expressed in t/ha. Statistical analysis of the data was carried out using standard analysis of variance (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Moisture management practices

Soil microbial parameters *viz.*, dehydrogenase activity and microbial biomass carbon were significantly influenced by moisture management practices (Table 1). Flat bed with 5.0 t/ha crop residue proved significantly superior over flat bed and flat bed with 2.5 t/ha cop residue in terms of dehydrogenase activity and it was found to be statistically similar with narrow bed and furrow with 2.5 t/ha crop residue. However, in terms of microbial biomass carbon flat bed with 2.5 t/ha cop residue proved significantly superior over all other moisture management practices. Addition of crop residue conserved soil moisture by reducing evaporation losses and also added organic carbon to the soil which results in the better aeration and microbial activity in the soil (Chakarabarti et al., 2000; Singh et al., 2009). Moisture management practices significantly influenced the plant height, dry matter accumulation and total number of tillers of pearl millet (Table 1). Flat bed with 5.0 t/ha crop residue recorded signi-ficantly higher plant height (271.0 and 273.3 cm) and total number of tillers per metre row length (20.0 and 20.1) during both years as compared to flat bed but it was found to be statistically at par with narrow bed and furrow with 2.5 t/ha crop residue and flat bed with 2.5 t/ha crop residue. However, dry matter accumulation per plant was significantly enhanced with flat bed with 5.0 t/ha crop residue by 35.9 and 12.7% in 2012 and 33.7 and 12.2% in 2013, respectively, over flat bed and flat bed with 2.5 t/ha cop residue. Under moisture stress conditions in search of moisture, the flat bed planted pearl millet recorded significantly higher value of rooting parameters (root length, root volume and root dry weight) as compared to crop residue applied treatments (Table 2). The improvement in growth parameters of pearl millet planted under residue applied moisture management practices might be due to the fact that residue cover helped to conserve soil moisture available through rainfall (Mulumba and Lal, 2008) and continuously provided t the needs of crops. Adequate availability of moisture to plants resulted in cell turgidity and eventually high meristematic activity, leading to more foliage development, greater photosynthetic activity and consequently higher growth and development. Moreover, applied residue as moisture management practice also enhanced the nutrient supply through decomposition of organic residue coupled with favorable moisture condition creating conducive environment for plant growth and development (Parihar et al., 2012; Singh et al., 2012; Dass et al., 2013).

Moisture management practices also had significant effect on yield attributes and yield of pearl millet (Tables 3 and 4). Flat bed with 5.0 t/ha crop residue, narrow bed and furrow with 2.5 t/ha crop residue and flat bed with 2.5 t/ha crop residue remained at par with each other and proved significantly better over flat bed planted pearl millet in terms of number of earheads per metre row length and length of earhead. Flat bed with 5.0 t/ha crop residue being at par with narrow bed and furrow with 2.5 t/ha crop residue produces significantly higher grain weight per head (19.05 and 20.12 g), which was higher by 25.2 and 9.8% in 2012 and 25.1 and 9.8% in 2013 over flat bed and flat bed with 2.5 t/ha crop residue, respectively. However, there was no significant difference observed in test weight during both years. The favourable improvements in yield attributes was due to the favourable effect of moisture management practices on growth parameters, leading to greater nutrient uptake, efficient partitioning of metabolites and adequate accumulation and translocation of photosynthates. Adequate supply of moisture in general is known to enhance the growth and dry matter production of crops directly and indirectly by increasing the availability and utilization

Table 1. Effect of moisture management and zinc fortification on soil microbial properties and growth parameters of pearl millet.

Treatment	Dehydrogenase activity (µg TPF/g soil/day)		Microbial biomass carbon (µg C/g soil)		Plant height (cm)		Dry matter accumulation (g/plant)		Total number of tillers per metre row length	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Moisture management										
Flat Bed	23.6	23.8	62.8	64.7	235.0	238.9	59.58	65.46	17.3	17.4
Flat Bed + 2.5 t residue/ha	30.2	31.6	84.8	90.7	255.6	259.8	71.87	78.04	19.1	19.3
Flat Bed + 5.0 t residue/ha	35.1	36.8	97.7	105.6	271.0	273.3	80.97	87.53	20.0	20.1
NBF + 2.5 t residue/ha	33.0	34.5	88.6	95.6	263.2	267.4	77.43	83.93	19.7	19.8
Sem±	0.42	0.42	1.46	1.42	5.0	4.8	2.33	2.39	0.46	0.35
CD (P=0.05)	1.46	1.44	5.06	4.90	17.3	16.7	8.07	8.28	1.60	1.23
Zinc fortification to pearl millet (kg/ha)										
0	24.8	25.8	79.2	84.3	245.7	250.0	66.82	72.62	18.0	18.0
2.5	31.4	32.6	84.2	89.9	259.0	262.2	73.76	80.08	19.3	19.4
5.0	35.3	36.7	87.0	93.3	263.9	267.4	76.81	83.52	19.8	20.0
SEm±	0.26	0.39	0.46	0.70	4.1	2.6	1.82	1.33	0.33	0.29
CD (P=0.05)	0.78	1.17	1.39	2.10	12.1	7.7	5.46	3.99	0.99	0.88
Zinc fortification to chickpea (kg/ha)										
0	-	28.4	-	86.7	-	254.9	-	75.59	-	18.5
2.5	-	32.2	-	89.6	-	261.1	-	79.34	-	19.3
5.0	-	34.4	-	91.1	-	263.5	-	81.29	-	19.6
SEm±	-	0.25	-	0.50	-	1.9	-	0.87	-	0.21
CD (P=0.05)	-	0.70	-	1.42	-	5.4	-	2.46	-	0.59

Table 2. Effect of moisture management and zinc fortification on root length, volume and dry weight of pearl millet.

Treatment	Root length per plant (cm)		Root volume per plant (cm^3)		Root dry weight per plant (g)	
	2012	2013	2012	2013	2012	2013
Moisture management						
Flat Bed	402.4	395.0	11.34	11.13	7.34	7.18
Flat Bed + 2.5 t residue/ha	352.4	348.0	10.10	9.96	6.52	6.42
Flat Bed + 5.0 t residue/ha	328.2	327.7	9.39	9.34	6.17	6.10
NBF + 2.5 t residue/ha	340.5	337.7	9.69	9.66	6.37	6.30
Sem±	11.70	10.12	0.30	0.24	0.22	0.15
CD (P=0.05)	40.49	35.01	1.03	0.82	0.76	0.52
Zinc fortification to pearl millet (kg/ha)						
0	330.7	322.2	9.41	9.18	6.06	5.96
2.5	362.8	360.6	10.29	10.21	6.70	6.62
5.0	374.1	373.5	10.69	10.67	7.03	6.92
SEm±	9.66	6.94	0.23	0.20	0.18	0.13
CD (P=0.05)	28.97	20.80	0.68	0.61	0.54	0.40
Zinc fortification to chickpea (kg/ha)						
0	-	337.1	-	9.62	-	6.24
2.5	-	355.9	-	10.11	-	6.56
5.0	-	363.4	-	10.34	-	6.70
SEm±	-	6.00	-	0.12	-	0.08
CD (P=0.05)	-	17.06	-	0.35	-	0.23

Table 3. Effect of moisture management and zinc fortification on yield attributes of pearl millet.

Treatment	Number of earheads per meter row length		Length of earhead (cm)		Grain weight per earhead (g)		1,000-grain weight (g)	
	2012	2013	2012	2013	2012	2013	2012	2013
Moisture management								
Flat Bed	11.8	12.1	25.6	26.7	15.21	16.08	7.76	7.87
Flat Bed + 2.5 t residue/ha	13.7	14.0	28.4	29.4	17.35	18.33	8.20	8.30
Flat Bed + 5.0 t residue/ha	14.6	15.0	29.4	30.6	19.05	20.12	8.41	8.54
NBF + 2.5 t residue/ha	14.3	14.7	29.1	30.1	18.34	19.38	8.28	8.40
Sem±	0.35	0.40	0.62	0.57	0.47	0.45	0.16	0.15
CD (P=0.05)	1.21	1.37	2.15	1.98	1.61	1.57	NS	NS
Zinc fortification to pearl millet (kg/ha)								
0	12.8	13.3	26.9	27.7	16.52	17.35	7.96	8.09
2.5	13.8	14.1	28.3	29.5	17.72	18.74	8.20	8.30
5.0	14.2	14.4	29.1	30.3	18.22	19.34	8.34	8.44
SEm±	0.22	0.18	0.39	0.41	0.32	0.33	0.11	0.10
CD (P=0.05)	0.65	0.53	1.18	1.23	0.96	1.00	NS	NS
Zinc fortification to chickpea (kg/ha)								
0	-	13.6	-	28.1	-	17.89	-	8.16
2.5	-	14.0	-	29.4	-	18.60	-	8.30
5.0	-	14.2	-	30.0	-	18.94	-	8.37
SEm±	-	0.12	-	0.32	-	0.21	-	0.07
CD (P=0.05)	-	0.35	-	0.91	-	0.59	-	NS

Table 4. Effect of moisture management and zinc fortification on yield and harvest index of pearl millet.

Treatment	Yield (t/ha)						Harvest index (%)	
	Grain		Stover		Biological			
	2012	2013	2012	2013	2012	2013	2012	2013
Moisture management								
Flat Bed	1.89	2.02	6.48	6.81	8.36	8.84	22.54	22.98
Flat Bed + 2.5 t residue/ha	2.25	2.42	7.44	7.83	9.69	10.25	23.25	23.63
Flat Bed + 5.0 t residue/ha	2.52	2.72	8.21	8.65	10.72	11.37	23.52	23.90
NBF + 2.5 t residue/ha	2.45	2.65	8.05	8.47	10.50	11.12	23.30	23.77
Sem±	0.06	0.05	0.20	0.19	0.23	0.22	0.50	0.296
CD (P=0.05)	0.19	0.17	0.68	0.65	0.80	0.77	NS	NS
Zinc fortification to pearl millet (kg/ha)								
0	2.05	2.22	7.04	7.44	9.09	9.66	22.54	23.11
2.5	2.33	2.51	7.68	8.07	10.01	10.58	23.28	23.64
5.0	2.45	2.62	7.91	8.32	10.36	10.94	23.65	23.96
SEm±	0.04	0.04	0.14	0.12	0.17	0.14	0.38	0.351
CD (P=0.05)	0.12	0.13	0.43	0.37	0.50	0.41	NS	NS
Zinc fortification to chickpea (kg/ha)								
0	-	2.31	-	7.60	-	9.91	-	23.28
2.5	-	2.49	-	8.01	-	10.50	-	23.63
5.0	-	2.57	-	8.22	-	10.78	-	23.79
SEm±	-	0.03	-	0.08	-	0.10	-	0.180
CD (P=0.05)	-	0.09	-	0.24	-	0.30	-	NS

of nutrients (Tetarwal et al., 2012).

Grain, stover and biological yield were significantly higher with flat bed with 5.0 t/ha crop residue over flat bed and flat bed with 2.5 t/ha crop residue but remained at par with narrow bed and furrow with 2.5 t/ha crop residue. Flat bed with 5.0 t/ha crop residue enhanced the grain yield of pearl millet by 33.3 and 12.0% in 2012 and 34.7 and 12.4% in 2013 over flat bed and flat bed 2.5 t/ha crop residue, respectively. Harvest index was increased linearly with moisture management practices but has no significant improvement. The increase in grain yield of pearl millet with flat bed with 5.0 t/ha crop residue might be due to the better availability of moisture and addition of organic matter. Rapid decomposition of organic residue helped in greater availability of nutrients, which led to increase in growth and yield attributes and finally the grain yield. High and well distributed rainfall in 2013 results in the better growth of crop due to adequate availability of moisture throughout the growing season and produced higher grain yield as compared to 2012. Similar findings were also reported by Kumar and Gautam (2004) and Parihar et al. (2012).

Zinc fortification to pearl millet

Zinc fortification treatments had significant effect on soil microbial properties *viz*., dehydrogenase activity and microbial biomass carbon as compared to the control (Table 1). Application of 5.0 kg Zn/ha reported significantly higher dehydrogenase activity (35.3 and 36.7µg TPF/g soil/day) and microbial biomass carbon (87.0 and 93.3 µg C/g soil) over control and 2.5 kg Zn/ha during both the years. More favourable condition results in higher soil microbial activities during the second year in comparison with first. Zinc is an important component of several enzymes especially dehydragenase and RNA polymerase which results in higher soil microbial activities. Growth parameters of pearl millet namely plant height, dry matter accumulation and total number of tillers were improved significantly due to application zinc. Application of 5.0 kg Zn/ha being at par with 2.5 kg Zn/ha significantly enhanced the plant height and dry matter accumulation by 7.0 and 15.0% in 2012 and 7.0 and 15.0% in 2013, respectively, over control. Root length, volume and dry matter were significantly higher under 5.0 kg Zn/ha as compared to control during both years of experiment (Table 2). The favourable influence of applied zinc on different growth parameters of pearl millet and chickpea is ascribed to its involvement in various metabolic activities, controlling auxin levels and nucleic acids (Marschner, 1995). Zinc is also an essential component of enzymes responsible for assimilation of nitrogen which helps in chlorophyll formation and plays an important role in nitrogen metabolism contributing towards increase in growth and development of plant (Jakhar et al., 2006; Badiyala and Chopra, 2011).

Yield attributes (number of earheads per metre row length, length of earhead and grain weight per earhead) and yield (grain, stover and biological) of pearl millet were enhanced significantly with zinc fortification treatments (Tables 3 and 4). Application of 5.0 kg Zn/ha to pearl millet significantly increased the number of earheads per metre row length by 10.9 and 8.3%, length of earheads by 2.2 and 2.6 cm and grain weight per earhead by 10.3 and 11.5%, respectively during 2012 and 2013, over control. Zinc fortification treatments failed to have any significant effect on test weight of pearl millet during both years of study. As already discussed in preceding paragraph, zinc plays an important role in nitrogen metabolism and formation of chlorophyll and carbohydrate, which leads to maintaining photosynthetic activity for longer period and finally results in increasing the yield attributes of the crop (Mehta et al., 2008; Ram Pratap et al., 2008).

Results further revealed that increasing levels of zinc linearly increased the grain, stover and biological yield of pearl millet but the response was significant only up to 2.5 kg Zn/ha. Application of 5.0 kg Zn/ha recorded significantly higher grain (2.45 and 2.62 t/ha), stover (7.91 and 8.32 t/ha) and biological yield (10.36 and 10.94 t/ha) and it enhanced the grain yield by 19.5 and 18.0%, stover yield by 12.4 and 11.8% and biological yield by 14.0 and 13.3%, respectively, in 2012 and 2013 over control. The effect of different treatments of zinc fortification remained non-significance on harvest index during both years of experiment. The cumulative beneficial effect of growth and yield attributing characters was finally reflected in grain yield of pearl millet. These results are in close conformity with that of Mehta et al. (2008) and Ram Pratap et al. (2008).

Residual effect of zinc fortification

The residual effect of preceding zinc fortification treatments applied to chickpea was examined during second year of study and results were found to be significant on soil microbial properties. The residual effect of 5.0 kg Zn/ha recorded significantly higher dehydrogenase activity and microbial biomass carbon as compared to control and 2.5 kg Zn/ha. Growth parameters of pearl millet namely plant height, dry matter accumulation, total number of tillers and rooting characteristics were also influenced significantly with zinc treatments applied to preceding chickpea crop.

Application of 5.0 kg Zn/ha to chickpea crop significantly enhanced the plant height, dry matter accumulation per plant, total number of tillers per metre row length, root length, root volume and root dry weight by 3.4, 7.5, 5.9, 7.8, 7.5 and 7.4%, respectively, over control and was found to be statistically similar with 2.5 kg Zn/ha.

Yield attributes and yield of pearl millet were also influenced significantly by residual effect of zinc fortification.

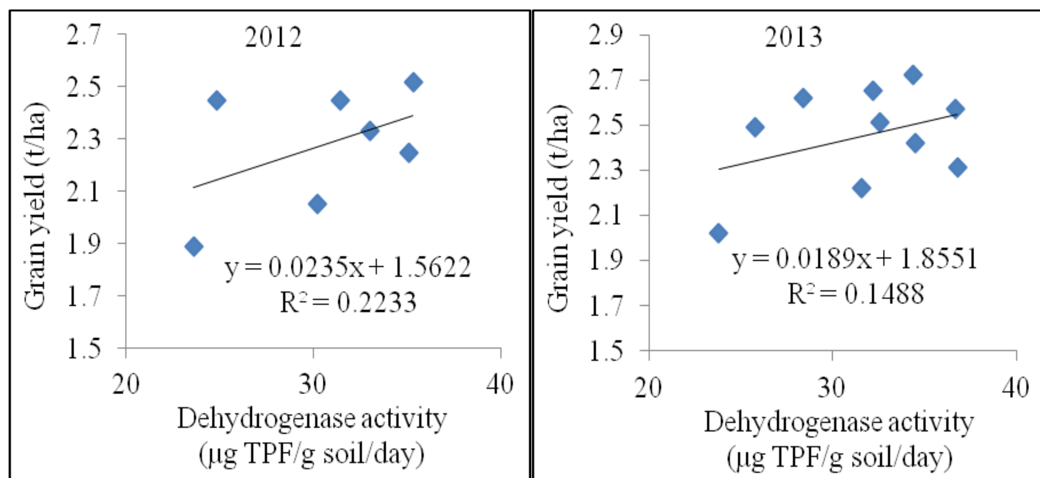

Figure 3. Correlation between pearl millet yield (y-axis) and dehydrogenase activity(x-axis) under moisture management and zinc fortification.

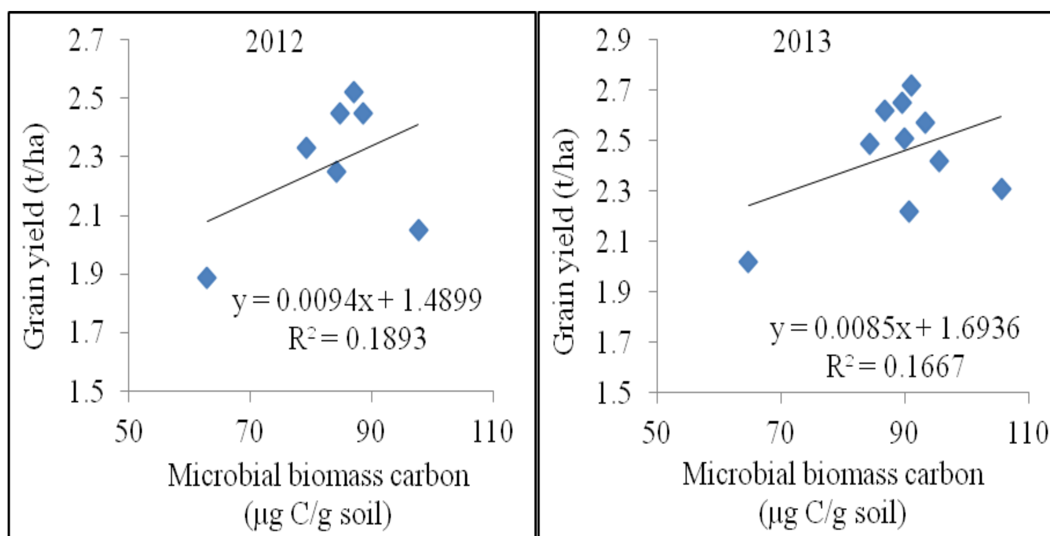

Figure 4. Correlation between pearl millet yield (y-axis) and microbial biomass carbon (x-axis) under moisture management and zinc fortification.

Wherein, application of 5.0 kg Zn/ha remained at par with 2.5 kg Zn/ha and produced significantly higher number of earheads per metre row length (14.2), length of earhead (30.0 cm) and grain weight per earhead (18.94 g) over control. Residual effect of 5.0 kg Zn/ha recorded significantly better grain (2.57 t/ha), stover (8.22 t/ha) and biological yield (10.78 t/ha), which were 11.3, 8.2 and 8.8% higher than the control. The application of zinc to chickpea crop improved the soil status of DTPA extractable zinc in the soil and increased supply and uptake by the succeeding pearl millet crop resulting in improvement in growth parameters and yield attributes. Thus, positive impact on these characters led to significant improvement in yield of succeeding pearl millet. Jain and Dahama (2005) and Sammauria and Yadav (2008) has also reported similar results with regard to residual effect of zinc.

Correlation studies

Regression analysis between yield and soil microbial properties of pearl millet showed positive but non-significant correlation (Figures 3 and 4). Whereas, regression analysis between yield and major yield attributes of pearl millet showed highly significant and positive correlation of pearl millet yield with number of earheads per metre row length, length of earhead and grain weight per earhead during both years of study (Figures 5 to 7).

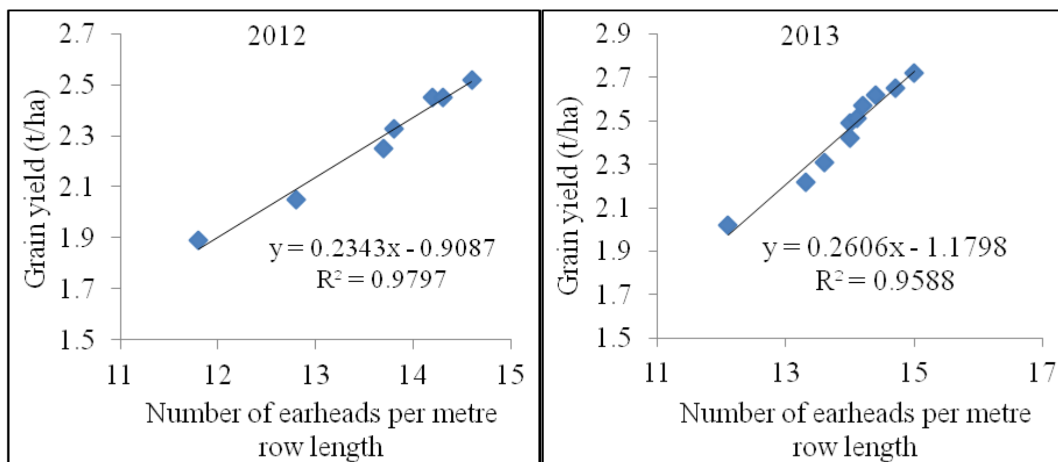

Figure 5. Correlation between pearl millet yield (y-axis) and number of earheads per metre row length (x-axis) under moisture management and zinc fortification.

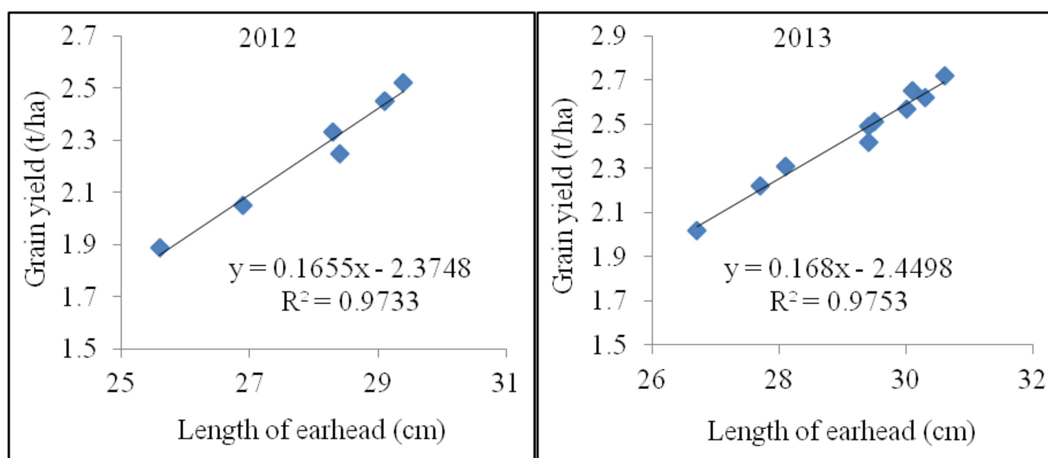

Figure 6. Correlation between pearl millet yield (y-axis) and length of earhead (x-axis) under moisture management and zinc fortification.

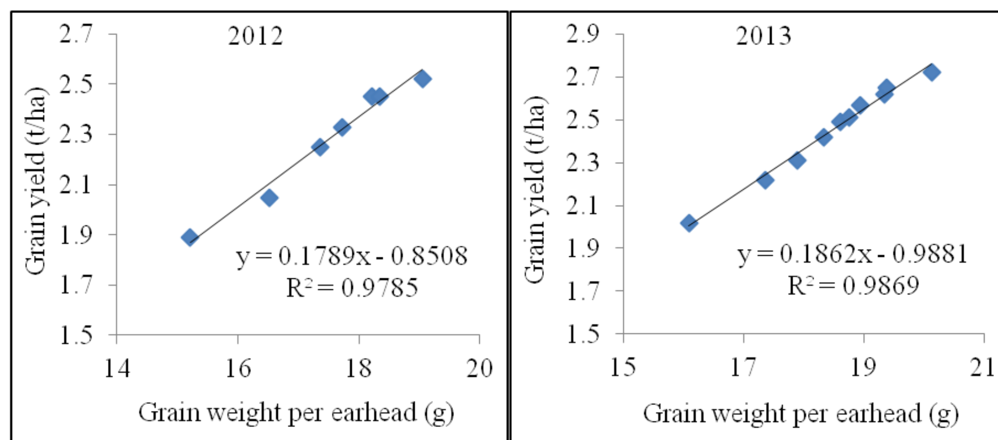

Figure 7. Correlation between pearl millet yield (y-axis) and grain weight per earhead (x-axis) under moisture management and zinc fortification.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Resistance pattern of uropathogenic bacteria in males with lower urinary tract obstruction in Kumasi, Ghana

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We described the antimicrobial susceptibility pattern of pathogenic bacteria causing urinary tract infections among males with lower urinary tract obstruction (LUTO) at the Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana. Between January and December 2009, a cross sectional hospital based study was conducted for 102 subjects. Patients presenting with symptoms of LUTO and who were on short admission (at most 48 h) at the accident and emergency unit of KATH were identified as potential study subjects. All the patients presented with acute retention of urine at the emergency unit of KATH and urethral catheterization was attempted to relieve them of the retention under sterile conditions. Urine specimen were collected into sterile urine containers immediately after the catheterization (irrespective of type) and sent to the microbiology laboratory at KATH for routine examination, culture and antimicrobial sensitivity testing. The mean age for males with LUTO was 62 years (Range: 2-93 years). The overall prevalence of pathogens was highest among the >70 years age group (40.2%). Whilst *Klebsiella* spp. (43.5%) and *Pseudomonas* spp. (50.0%) were the most predominant species within the >70 years age group, *Escherichia coli* (45.3%) and *Staphylococcus aureus* (57.1%) were the most predominant species within the <50 and 50-70 age groups. With respect to antibiotic sensitivity, *Salmonella* spp. showed the highest susceptibility rate of 15.8% whilst *Klebsiella* spp. was the least susceptible (9.2%) to antimicrobial agents used. The highest and least multi-drug susceptible bacterial isolates were *E. coli* and *Citrobacter freundii* respectively. This study's results indicate that antibiotics commonly used in UTIs are still effective, but species distribution and their susceptibility to antibiotics are changing. The caution therefore is that antimicrobial susceptibility testing needs to be done to help select the appropriate antibiotic for effective treatment of diseases.

Key words: Antibiotic, asymptomatic, bacteriuria, acute, cystitis, urosepsis, catheter, antimicrobial susceptibility.

INTRODUCTION

Antimicrobial resistance (AMR) is resistance of a micro-organism to an antimicrobial compound. Resistant organisms including bacteria, fungi, viruses and some parasites are able to withstand attack by antimicrobial compounds,

such as antibiotics, antifungals, antivirals and antimalarials, so that standard treatments become ineffective and infections persist increasing risk of spread to others. The evolution of resistant strains is a natural phenomenon that happens when microorganisms are exposed to antimicrobial drugs, and resistant traits can be exchanged between certain types of bacteria. The misuse of antimicrobial medicines and poor infection control practices accelerates this natural phenomenon. Many of the drug treatment breakthroughs of the last century could be lost through the spread of antimicrobial resistance. As a result, many infectious diseases may one day become uncontrollable and could rapidly spread throughout the world (Bhullar et al., 2012; WHO, 2012).

Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death. The death rate for patients with serious infections caused by resistant pathogens is about twice that in patients with infections caused by non-resistant bacteria (WHO, 2012). New resistance mechanisms, such as enzymes produced by the bacteria that destroy last generation antibiotics, have emerged among several Gram-negative bacilli and have rapidly spread among many countries. This can render ineffective powerful antibiotics, which are often the last defense against multi-resistant strains of bacteria. This new resistant mechanism is encountered in ordinary human pathogens (for example, *E. coli*) that cause common infections such as urinary tract infection (Bhullar et al., 2012; Kuo, 1999).

Lower urinary tract obstruction (LUTO) and urinary tract infections (UTIs), bladder cancer, incontinence, etc. are more common in elderly patients. UTI is currently one of the most common type of bacterial infection in humans. It involves any part of the urinary system, including urethra, bladder, ureters and kidney. It has been reported that about 150 million people around the globe are diagnosed each year with UTI and this cost in excess of six billion dollars (Gupta et al., 2001). UTI is mostly caused by bacteria though viruses and fungi are rare aetiologic agents (Griebling, 2007). Gram negative bacteria such as *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Proteus* spp., *Neisseria gonorrhoea* etc are usually the predominant causative agents of UTI (Al Sweih et al., 2008). However, other non-Gram negative bacteria such as *Staphylococcus* spp., *Streptococcus* spp., *Chlamydia trachomatis,* etc. have also been identified as causative agents (Griebling, 2007). The emergence of antibiotic resistance in the management of UTI is a serious public health concern, particularly in the developing world where apart from high level of poverty, ignorance and poor hygienic practices, there is also high prevalence of fake and spurious drugs of questionable quality in circulation (Abubakar, 2009).

This study was conducted to determine the etiological agents of UTI in men presenting with LUTO at KATH and their antimicrobial susceptibility and resistance patterns.

MATERIALS AND METHODS

Subjects

This was a cross-sectional hospital based study conducted for 102 subjects between January and December 2009. Patients with symptoms of LUTO or who had acute urine retention and were on short admission (up to 48 hours) at the Accident and Emergency Unit (AEU) of KATH were identified as potential study subjects. Prior to the study, ethical approval was sought from the Kwame Nkrumah University of Science and Technology (KNUST) School of Medical Sciences (SMS)/KATH Committee on Human Research Publications and Ethics (CHRPE). The participation of the respondents was voluntary and informed consent was obtained from each participant.

Inclusion and exclusion criteria

All patients admitted to the AEU with acute urine retention received urethral catheterization to relieve them of the retention under sterile conditions. Suprapubic Cystostomy (SPC) was done for a number of patients whose initial urethral catheterization failed. Urine specimen for routine examination, culture and sensitivity tests was collected into sterile urine containers immediately after the catheterization (irrespective of type). Subjects whose urinalysis revealed pyuria and positive urine culture and who had voluntarily agreed to participate in the study were enrolled. Subjects whose urine culture had multiple bacteria growth were considered to be contaminants and were excluded from the study.

Specimen collection

The importance of clean catch urine specimen was explained to consented study subjects. 10 ml of urine was collected from study subjects for routine examination, culture and sensitivity tests. The urine specimens were collected from the distal end of the catheter immediately after the catheterization (irrespective of type) into sterile urine containers.

Specimen processing

Collected urine specimens were transported immediately (within 30 min after collection) to the microbiology laboratory of KATH for routine examination, culture and sensitivity testing. Samples were processed within 30 min of arrival into the laboratory. Specimens were initially processed appropriately for culture and sensitivity before the routine examinations were done. Sample processing was done by following standard procedures and processes within the laboratory.

Urine culture

The urine specimens were gently mixed to avoid foaming. A calibrated loop (1 µl) full of the unspun urine was inoculated onto

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Table 1. Prevalence of the isolates among study population stratified by age.

Bacteria	Total number isolated (n=102)	Patient age groups (years)		
		< 50 (n = 26)	50 -70 (n = 38)	> 70 (n = 41)
All pathogens	102 (100.0%)	27 (26.5%)	36 (35.3%)	39 (38.2%)
E. coli	53 (52.0%)	8 (15.1%)	24 (45.3%)	21 (39.6%)
S. aureus	14 (13.7%)	8 (57.1%)	4 (28.6%)	2 (14.3%)
Klebsiella spp.	23 (22.5%)	7 (30.4%)	6 (26.1%)	10 (43.5%)
Pseudomonas spp.	8 (7.8%)	2 (25.0%)	2 (25.0%)	4 (50.0%)
Salmonella spp.	1 (1.0%)	1 (100.0%)	-----	-----
Proteus spp.	2 (2.0%)	-----	-----	2 (100.0%)
C. freundii	1(1.0%)	1 (100.0%)	-----	-----

Data are presented as absolute count and percentages. ------: 0(0.0%)

a CLED agar (Difco Laboratories GmbH, Augsburg, Germany) and streaked out for single colonies. These plates were then incubated under aerobic conditions (35-37°C) for 16-24 h. The plates were examined after the incubation period for significant growth. Biochemical tests such as catalase tests, coagulase tests and oxidase tests were performed to help identify the bacterial pathogens using Becton Dickinson's Phoenix Spec Nephelometer and BBL Crystal semi-auto reader for bacterial pathogens at the Microbiology Laboratory of Kumasi Centre for Collaborative Research in Tropical Medicine (Malaria Research Centre, Agogo, Asante Akim North). All the microbiology procedures were quality controlled using American Type Culture Collection (ATCC) strains (Cheesbrough, 2009).

Antibiotic susceptibility testing

The antibacterial susceptibility testing of the pathogenic isolates was done using the Kirby-Bauer disk diffusion method (Bauer et al., 1966) following the definition of the Clinical and Laboratory Standards Institute using antibiotics containing discs from Oxoid. Mueller-Hinton agar (Difco Laboratories GmbH, Augsburg, Germany) was prepared following the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2006).

The sensitivity test was performed based on the guidelines provided by CLSI (Clinical and Laboratory Standards Institute, 2006). The mean of triplicate results was taken as the zone diameter. The antibiotics discs and the concentration used were ampicillin 25 µg, naladixic acid 30 µg, cefuroxime 25 µg, ciprofloxacin 25 µg, chloramphenicol 30 µg, ofloxacin 5 µg, erythromycin 5 µg, gentamicin 25 µg, amikacin 30 µg, nitrofurantoin 20 µg, ceftriazone 25 µg, streptomycin 25 µg, tetracycline 25 µg, cefotaxime 25 µg, imipenem 25 µg, meropenem 25 µg, flucloxacillin 25 µg, augmentin 25 µg and cloxacillin 25 µg. Isolates were classified as either resistant or intermediate sensitive or sensitive based on the CLSI definition (Clinical and Laboratory Standards Institute, 2006) and in accordance with WHO requirements (Onanuga et al., 2005). Resistant and intermediate isolates were grouped together for analysis in this study. An isolate was considered multi-drug resistant if it was resistant to at least three of the antibiotics tested (Santos et al., 2008). Quality control of the susceptibility discs were performed using reference strains of *Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853)*, *Staphylococcus aureus (ATCC 25923)* and *E. faecalis (ATCC 29212)* of known sensitivity.

Urine microscopy

The remaining urine specimens were transferred into centrifuge tubes and spun at 4000 relative centrifugal force for 5 min. Each supernatant was decanted and the sediment remixed by tapping the bottom of the tube. A drop of well mixed sediment was transferred unto a microscopic slide, cover-slipped and examined microscopically as described earlier. The pellet was screened microscopically for epithelia, bacteria, *Trichomonas vaginalis*, *Schistosoma* spp. etc (Cheesbrough, 2009).

Bio-data analysis

Data generated in this study were entered on Microsoft Excel, cleaned and exported into Graph Pad Prism version 5.00 (Graph Pad software, San Diego California USA) for windows for the statistical analysis.

RESULTS

A total of 102 suspected UTI patients participated in the study. The mean age of the study patients was 62 years (Range: 2-93 years). The overall prevalence of pathogens was highest among the >70 years age group (40.2%). Whilst *Klebsiella* spp. (43.5%) and *Pseudomonas* spp. (50.0%) were the most predominant isolates within the >70 years age group, *E. coli* (45.3%) and *S. aureus* (57.1%) were the most predominant isolates within the <50 and 50-70 age groups (Table 1).

Susceptibility results of the isolates to the various antibiotics are presented in Table 2. Whilst *Salmonella* spp. showed the highest susceptibility rate of 15.8%, *Klebsiella* spp. was the least susceptible (9.2%) to antimicrobial agents used. Compared to the other isolates, *E. coli* was found to be susceptible to most of the antibiotics with about half of it susceptible to nitrofurantoin. With the exception of *Citrobacter freundii* which was completely resistant to all the antibiotics except imipenem and meropenem, at least one of all other isolates was susceptible to nitrofurantoin. The only isolated *Salmonella* spp. was susceptible to only nitrofurantoin, gentamycin and ciprofloxacin (Table 2).

All the organisms except *Citrobacter freundii* showed a certain degree of susceptibility to nitrofurantoin (Table 2). The Gram-negative bacteria constituted the largest group (6 out of 7) with a prevalence of 85.70%, while Gram-positive

Table 2. Susceptibility of the isolates to routinely prescribed antibiotics.

Antibiotic	Susceptibility of the isolates to antibiotics (%)						
	EC (n=53)	**CF (n=1)**	**KL (n=23)**	**PR (n=2)**	**PS (n=8)**	**SAL (n=1)**	**SA (n=14)**
NIT	31(58.5)	-----	13(56.5)	1(50.0)	2(25.0)	1(100.0)	1 (7.1)
GEN	16(30.2)	-----	6(26.1)	-----	2(25.0)	1(100.0)	10 (71.4)
CEF	16(30.2)	-----	6(26.1)	1(50.0)	-----	-----	5 (35.7)
CIP	7(13.2)	-----	-----	1(50.0)	2(25.0)	1(100.0)	3 (21.4)
AMI	7(13.2)	-----	6(26.1)	-----	4(50.0)	-----	-----
ERY	-----	-----	-----	-----	-----	-----	4(28.6)
CTR	5(9.4)	-----	3(13.0)	1(50.0)	-----	-----	-----
CFOT	4(7.5)	-----	3(13.0)	2(100.0)	1(12.5)	-----	-----
SEPT	1(1.9)	-----	-----	-----	2(25.0)	-----	-----
IMI	-----	1 (100.0)	-----	-----	1(12.5)	-----	-----
NALD	6(11.3)	-----	-----	-----	-----	-----	-----
CEFTA	1(1.9)	-----	-----	-----	-----	-----	-----
FLUC	-----	-----	-----	-----	-----	-----	3(21.4)
OFL	-----	-----	-----	-----	-----	-----	1(7.1)
AUG	1(1.9)	-----	-----	-----	-----	-----	-----
MERO	-----	1 (100.0)	1(4.3)	-----	-----	-----	-----
AMP	-----	-----	-----	-----	-----	-----	1(7.1)
CLOX	-----	-----	-----	-----	-----	-----	12(85.7)
CHL	1(1.9)	-----	-----	-----	-----	-----	-----
SM	9.5%	10.5%	8.7%	15.7%	10.5%	15.8%	15.0%

Data are presented as number (percentage of isolates per the total in each category). EC: *E. coli*, CF: *C .freundii*. KL: *Klebsiella* spp. PR: *Proteus* spp. PS: *Pseudomonas* spp. SAL: *Salmonella* spp. SA: *S. aureus*. NIT: nitrofurantoin. GEN: gentamicin. CEF: cefuroxime. CIP: ciprofloxacin. AMI: amikacin. ERY: erythromycin. CTR: ceftriaxone. CFOT: cefotaxime. SEPT: streptomycin. IMI: imipenem. MERO: meropenem. NALD: nalidixic acid. FLUC: flucloxacillin. AUG: augmentin. MER: meropenem. AMP: ampicillin. CLOX: cloxacillin. CHL: chloramphenicol. SM: Susceptibility mean. -----:0.0% susceptibility.

Table 3. Degree of susceptibility and resistance to the various antibiotics.

Bacteria	Antibiotics (n = 19)	
	Proportion of antibiotics to which bacteria were sensitive	**Proportion of antibiotics to which bacteria were resistant**
E. coli	12 (63.2%)	7 (36.8%)
C. freundii	2 (10.5%)	17 (89.5%)
Klebsiella spp.	7 (36.8%)	12 (63.2%)
Proteus spp.	5 (26.3%)	14 (73.7%)
Pseudomonas spp.	7 (36.8%)	12 (63.2%)
Salmonella spp.	3 (15.8%)	16 (84.2%)
Staphylococcus aureus	9 (47.4%)	10 (52.6%)

Data are presented as absolute count and percentages.

bacteria constituted 14.30% of the total isolates. The proportions of the isolates showing multidrug resistance are shown in Table 3. The highest and least multi-drug susceptible bacterial isolates were *E. coli* and *C. freundii* respectively. A reverse trend was however observed for those two organisms in terms of multi-drug resistance. None of the isolates was sensitive to all the antibiotics tested and none, except *E. coli* was resistant to less than 10 of the 19 different antibiotics tested (Table 3).

DISCUSSION

Globally, urine culture has been identified as the gold standard for infection assessment whereas the susceptibility

testing also helps in antibiotic selection for therapeutic guidance. The various bacteria isolated from the urine samples were *E. coli* (52.0%), S*. aureus* (13.7%), *Klebsiella* spp. (22.5%), *Pseudomonas* spp. (7.8%), *Salmonella* spp. (1.0%), *Proteus* spp. (2.0%) and *C. freundii* (1.0%). These isolates represent clinically significant pathogens which are most often isolated from urine cultures. The significant bacteriuria identified in the urine samples of our study patients depicts a good clinical correlation between clinical and microbiological diagnosis as reported in western Nepal (Das et al., 2006).

The bacterial isolates in our study were mostly the Gram negative bacteria (85.70%) whereas the Gram positive bacteria accounted for 14.3%. This finding is consistent with a similar study conducted by Bahadin and colleagues (2011). This study also reported that the uropathogenic bacteria isolated from urine cultures were mainly the Gram negative type due to a number of properties that enables them to attach and invade urothelium as compared to the Gram positive bacteria (Bahadin et al., 2011).

E. coli (52.0%), the most common isolate was sensitive to 12 (63.2%) antibiotics but resistance to 7 (36.8%) antibiotics, largely the most common microbial agent (Table 3). *C. freundii* was sensitive to only two antibiotics (imipenem and meropenem). The highest degree of antimicrobial resistance was identified with *C. freundii* (89.5%) followed by *Salmonella* spp. (84.2%), *Proteus* spp. (73.7%), *Klebsiella* spp. and *Pseudomonas* spp. (63.2% each), *S. aureus* (52.6%) and *E. coli* (36.8%).

The high rate of resistance to the penicillin based antibiotics (such as ampicillin, amoxicillin, flucloxacillin, oxacillin, cloxacillin, etc.), tetracycline, aminoglycosides (such as streptomycin, gentamicin, etc.), etc. observed in this study may reflect the fact that these are the most commonly prescribed antibiotics at the hospital and also the most easily available in the community without prescription. The degree of resistance however reduced among quinolones (ciprofloxacin, nalidixic acid) and cephalosporins (including cefuroxime, ceftriaxone, cefotaxime, etc.). In their study of resistance to antimicrobial drugs in Ghana using bacterial isolates from a number of clinical specimens including urine, Newman and her colleagues reported similar findings from a study conducted in Ghana (Newman et al., 2011). They reported high resistance for ampicillin, chloramphenicol and cotrimoxazole whereas multi-drug resistance was observed for a combination of ampicillin, tetracycline, chloramphenicol and cotrimoxazole. Navaneeth and colleagues reported a similar resistant rate (80.0%) for *Proteus* spp. to a number of common antibiotics in India (Navaneeth et al., 2002). Resistance to antimicrobial agents has been noted since their first use and has gradually become a global challenge. A study done pointed out that high antimicrobial resistance rates in tertiary hospitals especially where both inpatients and outpatients are involved, could be due to some of those patients having complicated UTIs or having failed in previous UTI treatment (Mazzulli, 2002).

Conclusion

Overall susceptibility testing demonstrated a significantly reduced usefulness of common antibiotics and further strengthens the need for a re-evaluation of common antibiotics used in the therapeutic management of patients with urinary tract infection. This study's results do indicate that antibiotics commonly used in UTIs are still effective, but species distribution and their susceptibility to antibiotics are rapidly changing. The important caution therefore is that antimicrobial susceptibility testing may have to be done in order to help select the appropriate antibiotic for effective treatment of diseases.

Conflict of Interests

The authors have not declared any conflict of interests.

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Comparative studies on five culture media for bacterial isolation

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The research made comparative studies on five media for bacterial isolation. This study aimed at capturing important comparative data in the various types of media for growth efficiency and specific bacteria identification in clinical microbiology. The sources of the samples were urine, nasal swabs and stool. The totality of 15 samples was plated monthly and 120 samples were studied during an eight month period. The mean bacterial load from the cultures grown over the period from each source was calculated and used for comparative growth efficacy. Dominant colonies were characterized and identified based on morphological features and biochemical tests. A 0.1 ml of 10^{-3} of each bacteria isolate was evaluated for growth potential in triplicate on three different special purpose media. The mean bacteria load from the triplicate cultures was calculated. Salmonella-Shigella agar (SSA); a selective medium had the highest number of bacterial colonies of 2.98 x 10^5 CFU/ ml followed by the enrichment medium; blood agar that had 2.96 x 10^5 CFU/ ml and MacConkey agar (MCA) with 2.93 x 10^5 CFU/ ml. Biochemical identification and characterization of four dominant isolates confirmed the presence of *Staphylococcus aureus, Escherichia coli, Salmonella* and *Shigella.* Growth potential of each medium on the bacterial isolates showed that MacConkey agar recorded the highest growth potential of 8.9 x 10^5 CFU/ ml for *E. coli* followed by Blood Agar that gave 8.8 x 10^5 CFU/ml for *Shigella.* The third highest growth potential of 8.6 x 10^5 CFU/ ml was recorded in nutrient agar against *S. aureus.* Statistically, there exists a significant difference among the mean of the five media in their support for bacteria growth at α =0.05.

Key words: Bacterial isolation, isolates, *Escherichia coli*, *Shigella*, Blood agar.

INTRODUCTION

Different types of bacteria that cannot be covered by a single growth medium are found in one sample; therefore, it is important to compare the growth efficacy of routinely used media in clinical microbiology. Some experts in clinical microbiology consider the microbial density to be critical in predicting wound healing and infection while others consider the types of microorganisms to be of greater importance. Infections in clinical microbiology are frequently polymicrobial involving numerous microorganisms that are potentially pathogenic (Bowler, 1998; Bowler and Davies, 1999; Summanen et al., 1995). There has been a debate about the sampling

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technique required to provide the most meaningful data in polymicrobial infection (Bowler et al., 2001). Thus, concern among health care practitioners regarding rapid specific bacteria identification and growth efficiency in clinical microbiology is justifiable. Regarding the role of the microbiology laboratory, consideration must be given to meaningful comparative data in the various types of media for specific bacteria identification in clinical microbiology. Hence, this study aims to capture important comparative data in five types of media for growth efficiency and specific bacteria identification in clinical microbiology.

Dependability on media for isolation of specific bacteria is an important problem for all bacteriology laboratories. Individual enrichment and plating media have been investigated in numerous studies; Orji et al. (2007) reported a significant increase in bacterial isolation when solid media culture was pre-enriched than when the former was used alone. Dunn and Martin (1971) reported that shigellae were best isolated by direct inoculation, whereas salmonellae were isolated in greater numbers after tetrathionate (without Brilliant Green) enrichment with subsequent culturing on the plating medium. Furthermore, Cassar and Cuschieri (2003) studied "Comparison of *Salmonella* Chromogenic Medium (SCM) with desoxycholate citrate lactose sucrose agar (DCLS)". They reported that the sensitivity of SCM was significantly higher after enrichment. In addition, the specificity of SCM was also significantly higher than that of DCLS agar both before and after enrichment. Neil et al. (2014) carried out "Comparison of Blu-ray Disc (BD) MAX Enteric Bacterial Panel (EBP) to Routine Culture Methods for Detection of Campylobacter, Enterohemorrhagic *Escherichia coli* (O157), Salmonella, and Shigella Isolates in Preserved Stool Specimens". The study found that EBP demonstrated superior sensitivity and reliably detected Salmonella, enterohemorrhagic Escherichia coli (EHEC O157), Shigella, and Campylobacter at concentrations 1- to 2-$\log_{10}$ lower than those needed for culture detection. Alo et al. (2013) carried out "a comparative analysis between solid media and liquid media supplementation". The study concluded that the use of broth media to supplement solid media increased the sensitivity of semen culture and higher bacterial isolates were recovered.

Enrichment methods were reported to produce twice the number of pathogens as direct streaking in a study comparing xylose lysine deoxycholate agar, Hektoen enteric agar, Salmonella agar and Eosin methylene blue agar with stool specimes carried out by Taylor and Schelhart (1971)

Various growth media such as blood agar, and MacConkey agar is used for the isolation of gram-negative rods. It also inhibits the growth of gram positive cocci. Blood agar is used to detect the haemolytic streptococci. Instead of using the above mentioned media, some laboratories use single non-inhibitory medium such as cystine lactose electrolyte deficient medium (CLED). Ramzan et al. (2004) carried out comparative study of various growth media in isolation of urinary tract pathogens in which they reported that since different types of organisms are responsible for urinary tract infection, the whole range of pathogens cannot be covered by a single growth medium, therefore, they used blood agar, MacConkey agar and cystine lactose electrolyte deficient medium (CLED). Manipulation of different media and methods for cost-effective characterization of *E. coli* strains collected from different habitats (Arshad et al., 2006) can be done effectively by membrane filtration utilizing three types of selective media and differential agar media (MacConkey, Eosin metylene blue and endo agar) without importing expensive diagnostic kits. The main objective of this study was to provide important comparative information regarding how to choose an appropriate medium for growth of clinical bacterial isolates. This study aims to capture important comparative data in five types of media for growth efficiency vis-a-vis growth potential of each medium in the isolation of *E. coli, S. aureus, Salmonella* and *Shigella.*

MATERIALS AND METHODS

Source of microorganisms

Bacterial populations used in the study were collected from the Anambra State University Medical Centre Laboratory. Samples of urine, nasal swab and stool were collected at random from routine samples submitted for analysis in the Laboratory during the eight month period of the study starting from April, 2011- November, 2011.

Sample processing and Isolation procedure:

Serial dilutions, ratio 1:10 were prepared for each sample. One gram of stool sample was each time suspended in 10 ml of sterile phosphate buffer (pH 7.2) before it was used for serial dilution. Each month, samples were collected from the three sources and inoculated in duplicates on five separate media with 0.1 ml of 10^{-3} dilution. The totality of 15 samples was plated monthly and 120 samples were studied during the period. The media used were all purpose [Nutrient agar (NA)], selective; MacConkey agar (MCA), Mannitol-salt agar (MSA) and Salmonella-Shigella agar (SSA) and Enrichment medium was Blood agar (BA). The plates were incubated for 24 h at 37°C. The mean bacterial load from the cultures made over eight month period from each source was calculated. The media were prepared according to manufacturers' instructions, sterilized and poured onto sterilized Petri dishes.

Evaluation of growth potential of all purpose, selective and enrichment media

Dominant colonies were obtained and used for development of pure cultures which were characterized and identified based on morphological features and biochemical tests (Manaal et al., 2011). Stock cultures of the pure isolates were stored. Subsequently, sub-culturing and reactivation in broth cultures were carried out. Ratios

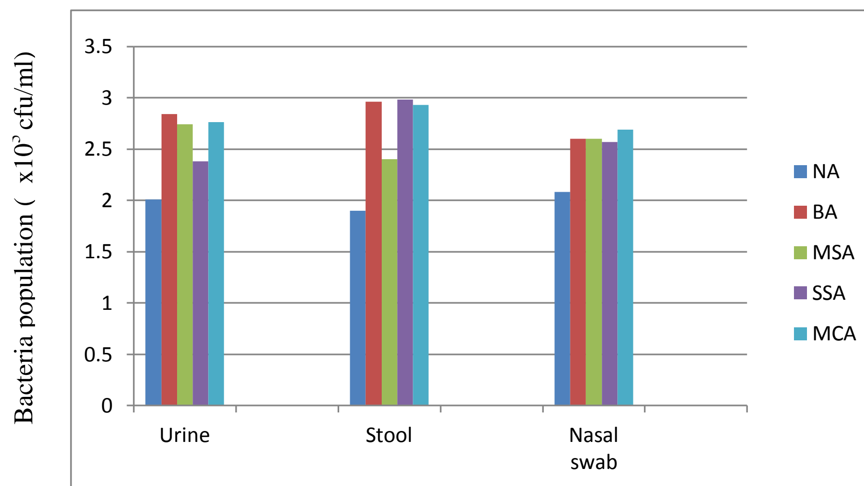

Figure 1. The mean bacterial load from urine, stool and nasal swab samples cultured on different media. NA = Nutrient agar; BA = blood agar; MSA = mannitol salt agar; SSA = Salmonella-Shigella agar; MCA = MacConkey agar.

1:10 dilutions of the reactivated bacteria isolates were made. A 0.1 ml of 10^{-3} of each bacteria isolate was evaluated for growth potential in triplicate on three different special purpose media. The mean bacteria load from the triplicate cultures was calculated.

Statistical analysis

Analysis of variance (ANOVA) for growth efficiency of the types of media in relation with growth potential of each medium in the isolation of *E. coli, S. aureus, Salmonella* and *Shigella* was carried out to deterimine if significance existed among the media in their support for bacteria growth. Tukey test was used to show which media were different. Also standard deviation of bacterial load from urine, stool and nasal swab samples cultured on the five media and standard deviation of growth potentials of the media on four dominant bacterial isolates were calculated.

RESULTS

The mean bacterial load from urine, stool and nasal swab samples cultured on different plating media showed that SSA; a selective medium had the highest number of bacterial colonies of 2.98 x 10^5 CFU/ ml followed by the Enrichment medium (blood agar) that had 2.96 x 10^5 CFU/ ml and MCA with 2.93 x 10^5 CFU/ ml; all were stool samples while Nutrient agar had the least number of bacteria count ranging from 1.90 - 2.08 x 10^5 CFU/ ml (Figure 1). Biochemical identification and characterization of four dominant isolates confirmed the presence of *S. aureus, E. coli, Salmonella* and *Shigella*.

Determination of the growth potential of each medium on the bacterial isolates showed that MacConkey agar recorded the highest growth potential of 8.9 x 10^5 CFU/ ml for *E. coli* followed by BA that gave 8.8 x 10^5 CFU/ ml for *Shigella.* The third highest growth potential of 8.6 x 10^5 CFU/ ml was recorded in NA against *S. aureus* (Figure 2).

Comparative study of the growth potential of the different media is useful in getting information on the microbial density of infection, types of microorganisms and polymicrobial nature of infection.

Statistical analysis using ANOVA showed that there exists a significant difference among the mean of the five media in their support for bacteria growth at α =0.05 and significant value was 0.002. Therefore, null hypothesis was rejected and the rejection of the null hypothesis implies that among the media, there were at least two that had different means. The Turkey test was used for interpretations of multiple comparisons and to show which media were different. Inference showed that the difference in mean performance of each of the following pairs of media is significant; NA and BA, NA and MSA, NA and SSA, NA and MCA. Blood agar, MSA, SSA and MCA with mean 2.800, 2.5667, 2.6433 and 2.7933 respectively performed better than NA with mean 1.9967.

ANOVA result for growth potentials of the different media on four dominant bacterial isolates failed to reject null hypothesis because it had significant value of 0.233. It is not less than significant level, 0.05. Hence, it was concluded that the mean of the five sample media were the same. The standard deviation of the bacterial load from urine, stool and nasal swab samples cultured on the five media was 0.34529 and Standard Deviation of growth potentials of the media on four dominant bacterial isolates was 3.29515.

DISCUSSION

Different types of bacteria are found in urine, stool and nasal cavity, although some of these bacteria are

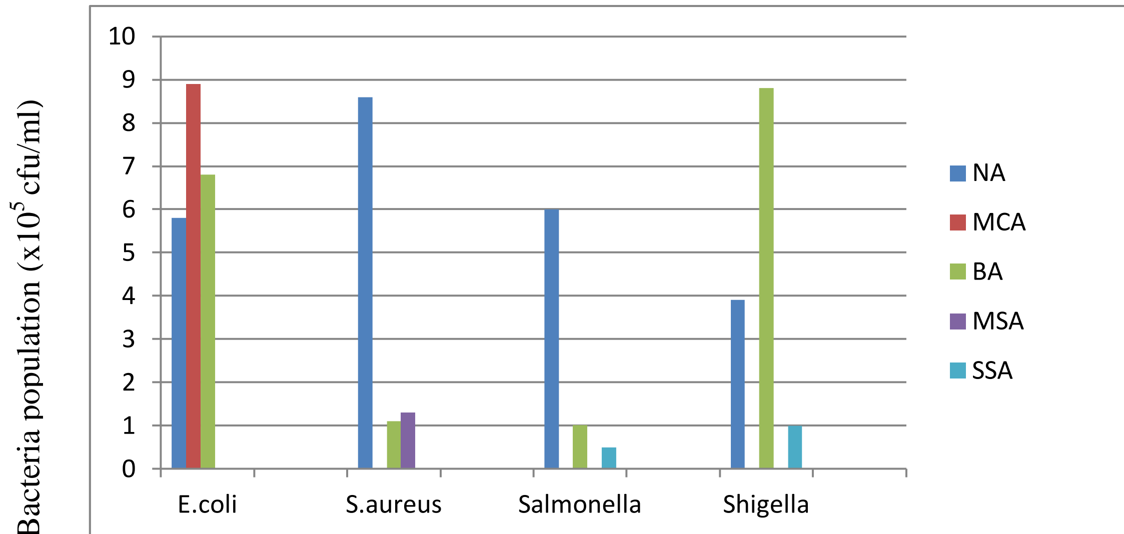

Figure 2. Growth potentials of the different media on four dominant bacterial isolates. NA = Nutrient agar; BA = blood agar; MSA = mannitol salt agar; SSA = Salmonella-Shigella agar; MCA = MacConkey agar.

asymptomatic. Nonetheless, the different types of bacteria found in one sample cannot be covered by a single growth medium; therefore, the observation of this study on mean bacterial load from urine, stool and nasal swab samples cultured on different media lends more weight to the report of Ramzan et al. (2004).

The different sources of the isolates was in line with those of Abdulhadi et al. (2008), who reported that microorganisms colonize different habitats and that the nose is colonized by different microorganisms including *S. aureus*. Similarly, study done by Manaal et al. (2011) reveals *E. coli* as the main causal agent of uniary tract infection and has been isolated from urine. This also agrees with findings of Nicolle (2008). Other studies have revealed the isolation of Salmonella and Shigella from stool specimens. Several different plating media were used for their isolation and Salmonella and Shigella agar was included (Isenberg, 1992; Taylor and Schelhart, 1971; Vandeizant and Splittsoesser, 1992).

The observation that selective and enrichment media are best for isolation of bacteria during routine laboratory investigations lends more weight to previous studies that reported the use of at least one selective media with other plating media during routine laboratory work investigation (Rubina et al., 2006; Olle et al., 2002; Taylor and Schelhart, 1971; Cassar and Cuschieri, 2003).

Similarly, the report of this study that MacConkey agar recorded the highest growth potential for *E. coli* supports the observation of previous researchers (Olle et al., 2002).

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Biodegradation study of γ-hexachlorocyclohexane using selected bacteria isolated from agricultural soil

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Bacterial isolates RP-1, RP-3 and RP-9 were isolated from agricultural soil using enrichment culture technique and screened positive for lindane degradation. RP-1, RP-3 and RP-9 were found to utilize and degrade higher concentrations (100 ppm) of lindane. RP-1 and RP-3 showed 69.5 and 65% lindane degradation after 10 days of inoculation where as RP-9 degraded 62% of lindane after 15 days. The estimated Cl^- ion release was 49, 42 and 39 mg/mL, respectively for the three bacterial isolates. Gas chromatography was used for analysis of metabolite formed during lindane degradation and different parameters of degradation kinetics were calculated using first order kinetic equation. A drastic decrease in degradation rate was observed at initial lindane concentrations higher than 200 mg/l in the mineral media. The calculated half-life periods for RP-1, RP-3 and RP-9 were found to be 3.85, 2.77 and 4.00 days, respectively. All three isolates showed maximum degradation activity at: incubation period; 10-15 days, incubation temperatures; 30°C, pH; 7.0, shaking speed 120 rpm, initial substrate concentration; 100 mg/l. Galactose and succinate enhanced the degradation rate up to 10% whereas maltose, lactose and xylose decreased the degradation level up to 40%. Addition of glucose as a co-substrate was found highly favorable for enhancement of lindane degradation.

Key words: Enrichment culture, colorimetric assay, lindane, degradation, gas chromatography.

INTRODUCTION

The use of pesticides has increased dramatically during the last two decades at global level, due to their promising effects in agricultural and other related areas. Some of these are extremely resistant to biodegradation by native flora when compared with the naturally occurring compounds that are readily degraded upon introduction into the environment. Therefore, pesticides residues and their transformation products are frequently found in the environmental matrices. Despite concerns regarding their toxicity to humans and wildlife along with their relative stability to sediments and soil, they are still widely used (Diez, 2010). Lindane or γ- hexa-chlorocyclohexane (γ-HCH) has been used historically as a broad spectrum pesticide in agricultural, livestock, forestry, veterinary and human health applications because of its low production cost and effective pesticide properties. The HCH formulation consists of γ-(10–12%), α- (60–70%), β- (5–12%) and δ- (6–10%) isomers

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and out of these only γ-HCH possesses insecticidal activity (Li et al., 2003). Therefore γ-HCH is generally purified with 99% purity; the remaining four isomers are discarded and released as HCH muck. Its residues have been detected in drinking water sources, beverages and in different food stuffs. The half-life period for lindane in soil and water was reported as 708 and 2,292 days, respectively. It has been classified as a persistent organic pollutant (POP), potent carcinogen and hazardous, by Stockholm Convention in 2009. The production and agricultural use of lindane had been banned in more than 50 countries due to its toxicity and long persistence in soil but the pharmaceutical use is permitted till 2015. Large amounts of HCH still remain at the production sites even when the units have been closed. The use of γ-HCH for control of agricultural pests has been discontinued, run-offs from the already contaminated agricultural soils or from the dumping sites of adjoining regions can result in high levels of contamination. The use of lindane in agriculture leads to 12-30% volatilization into atmosphere and comes back in the form of rain.

The HCH degradation occurs by stepwise removal of chlorine atom known as dechlorination and influenced by temperature, pH, oxygen and biomass concentration. Nagata et al. (1996a) proposed the degradation pathway of lindane using *Pseudomonas paucimobil*is. The γ- HCH is transformed to 2,5-dichlorohydroquinone via sequential reactions catalyzed by enzymes *Lin*A, *Lin*B and *Lin*C. The 2,5-dichlorohydroquinone in turn, is metabolized by enzymes *Lin*D, *Lin*E, *Lin*F, *Lin*GH and *Lin*J to succinyl-CoA and acetyl-CoA, that are further channeled and metabolized in the tricarboxylic acid cycle.

Though all HCH isomers are toxic, carcinogenic, endocrine disrupters are known to exert damaging effects on the reproductive and nervous systems in mammals, it is ubiquitously used in tropical countries to reduce vector-transmitted diseases, to protect livestock and to increase agricultural yields.

It produces histological alterations in cardiac tissue and cardiovascular dystrophy (Rajendran et al., 1999). Considering the various environmental impacts and persistence of lindane in the soil for a long time and its toxicity, threats of environmental contami-nation are of great concern. As lindane is highly recalcitrant and toxic compound which is degraded at a low rate, the present study is an attempt to isolate and characterize the potent bacterial strains from field soil, involved in biodegradation of lindane.

MATERIALS AND METHODS

Lindane (γ-HCH, 97.2% purity) technical grade was procured from Sigma Aldrich (USA). A stock solution of lindane, prepared at a concentration of 4 x 10^3 mg/L in acetone (Fisher scientific India Pvt. Ltd.) was added to mineral salt medium according to the requirements. All other chemicals were of analytical grade, obtained from Hi-media, Merck and Qualikam India. *Sphingomanas japonicum* (MTCC No. 6362) procured from IMTECH, Chandigarh, was used as a reference culture for all the biodegradation studies.

Soil sampling

For isolation of lindane degrading bacteria, soil samples were collected from different agricultural field sites of Haryana, India which had a long history of pesticide application (more than 15 years). Soil cores (0-20 cm) taken from selected spots were collected in sterile plastic bags and stored at 4°C until microbial isolation. Physiological characterization of soil samples was carried out using Soil Testing Kit (K052), Hi-media, India.

Enrichment and isolation of bacterial

Bacterial isolation was carried out by enrichment culture technique using mineral broth (MSM) containing (per liter) potassium dihydrogen phosphate; 0.85 g, dipotassium hydrogen phosphate; 2.17 g, disodium hydrogen phosphate; 3.34 g, ammonium chloride; 0.1 g, magnesium sulphate; 0.5 g, calcium chloride; 0.5 g, ferrous sulphate; 0.01 g, sodium molybedate; 0.01 g at a pH of 7.2 ± 0 5 (Sahu et al., 1990). Two grams of collected soil sample was added to 100 mL of sterile medium supplemented with 10 mg/L of lindane, after sterilization. The flask were mixed thoroughly and incubated at 30°C for 7 days on rotary shaker at 120 rpm. Subsequently 1 mL of the inoculum having 5 x 10^3 CFU/ mL, from the flasks were transferred to sterile medium (100 mL) containing same lindane concentration. The pesticide concentration was increased from 10 to 100 mg/L in a stepwise manner, transferring the inocula to fresh media each time. After acclimatization, bacterial colonies were isolated by serial dilution and spread plated onto mineral agar plates. These were incubated under aerobic conditions at 30°C for 24 h and colonies with different morphology were sub cultured on fresh agar plates in the form of single culture and preserved at 4°C.

Screening and selection of lindane degrading bacteria

γ-HCH utilization assay

For this, spray plates were prepared with 1.5% agar in mineral medium on Petri dishes and culture was streak on to the plates. The surface of the preset agar plates was sprayed with 0.5% of lindane in acetone. The plates were incubated for seven days in at 28 ± 2°C. The formation of lindane clearance zone surrounding bacterial colonies indicated utilization of lindane by that culture. The pure isolates were grown into mineral broth supplemented with 100 mg/L of lindane, for two to three days at 30°C.

Dechlorinase enzyme assay

For detecting haloalkane dehalogenase activity colorimetric assay was performed in a 96 well microtiter plate using the method given by Holloway et al. (1998), with slight modifications. The assay buffer contains 0.5 mM HEPES (pH 8.2), 10 mM sodium sulphate and 0.5 mM EDTA. Phenol red (dissolved in acetone) was added to the buffer before addition to 96 well plate to give a final concentration of 20 µg/mL. Each well was filled with 194 µLof buffer and 3 µL of lindane stock solution (12.5 mg/mL of acetone) prior to the addition of 6 µL of cell free extract. The organisms were also tested for dechlorination of dichloroethane (DCE). The microtitre plates were kept covered to prevent substrate volatilization and evaporation of the reaction mixture. A visual color change from red to yellow was indicative of lindane and DCE dechlorination which arises due to decreasing pH. Wells with blank samples were having only HEPES buffer instead of cell free extract.

Table 1. Morphological, physiological and biochemical characteristics of potent lindane degrading bacterial isolates RP-1, RP-2 and RP-3.

Characteristic	RP-1	RP-3	RP-9	Characteristics	RP-1	RP-3	RP-9
Morphological				**Biochemical**			
Cell Shape	Oval	Rods	Round	Gelatin liquefaction	-ve	-ve	-ve
Cell Size	0.3 µm	0.2 µm	0.3 µm	Starch hydrolysis	+ve	+ve	-ve
Gram stain	+ve	+ve	-ve	Casein hydrolysis	-ve	+ve	+ve
Motility	+ve	+ve	-ve	Catalase	+ve	+ve	+ve
Capsule	-ve	+ve	+ve	Methyl red	+ve	+ve	_ve
Colony morphology	Orange small	White opaque	Yellow, raised	Vogues Proskeur	+ve	-ve	+ve
Pigmentation	+ve	-ve	-ve	Sorbitol utilization	+ve	-ve	-ve
Physiological				Citrate utilization	-ve	+ve	+ve
Growth at 5°C	-ve	-ve	-ve	Lysine utilization	-ve	+ve	-ve
at 30°C	+ve	+ve	+ve	Orinithine utilization	-ve	+ve	-ve
at 45°C	_ve	-ve	+ve	Urease utilization	-ve	-ve	-ve
Growth at 1% NaCl	+ve	+ve	+ve	Phenylalanine deamination	-ve	-ve	-ve
at 3% NaCl	+ve	+ve	+ve	Nitrate reduction	+ve	+ve	+ve
at 5% NaCl	+ve	+ve	+ve	H_2S Production	-ve	+ve	-ve
at 7% NaCl	-ve	+ve	-ve	Triple Sugar Iron Test	+ve	+ve	-ve
at 10% NaCl	-ve	-ve	-ve	Adanitol	-ve	+ve	-ve
Growth at pH 3	-ve	-ve	-ve	**Carbohydrate test**			
at pH 5	-ve	+ve	+ve	Mannitol	+ve	-ve	-ve
at pH 8	+ve	+ve	+ve	Xylose	+ve	-ve	+ve
at pH 11	-ve	-ve	-ve	Galactose	-ve	-ve	+ve
Degradation of: Aesculin	-ve	+ve	+ve	Dextrose	-ve	+ve	+ve
Tween 20	-ve	+ve	-ve	Sucrose	-ve	+ve	-ve
Tween 80	+ve	+ve	+ve	Glucose	-ve	+ve	-ve
				Rhamnose	+ve	+ve	-ve
				Lactose	-ve	+ve	+ve
				Arabinose	+ve	-ve	+ve

Substrate tolerance

Different stocks solutions of lindane were prepared with varying concentrations. Then lindane utilizing cultures RP-1, RP-3 and RP-9 were inoculated in half strength mineral medium containing various concentrations of lindane as a sole carbon source (20, 40, 60, 80, 100, 120 ppm) and incubated at 30°C at 120 rpm. OD_{600} was taken periodically up to one month by taking inocula from all flasks having different concentration of lindane to check the viability count of bacteria which is directly related to the substrate tolerance. Subsequently chloride release was estimated to check degradation potential of the isolates at higher concentrations.

Morphological and biochemical study of important isolates

Different morphological (cell shape, cell size, gram stain, motility, capsule formation, colony morphology pigmentation) and biochemical studies (*viz.* gelatin liquefaction, starch and casein hydrolysis, catalase test, methyl red and Vogues Proskeur, sorbitol, adanitol, citrate, lysine, orinithine and urea utilization, phenylalanine deamination, nitrate reduction, H_2S production, triple sugar iron test and various carbohydrate fermentation test like mannitol, xylose, galactose, sucrose glucose, rhamnose, lactose and arabinose) were carried out for the promising lindane utilizing isolates with the standard protocols given in Microbiology: A Laboratory Manual (Cappuccino and Sherman, 2010) (Table 1). For carbohydrate fermentation, 1% of each substrate was added to the medium as a carbon source.

Biodegradation studies

The potent isolates were inoculated into mineral broth supplemented with lindane (100 mg/L) and kept on rotary shaker at 120 rpm at 30°C up to one month to determine their degradation potential quantitatively and qualitatively. Quantitative dechlorination rate was determined by estimation of chloride ions released into the medium by inoculatory strains using indirect Argentometric method given by Greenberg et al. (1992). Individual samples were withdrawn at 0, 2, 4, 6, 8, 10, 13 and 15 days of incubation and were analyzed for Cl^- estimation. The biodegradation potential of the selected strains was evaluated by analyzing the residual lindane in the medium which was calculated using the following formula:

$$\text{Residual lindane (\%)} = (C_t/C_0) \times 100$$

where, C_0 = initial concentration of lindane in the medium; C_t = lindane concentration at time t.

Statistical analysis for degradation kinetics

All the degradation experiments were carried out in triplicates. The

Figure 1. Plate clearance assay: Lindane utilization on spray plates by bacterial isolates.

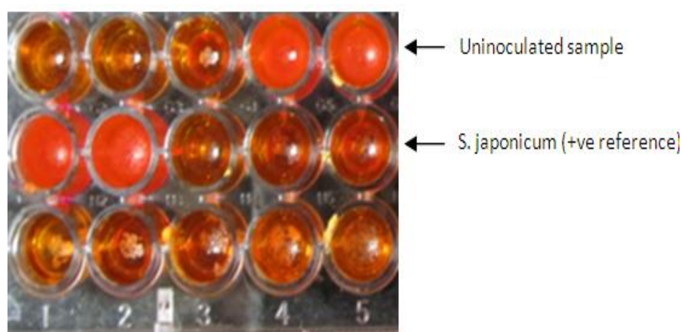

Figure 2. Colorimetric assay: Dechlorinase activity assay for lindane degrading bacterial isolates

isolates RP-1, RP-3 and RP-9 were inoculated into sterile basal mineral broth (50 mL) having different concentrations of lindane (50, 100, 150, 200, 250 and 300 ppm) and were incubated on a rotary shaker (120 rpm) at 30°C. Analysis of chloride ion release and residual lindane was carried out and uninoculated mineral medium was taken as the control. First order kinetic equation was used for calculating different parameters of lindane degradation by fitting experimental data:

$$C_t = C_0 e^{-kt}$$

$$\ln C_t = C_0 - kt$$

Where, C_0 = Initial lindane concentration; C_t = lindane concentration after reaction time t; K = degradation constant; T = reaction time.

Half- life for lindane biodegradation was calculated using the following formula:

$$T_{1/2} = \ln 2/K$$

Physiological studies of lindane biodegradation

Substrate tolerance studies using different concentration of lindane (20, 40, 60, 80, 100 and 120 ppm) as a sole carbon source has been carried out for the said isolates. Different physiological parameters, that is, incubation time, temperature, pH, initial lindane concentration, shaking speed, on biodegradation rate has been optimized for the potent lindane degrading isolates.

Effect of different carbon sources

Effect of various carbon sources on lindane degradation *viz.* glucose, maltose, galactose, succinate, xylose, lactose as degradation activators and deactivators was also studied. For this experiment, mineral medium was supplemented with 1% of each carbon substrate, inoculated with 1 mL of particular bacterial inoculum and incubated at 30°C on shaker for 15 days.

GC- analysis

Residual lindane in the culture was determined qualitatively by GC-ECD method using Gas chromatograph (Shimadzu -2010 Plus model) equipped with ECD detector and DB-1701 (30 µm x 0.25 µm x 0.25 µm) column. For GC-ECD analysis, the residual lindane and the degradation product formed were extracted twice in 1 mL of hexane (HPLC Grade, Qualikam Chemicals, India). Elution were as follows: Helium as carrier gas, detector temperature; 350°C, oven temperature conditions; 90°C for 2 min, increase to 250°C at 5°C/min, increase to 250°C at 30°C/min and held for 5 min. Preliminary test with known standards showed the method capable of detecting about 1 µg/mL of lindane in the injected sample (2 µL). All the 9 isolates obtained after testing their dechlorinase activity were subjected to the same protocol along with *S. japonicum* as reference.

RESULTS AND DISCUSSION

Different physico-chemical characteristics of soil samples were analysed: pH; 7.4, organic carbon 61.1 ppm, available phosphate; 16.5 ppm, potassium; 49 ppm, ammonical nitrogen 20.8 ppm, nitrate nitrogen 10 ppm; sand 71%, silt 13% and clay 16%.

Isolation and screening of lindane degrading bacterial isolates

A total of 78 bacterial isolates were obtained which were tested for lindane utilization and dechlorinase enzyme activity. Plate clearance assay and dechlorinase enzyme assay were performed to confirm lindane degrading activity. Nine isolates were found positive for lindane utilization when screened on γ-HCH spray plates. They showed a prominent clearance zone after 9-12 days of incubation (Figure 1). Similar type of lindane degradation zones has been observed for other organisms e.g. bacterium *Pesudomonas paucimobiliis* (Senoo and Wada, 1989), fungus *Conidiobolus* 03-1-56 (Nagpal et al., 2008) and yeast *Rhodotorula* sp. VITJzNo3 (Salem et al., 2013). These isolates were further screened for enzyme activity using colorimetric assay. The phenol red indicator turned yellow when cell free extract of these nine isolates along with *S. japonicum* (as reference) were incubated in the presence of lindane (Figure 2). All these

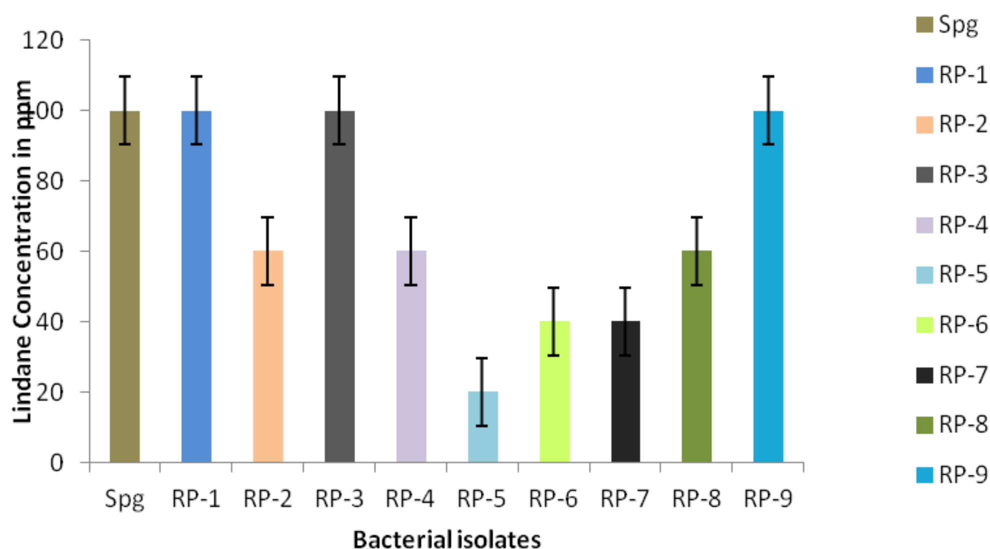

Figure 3. Lindane tolerance limit of different isolates viz. RP-1 to RP-9 along with the standard *S. japonicum*.

isolates were also found to be positive for dechlorination of dichloroethane (DCE). The colour change is due to decrease in pH. *S. japonicum* possess genes linA and linB which have been classified as haloalkane dehalogenases, responsible for the dechlorination of compounds like HCH and DCE. Dechlorination by linA results in accumulation of hydrochloric acid which in turn is responsible for lowering of pH (Nagata et al., 1997). When whole cells were inoculated into HEPES buffer containing lindane and incubated, no colour change was observed suggesting that enzymes having dechlorinase activity may be intracellular in nature (Thomas et al., 1996). It has already been reported in the case of *S. paucimobilis* UT26 that linA and linB genes are not excreted but are located in periplasmic space (Nagata et al., 1996a). However there is possibility that the same is not true for all lindane degrading micro-organisms, that is, some degraders may have dechlorinase enzymes present extracellularly.

Substrate tolerance

Both genetics and physiologies of microorganisms are involved in making them resistant/tolerant against any pesticide. It is observed that the tolerant microorganisms to lindane or any pesticide have biodegrading potential to break it down into smaller products which are later utilized by these organisms as carbon and nitrogen sources (Bellinaso et al., 2003). In this study, total ten isolates (along with the reference *S. japonicum*) were checked for their potential to withstand higher concentrations of the substrate by inoculating into mineral broth having different concentration of γ-HCH. After one month, *S. japonicum* and three isolates RP-1, RP-3 and RP-9 were found to tolerate 100 ppm of lindane where as RP-2, RP-4 and RP-8 could tolerate up to 60 ppm. RP-6 and RP-7 were able to grow in medium having only 40 ppm of the active substrate and RP-5 showed growth only in very low concentration up to 20 ppm. Higher concentrations of lindane were found detrimental to growth of most of the bacterial isolates. Similarly, higher concentrations of toxic contaminants like cadmium have been reported to diminish bacterial growth (Kumar et al., 2010). After these observations, RP-1, RP-3 and RP-9 were selected to further biochemical, morphological and biodegradation studies (Figure 3 and Table 1)

Biodegradation studies

When the bacterial isolates were grown in mineral medium containing 100 mg/l of lindane, they were found to utilize lindane as a sole carbon source. There was a lag phase of two days before the bacterial growth started. Figure 4 shows that maximum OD_{600} was reached after 7 days of incubation for *S. japonicum* and RP-1 whereas in the case of RP-3 and RP-9, it was reported after 10 days of incubation. The substrate disappearance started after 3 days of incubation, that is, when the OD_{600} reached above 1.0 for all the three isolates along with reference. Extent of lindane mineralization was estimated by quantifying the release of inorganic chloride ions and analyzing the percent residual lindane in the medium. RP-1 and RP-3 showed 69.5 and 65% lindane degradation after 10 days of inoculation and their degradation rate was stable up to 15 days. RP-9 was able to degrade 62% of lindane after 15 days of inoculation and after this period the rate of degradation started decreasing for all the three bacteria (Table 3).

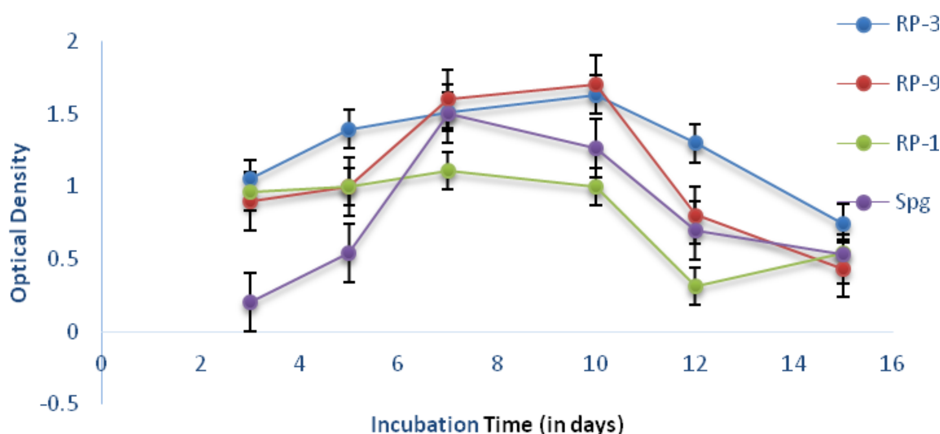

Figure 4. Effect of incubation time on bacterial growth.

Table 2. Differnt kinetic parameters for lindane degradation by bacterial isolates RP-1, RP-3 and RP-9.

Initial lindane concentrations (mg/l)	Kinetics equation of lindane degradation	Rate constants of lindane degradation								
		RP-1			RP-3			RP-9		
		R^2	$K(d^{-1})$	$T_{1/2}$ Days	R^2	$K(d^{-1})$	$T_{1/2}$ Days	R^2	$K(d^{-1})$	$T_{1/2}$ Days
50	$C = 4.853^{-0.435t}$	0.875	0.442	1.35	0.856	0.310	0.89	0.854	0.278	1.75
100	$C = 5.108^{-0.419t}$	0.890	0.165	2.14	0.945	0.069	1.48	0.910	0.265	2.00
150	$C = 5.532^{-0.401t}$	0.756	0.245	2.55	0.812	0.442	1.98	0.879	0.245	2.55
200	$C = 5.846^{-0.356t}$	0.873	0.300	**3.85**	0.964	0.300	**2.77**	0.867	0.400	**4.00**
250	$C = 6.024^{-0.140t}$	0.725	0.053	7.86	0.734	0157	5.80	0.723	0.153	6.90
300	$C = 6.354^{-0.090t}$	0.665	0.157	11.00	0.723	0.195	8.90	0.655	0.257	10.00

Where R^2 = Regression coefficient; K = rate constant of degradation; $T_{1/2}$ = half life period.

The concentration of Cl^- in the medium increased during for first 15 days and after this period bacterial count as well as concentration of Cl^- started diminishing which dissipated a linear relationship between growth and release of chloride ion or lindane mineralization.

Kinetics of lindane degradation

The degradation of lindane is dependent on the substrate concentration, which can be well explained by first order equation. The degradation kinetic of lindane was also studied in other organisms by different researchers, where the calculated half-life of lindane were as follows: 1.66 days for *Rhodotorula* sp. VITJzN03 (Salam et al., 2013); 4.78 days for *Anabeana azotica* (Zhang et al., 2012). In the present study, first order kinetic equation was used for calculating different parameters of degradation kinetics by fitting the triplicate values of experimental data. Degradation rate constant and half life period were calculated for different concentration of lindane by using first-order reaction model (Table 2). There was noticeable effect on lindane degradation with increasing initial concentration from 50 to 200 mg/L, however maximum degradation rate was observed for 100 mg/L of lindane. A drastic decrease in degradation rate was observed at concentrations higher than 200 mg/L, so this was taken as the optimum concentration for calculating half life period. The half-life periods calculated for RP-1, RP-3 and RP-9 were found to be 3.85, 2.77 and 4.00 days respectively. Previously, the degradation kinetic of lindane was also studied in other organisms isolated from different soils where the calculated half-life of lindane was described as follows: 3.66 days for *Rhodotorula* sp. VITJzN03 (Salam et al., 2013) and 4.78 days for *Anabeana azotica* (Zhang et al., 2012). Clearly, the calculated half-life of lindane, in the present study is shorter than in the earlier findings indicating the astonishing potency of the strain in lindane degradation.

Physiological studies for lindane biodegradation

Cultural conditions such as growth period, incubation

Table 3. Degradation of lindane (100 mg/L) by bacterial isolates RP-1, RP-3 and RP-9.

Strain	3 days		5 days		10 days		15 days		20 days	
	Cl^- release (mg/mL)	Lindane degradation (%)	Cl^- release (mg/mL)	Lindane degradation (%)	Cl^- release (mg/mL)	Lindane degradation (%)	Cl^- release (mg/mL)	Lindane degradation (%)	Cl^- release (mg/mL)	Lindane degradation (%)
RP-1	0.08	4.90	0.23	27.00	0.49	69.5	0.45	66.90	0.33	52.80
RP-3	0.10	5.00	0.19	19.35	0.42	65.00	0.40	63.77	0.28	35.46
RP-9	0.04	2.95	0.20	21.54	0.38	61.08	0.39	62.00	0.30	30.00
Control	0.00	0.00	0.00	0.00	0.05	3.00	0.02	1.50	0.00	0.00

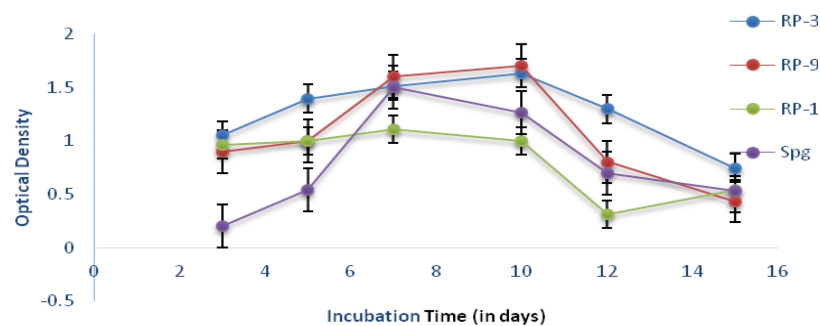

Figure 4. Effect of incubation time on bacterial growth.

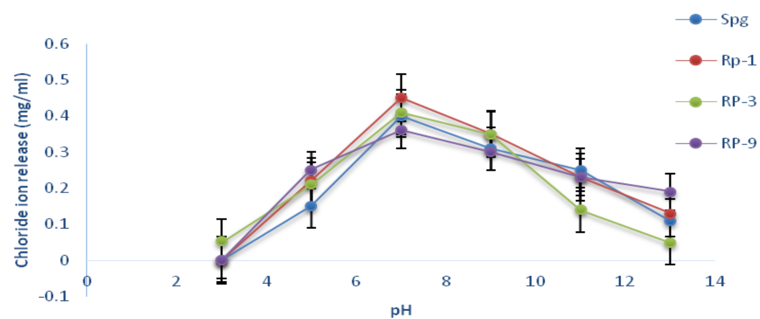

Figure 5. Effect of pH on lindane biodegradation by bacterial isolates RP-1, RP-3 and RP-9.

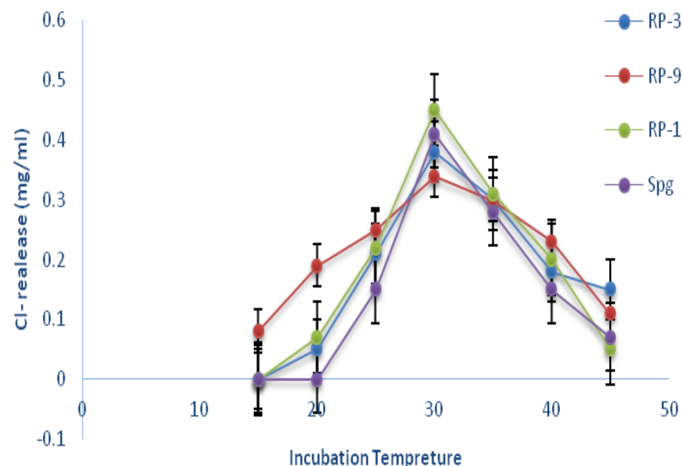

Figure 6. Effect of incubation temperature on lindane biodegradation by bacterial isolates RP-1, RP-3 and RP-9.

temperature, pH, revolution speed, substrate concentration have considerable effect on cell growth and degradation efficiency of micro-organisms (Kodama et al., 2001). For effective degradation study, the initial concentration of lindane was maintained at 50 mg/L in the optimization tests where pH, temperature, incubation time, shaking speed was also investigated. The effect of various parameters on lindane degradation by RP-1, RP-3 and RP-9 is depicted in Figures 4, 5 and 6. Strain RP-1, RP-3 and RP-9 showed better degradation at an initial pH of 7 which was considered as optimal pH. In the previous studies maximum lindane degradation was observed under a neutral pH of 7 (Siddique et al., 2002; Elcey and Kuhni, 2010). Salam et al (2014) also reported optimum pH as 7.0 for lindane degradation by Candida VITJzN04. In the case of temperature, maximum biomass and best course of degradation was observed at incubation temperature of 30°C.

Synchronous observations at 30°C were reported earlier in fungal strain *Rhodotorula* sp. VITJzN03 by Salem et al. (2013) and in bacterial strain *P. aeruginosa* (Zhang et al., 2012). To demonstrate the effect of agitation on degradation of lindane, experimental flasks were incubated at different shaking speeds ranging from 80-120 rpm. The degradation was more prominent at 120 rpm and hence was considered optimum. In the case of inoculum size, the larger the inoculum size, the greater the efficiency of degradation of toxic compounds (Guillen-Jimeneza et al., 2012). In our study, different inoculum size (50-300ml/L) was used as the initial substrate concentration and 100 mg/L of lindane was found optimum for degradation activity. There is possibility that low concentration of substrate might not be able for induce the enzymes of degradation pathway. Similar results were reported by Kumar et al. (2006) in the case of other pollutants. Beyond 100 mg/L, the degradation rate significantly reduced, this limited growth and lindane degradation at higher concentrations could be attributed to the toxicity at higher concentrations of lindane. In the case of incubation period, a time course of 10-15 days was found to be optimum for lindane degradation.

Effect of different carbon sources

Carbon sources, other than the target chemical, when present in medium may highly influence degradation rates. Accordingly, the effect of some of the common carbon sources was evaluated on the biodegradation of lindane.

Addition of different carbon sources in the inoculated medium showed prominent effect on lindane degradation. It was found that galactose and succinate enhanced the degradation rate up to 10% whereas maltose, lactose and xylose decreased the degradation level up to 40% as compared to the sample having no additional carbon source (Figures 7 and 8). Addition of glucose as a co-substrate was found highly favorable for enhancement of lindane degradation. Also, in a previous research, succinate and glucose were found favorable for degradation of chlorinated pesticide like chlorpyrphos (Singh et al., 2004; Anuja Goerge, 2005). The enhanced rate of lindane degradation after addition of different C-sources might be due to the cometabolic process. However, many C-source are also reported to decrease the degradation rate, which might be due to the mechanism of catabolite repression or decrease in the rate of transcription either due to supercoiling of promoter DNA or by decreased binding of transcription factors.

GC-analysis

Gas chromatography analysis with electron capture detector was carried out to check lindane degradation by the bacterial isolates. Hexane extract of pure lindane was run as standard and respective peak was obtained at retention time (RT) 7.458. Sphingomonas japonicum was taken as the reference culture for comparison of peaks obtained for metabolites released during the degradation of lindane as a substrate. For S. japonicum as well as other isolates, no peaks were observed for the chromatographs obtained with blank matrix (Figure 10). Different peaks were obtained only in samples having media as well as lindane and inoculated with different bacterial isolates. The RT- values had also been compared with the literature available on lindane degradation. Bacterial strain RP-1 showed 69.5% degradation which is equivalent to degradation rate of S. japonicum whereas RP-3 and RP-9 degraded at rate slower than the reference culture. Various peaks at different RT values were obtained in common for all the three isolates which were comparative to peaks observed in case of S. japonicum during lindane degradation (Figure 9).

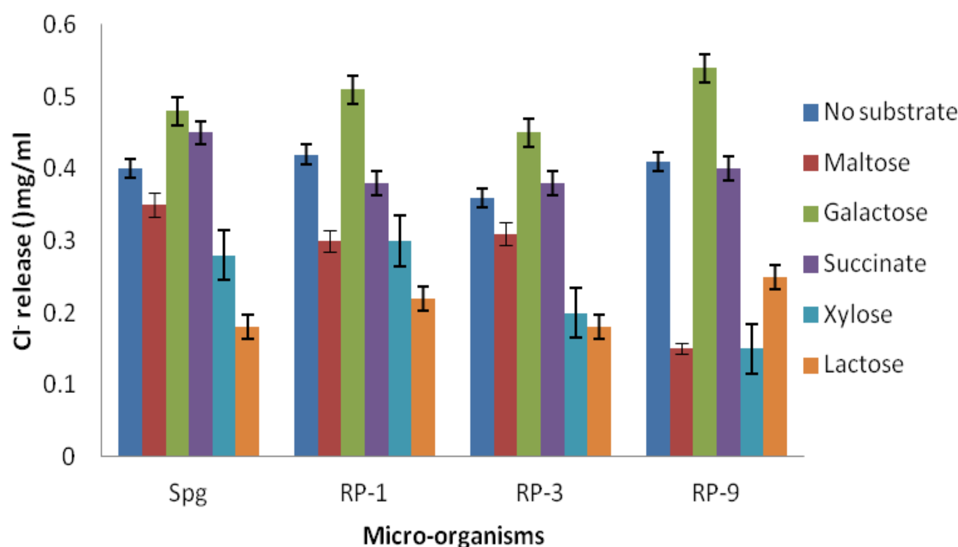

Figure 7. Effect of different carbon sources on lindane biodegradation by bacterial isolates RP-1, RP-3 and RP-9.

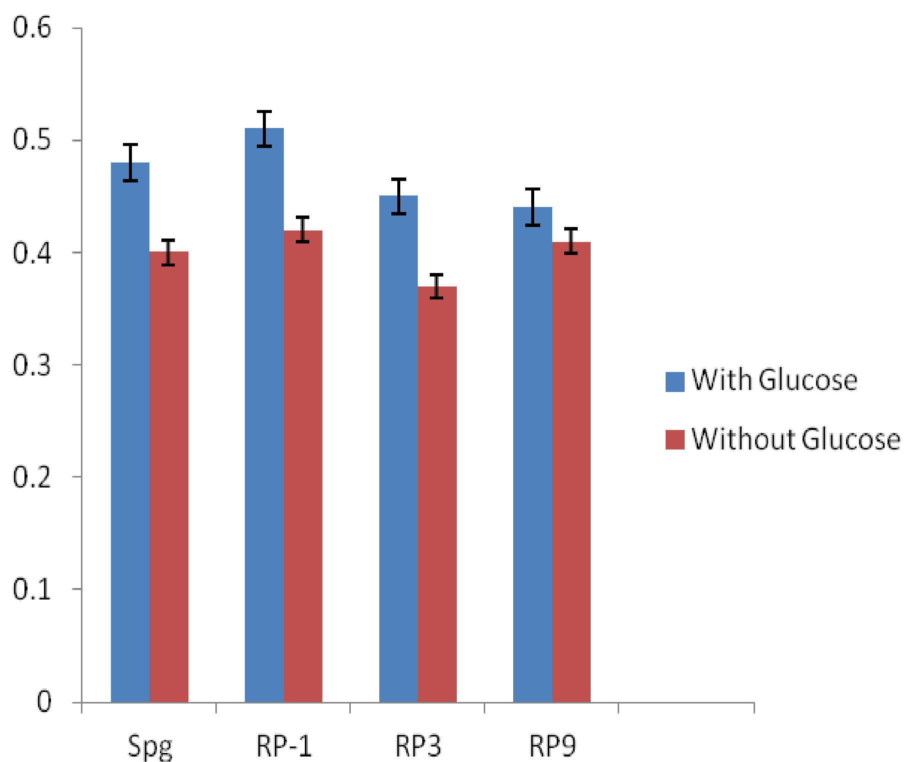

Figure 8. Effect of glucose as an activator on lindane biodegradation by bacterial isolates RP-1, RP-3 and RP-9.

Conclusion

Bacterial isolates RP-1, RP-3 and RP-9 isolated from agricultural soil have been found to possess the ability to utilize and degrade higher concentrations of lindane. Once molecular characterization studies are completed, these can be considered as potential microbes for field trials regarding bioremediation of γ-HCH contaminated

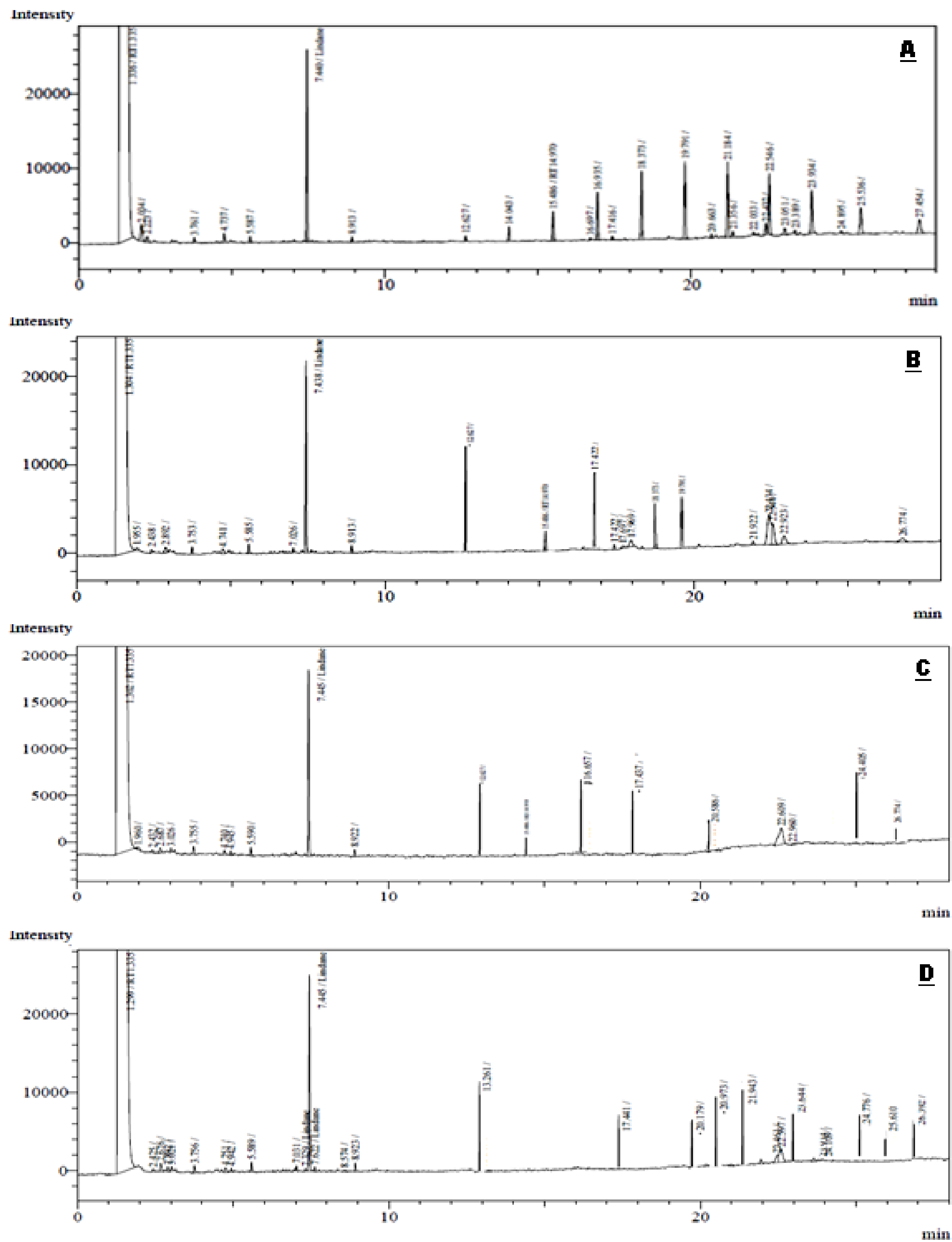

Figure 9. Gas chromatography/ECD analysis of hexane extract of (A) *S. japonicum* as reference for γ-HCH degradation (B) RP-1 (C) RP-3 (D) RP-9. Peaks at different RT representing formation of various metabolites during lindane degradation by the bacterial isolates.

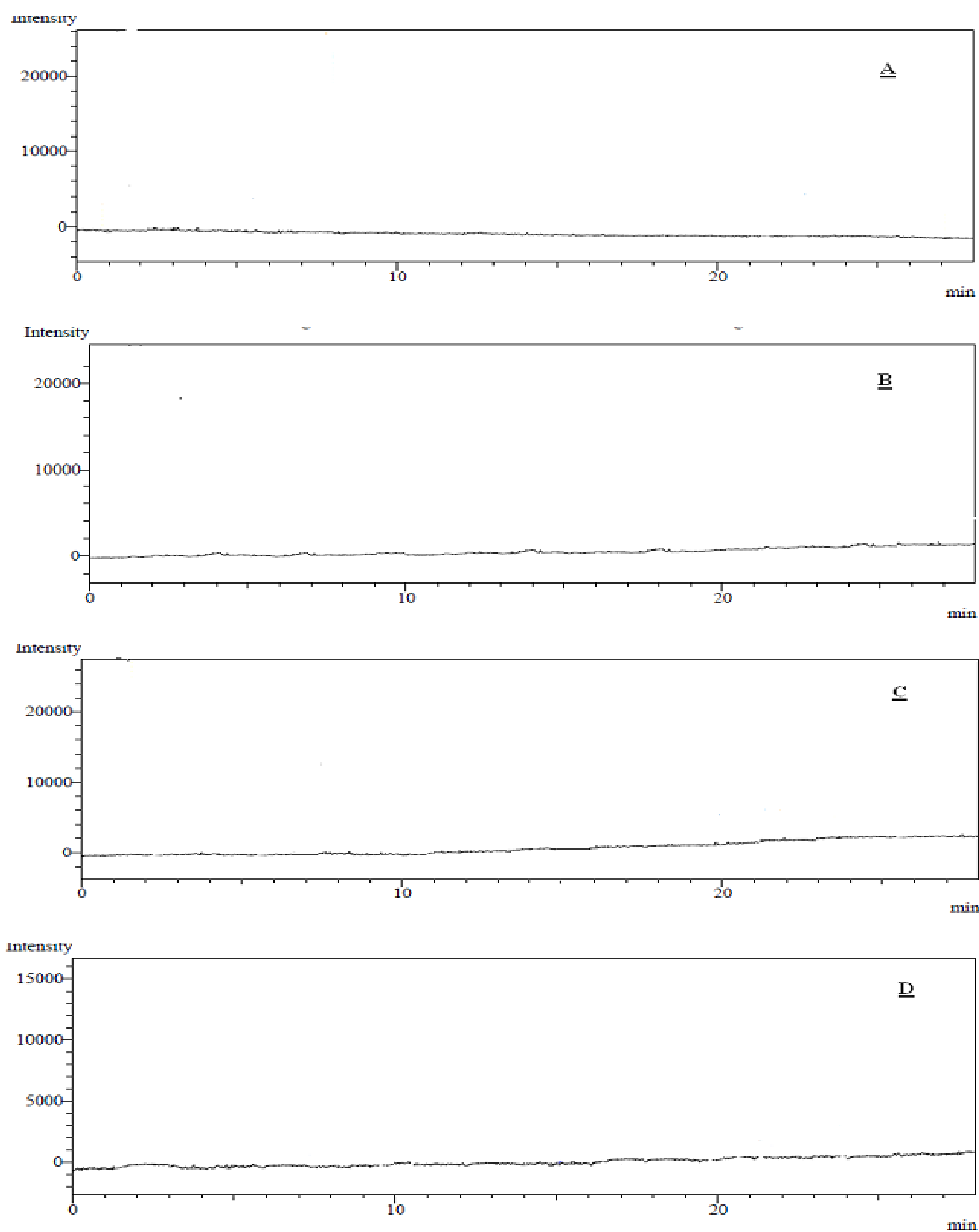

Figure 10. Gas chromatography/ECD analysis of matrix blanks of (A) *Sphingomonas japonicum* (B) RP-1 (C) RP-3 (D) RP-9.

soils. Further research on metabolic pathway elucidation at molecular level is in progress.

Conflict of Interest

The authors declare no conflict of interest.

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Full Length Research Paper

Action of sanitizers on *Staphylococcus aureus* biofilm on stainless steel and polypropylene surfaces

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The interest of researchers in various areas has resulted in the investigation of different biofilm systems using a wide range of techniques. Biofilms are microbial communities consisting of mono or multi-species sessile cells, embedded in a matrix of extracellular polymers (exopolysaccharides-EPS) adhering to surfaces. In the food industry, the existence of biofilms is quite problematic, being responsible for the economic loss and contamination of food. Consequently, research involving the characterization of the ability of microbial biofilm formation is relevant for the subsequent studies using sanitizing and antibiotic agents for prevention or remediation of surfaces with already formed biofilms. This multidisciplinary study led to the description regarding the effect of antimicrobial solutions of essential oils of *Syzygium aromaticum* and *Thymus vulgaris* and their combination on biofilm formed by *Staphylococcus aureus* ATCC 25923 on AISI 304 stainless steel and polypropylene surfaces. All sanitizing solutions showed antibacterial potential, being effective in reducing bacterial biofilms on these surfaces. The solution containing the combination of essential oils was the most efficient by reducing 7.38 and 6.58 Log CFU.cm^{-2} of cells adhering on the surfaces of AISI 304 stainless steel and polypropylene, respectively, after 5 min of contact. Essential oils of *S. aromaticum* and *T. vulgaris*, alone or in combination, are new alternatives for disinfection of industrial stainless steel and polypropylene surfaces contaminated by *S. aureus*.

Key words: Antimicrobial effect, microorganism, *Syzygium aromaticum*, *Thymus vulgaris*.

INTRODUCTION

Microorganisms have been evolving for approximately 4 billion years, and up to 2 billion years ago they were the only life forms on Earth. They comprise a taxonomic definition that congregates varied groups of organisms of microscopic size that live in nature as isolated cells or cell aggregates (Manfio, 2003). Most bacteria, when in their

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natural habitat, live in communities of varying degrees of complexity, associated with a variety of biotic and, or abiotic surfaces, usually forming a biofilm.

Biofilm is defined as a community of sessile microorganisms embedded in an extracellular polymeric matrix, that they produce themselves, characterized by cells irreversibly adhering to a surface or interface and which exhibits phenotypic alteration in relation to planktonic growth and gene transcription. A wide variety of microorganisms are able to adhere and form biofilms on biotic and abiotic surfaces, presenting certain advantages as compared to planktonic cells (Garrett et al., 2008).

A common model for biofilm formation suggests that the process occurs in five stages. First, the reversible attack of planktonic bacterial cells occurs, as they approach the solid surface by fluid flow or motility, which have dominion over the repulsive forces between the cell and the surface. In the second stage, the transition from reversible to non-reversible attack occurs by the production of extracellular polymers by the bacterium itself, and, or specific adhesins localized on the pili or fimbriae that interact with the surface. The third stage consists in the initial development of the architecture of the biofilm. The fourth stage refers to the development of microcolonies within the mature biofilm; on the other hand, extracellular polymeric substances, that serve as an adhesive matrix and nutrients, may continue to be formed, as well as water channels and pores. In the final stage, there is a dispersion of cells of the biofilm and the return of planktonic cells (Van Houdt and Michiels, 2005).

Despite these properties, the adhesion, activity and microbial growth is limited by chemical gradients resulting from the diffusion of nutrients and oxygen; characteristics of the microorganism, hydrophobicity, surface electric charge, flagellum, fimbriae and pili, the adherent material characteristics and the environment surrounding the microorganism, such as pH, temperature, agitation time and a variety of other factors (Chen et al., 2005).

In the food industry, biofilm formation leads to serious health and economic losses due to food contamination and equipment damage. Biofilms can develop the most varied surfaces, and those most used in food production plants is the AISI 304 stainless steel (American Iron and Steel Institute) and polypropylene. The microorganisms present in the biofilm catalyze chemical and biological reactions causing corrosion of metal pipes and tanks, reducing heat transfer due to the thickness of the biofilm, among others (Shafahi and Vafai, 2009).

Among these microorganisms, *Escherichia coli* and *Staphylococcus aureus* are usually found in nature, their presence in food being the consequence of the often precarious sanitary conditions of food production, due to contaminated handling or surfaces. In the food industry, biofilm formation leads to serious hygiene problems and economic losses, mainly due to food contamination, spoilage and damage to equipment. Once established, biofilms act as points of constant contamination, releasing pathogenic and/or spoilage microorganisms (Boari et al., 2009).

Surveys have been documented involving food contact surfaces and various microorganisms, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *E. coli* O157: H7, *S. aureus* and *Pseudomonas aeruginosa*, among others (Shi and Zhu, 2009).

In the quest for better understanding of the biofilm formation process on different surfaces, the search for, and research on sanitizing agents and antimicrobial alternatives should be generated. In this context, it has been observed that the essential oils found in condiment plant extracts have antibacterial, antifungal and antioxidant properties, which has aroused the interest of food industries (Kalemba and Kunicka, 2003).

According to Sikkema et al. (1994), essential oils accumulate in the cytoplasmic membrane and cause damage such as loss of function of selective barrier. In recent years, several reports have been published on the composition and biological properties of essential oils of several condiment plants, among them *Syzygium aromaticum*, *Thymus vulgaris, Cymbopogon citratus* and *Laurus nobilis (*Fabio et al., 2007; Oliveira et al., 2010).

Some researches emphasize the existence of differences in the chemical composition among the extracted oils of different species or varieties. These variations tend to influence the antimicrobial activity of the oils and usually depend on factors such as genetically determined properties, plant age, seasonal variation, water availability, environmental temperature at which the plant is found, nutrients available in the soil, altitude and UV radiation (Gobbo-Neto and Lopez, 2007).

The inhibitory effect of these oils on microorganisms is an alternative to reduce the use of chemical additives in food and for the formulation of new sanitizing agents. Various studies have shown that essential oils extracted from leaves and different parts of plant species have high antimicrobial activity (Gill and Holley, 2006).

Given the above, the objective of this study was to evaluate the action of sanitizing solutions formulated with essential oils of Clove (*S. aromaticum*) and Thyme (*T. vulgaris*) and their combinations, on bacterial biofilms formed by *S. aureus* on AISI 304 stainless steel and polypropylene surface.

MATERIALS AND METHODS

Microbiological analyzes were performed in the Food Microbiology Laboratory, Food Science Department, Universidade Federal de Lavras (UFLA), MG.

Microorganism used, standardization, inoculum preparation and storage

The microorganism used in the development of this study was *S. aureus* ATCC 25923. The culture of *S. aureus* was maintained at -18°C in microcentrifuge tubes with freezing medium [glycerol (150 mL), peptone (5 g), yeast extract (3 g), NaCl (5 g), H_2O (1.000 mL), pH 7.2 ± 7.4]. During the experiment, subcultures were made for

the maintenance of cultures. Aliquots were transferred to the micro-centrifuge tubes containing tryptic soy broth (TSB) and incubated at 37°C/24 h. After culturing, 1 mL of the culture was dispensed into sterile microcentrifuge tubes and centrifuged at 6,000 xg for 8 min in a microcentrifuge. After removing the supernatant, the content was again coated with freezing medium and stored at -18°C.

For reactivation and use of the strain, 10 µL of the culture was inoculated in tubes containing 3 mL of TSB and incubated at 37°C/24 h. After incubation, 20 µL of the inoculum was removed and transferred to 200 mL of TSB. The number of cells per mL was quantified using standard curve and the growth monitored by spectrophotometry at 600 nm and then counting in TSA. The bacterial culture was standardized to a concentration of 10^8 CFU mL.

Experimental model of biofilm formation

Preparation and cleaning of coupons

The bacterial adhesion was conducted on AISI 304 stainless steel and polypropylene coupons with 1 mm thickness and dimensions of 10 x 20 mm.

The AISI 304 stainless steel coupons were cleaned individually with 100% acetone, submerged in detergent, rinsed with sterile distilled water, dried and cleaned with 70% ethanol (v/v). After cleaning, the coupons were again washed with sterile distilled water, dried for 2 h in oven at 60°C and autoclaved at 121°C/15 min (Rossoni and Gaylarde, 2000). As for the polypropylene coupons, they were initially immersed in a solution of commercial 0.3% peracetic acid for 30 min under stirring at 50 rpm at 50°C. They were then soaked in sterile distilled water at 80°C for 5 min and at room temperature for 1 min under agitation of 50 rpm. The coupons were dried at 40°C for 2 h and autoclaved for 15 min at 115°C (Oulahal et al., 2008).

Adhesion of bacterial cells to surfaces

In two Petri dishes (140 mm diameter) 45 AISI 304 stainless steel coupons and 80 mL TSB were added and inoculated with 10^8 CFU mL of culture. In two other Petri dishes, the same procedures were employed, with 45 polypropylene coupons, with the aim of promoting the formation of biofilms on these surfaces. The plates were incubated at 37°C with orbital agitation of 50 rpm. After 48 h of incubation, the coupons were collected, washed with peptone water (0.1% w/v) five times and immersed in TSB contained in sterile plates. This procedure was performed five times in order to complete the formation of the biofilm after 10 days of incubation (Joseph et al., 2001).

Enumeration of adhered cells

For enumerating the adherent cells, one AISI 304 stainless steel and one polypropylene coupon was removed from each Petri dish every two days of incubation. These were washed with peptone water (0.1% w/v) five times to remove planktonic cells and the sessile cells were collected using a standard sterile cotton swab. The swabs were transferred to tubes containing peptone water (0.1% w/v) then agitated in a vortex for 2 min. After this procedure, serial dilutions of the samples were carried out in which 0.1 mL aliquots were plated and the number of viable cells quantified in TSA (Triptic Soy Agar), using the surface seeding technique. The plates were incubated at 37°C/24 h, conducting a standard plate count at the end of this period, and results were expressed in CFU cm^2 (Joseph et al., 2001).

Obtainment of essential oils

The essential oils of *S. aromaticum* and *T. vulgaris* were purchased through the Ferquima Ind. e Com. Ltda Company (Vargem Grande Paulista, São Paulo, Brazil), their physical and chemical parameters being described by the supplier, which produces and sells essential oils on an industrial scale.

Identification and quantification of chemical constituents

The essential oils chemical components were identified by gas chromatography coupled with mass spectrometry (GC–MS). A Shimadzu Gas Chromatograph (model GC 17A) equipped with a mass selective detector (model QP 5000) was operated under the following conditions: fused silica capillary column (30 m x 0.25 mm) coated with a DB-5 MS stationary phase; ion source temperature of 220°C; column temperature programmed at an initial temperature of 40°C, and increased by 3°C/min up to 240°C; helium carrier gas (1 ml/min); initial column pressure of 100.2 kPa; split ratio of 1:10 and volume injected of 1 µl (1% solution in dichloromethane). The following conditions were used for the mass spectrometer (MS): impact energy of 70 eV; decomposition velocity of 1000, decom-position interval of 0.50 and fragments of 45 Daltons and 450 Daltons decomposed. A mixture of linear hydrocarbons (C_9H_20; $C_{10}H_{22}$; $C_{11}H_{24}$;…$C_{24}H_50$; $C_{25}H_{52}$; $C_{26}H_{54}$) was injected under identical conditions. The mass spectra obtained were compared with those of the database (Wiley, 229), and the Kovat's retention index (KI) calculated for each peak was compared with the values according to Adams (2007).

Determination of the minimum inhibitory concentration of the essential oils

The minimum inhibitory concentration (MIC) of essential oils was determined using the technique of disk diffusion in agar proposed by NCCLS (M7-A6) (2003) with modifications. The essential oils were diluted in Dimethyl Sulfoxide (DMSO) at different concen-trations (0.5, 1.5, 2.5, 5.0, 10.0, 15.0, 25.0, and 50.0%) and with DMSO control. The bacterial inoculum (in TSB) were added to vials containing TSA, the cell concentration was standardized to approxi-mately 10^8 $CFU.mL^{-1}$, and the inocolum was poured directly into sterile Petri dishes (150 mm). After solidification, a volume of 5 µL of each essential oil was dispensed on filter paper discs 6 mm in diameter, which were placed on TSA inoculated. The plates were incubated in B.O.D. at 37°C for 24 h (Ogunwande et al., 2005). The diameters of the inhibition halos formed were measured using a caliper rule. The analyses were conducted in triplicate.

Preparation of sanitizing solutions

To perform the sessile cell sensitivity test, four sanitizing solutions were formulated containing saline (NaCl 0.85% w/v), ethanol (p.a.95% v/v) and essential oil as shown in Table 1.

All sanitizing solutions contained a total volume of 10 mL and the amount of essential oils used in the formulation of the sanitizing solutions was defined from the minimum inhibitory concentration test results previously performed by disk diffusion technique with modifications (Ogunwande et al., 2005).

Treatment of biofilms with sanitizing solutions containing essential oils at different contact times

On the tenth day of cultivation, polypropylene and steel coupons

Table 1. Composition of the sanitizing solutions.

Sanitizing solutions	Composition (mL)		
	Saline solution	Ethanol	Essential oil
Control	8.00	2.00	0.00
S. aromaticum	8.00	1.99	0.01
T. vulgaris	8.00	1.99	0.01
Combination*	8.00	1.99	0.005

*Combination of oils *of S. aromaticum* and *T. vulgaris* at a 1:1 proportion.

Table 2. Number of sessile cells (Log CFU.cm^{-2}) of *Staphylococcus aureus*, quantified during biofilm formation on of AISI 304 stainless steel and polypropylene surfaces, with incubation at 37°C and TSB culture medium as substrate.

Time (hours)	Stainless steel (log CFU.cm^{-2})	Polypropylene (log CFU.cm^{-2})
48	6.13 ± 0.42^{a}	6.13 ± 0.54^{a}
96	6.17 ± 0.33^{a}	6.12 ± 0.48^{a}
144	6.57 ± 0.44^{a}	6.56 ± 0.49^{a}
192	6.89 ± 0.17^{a}	6.64 ± 0.49^{a}
240	7.38 ± 0.12^{b}	6.58 ± 0.05^{a}

**Results are expressed as mean ± standard deviation. Means followed by different letters in the same line differ by the Tukey test at 5% probability.

were taken from each Petri dish, rinsed in 0.1% peptone water (v/v) five times to eliminate non-adherent cells, and immersed in the above sanitizing solutions for 5 and 10 min at room temperature. After the sanitizing, the coupons were rubbed with standardized sterile swabs. The swabs were transferred to tubes containing 0.1% peptone water (v/v) and then agitated in a vortex for two minutes. After this procedure, serial dilution was conducted, 0.1 mL aliquots were plated and the number of viable cells determined in TSA medium, using the surface seeding technique. The plates were incubated at 37°C for approximately 24 h, conducting the standard plate count at the end of this period with, results expressed in CFU cm^{2} according to the method previously described by Joseph et al. (2001) with modifications.

Experimental design and statistical analysis

A completely randomized design (CRD) was used in a 2 x 5 factorial outline with 3 replicates, the surface factor having 2 levels: stainless steel and polypropylene, the time factor with 5 quantitative levels: 48, 96, 144, 192 and 240 h. The enumeration of adhered cells on the stainless steel and polypropylene coupons after treatment with these sanitizing solutions at different contact times, used the CRD in a factorial scheme (4 x 2 x 2) with 3 replicates with the factor agents at four qualitative levels: control, *S. aromaticum*, *T. vulgaris* and combination, the factor surfaces with two qualitative levels: stainless steel and polypropylene, and the time factor with 2 quantitative levels: 5 and 10 min. The statistical analyses were conducted utilizing the SISVAR program version 4.6 (Ferreira, 2008), R Development Core Team programs (R Development Core Team, 2004). For comparison of the averages, the Tukey test at the5% of probability level was used.

RESULTS AND DISCUSSION

Table 2 shows the counts of sessile cells that adhered to the surfaces of the AISI 304 stainless steel and polypropylene coupons during the biofilm formation process.

The adhesion of bacterial cells depends on factors such as physiology and cell morphology and physico-chemical properties of the contact surface. Gram nega-tive micro-organisms have greater ease of adhesion on surfaces as compared to Gram positive, as they have pili, flagella and fimbriae, as well as an outer membrane. Micro-organisms electrically charged with negative charges have more difficulty to link directly to surfaces. The participation of a conditioning film formed by various compounds and molecules from the aqueous phase, will be decisive (Boari et al., 2009; Van der Mei et al., 2003).

It can be observed that the microbial cells adhered similarly to both surfaces up to 192 h and differed significantly only at the end of the biofilm formation process, that is, at 240 h. The adhesion of bacterial cells depends on factors such as physiology and cell morphology and physicochemical properties of the contact surface.

The ability of *S. aureus* to adhere to solid surfaces producing compounds by multilayered cells embedded in a exopolysaccharide matrix is considered one of the relevant aspects of the epidemiology of this bacterium (Cucarella et al., 2001; Flach and Karnopp, 2005). This organization is extremely beneficial to all species of microorganisms, because it provides protection against adversity such as dehydration, colonization by bacteriophages and antimicrobial resistance (Gilbert et al., 2003).

By the phenomenon known as passivation, chromium, due to its high affinity for oxygen, tends to combine with it, forming a thin layer of chromium oxide with an approximate thickness of 40 Å. This passive layer is responsible for the corrosion resistance and the hydrophobicity of stainless steel. In this context, in the case of initial adhesion, the more hydrophobic the bacterial cell, the greater its ability to bind directly to this surface. Similar considerations were observed by Meylheuc et al. (2006) and Sheng et al. (2007). Thus, surfaces considered hydrophobic, such as stainless steel, allow the adhesion to occur more easily than less hydrophobic or hydrophilic surfaces, which is evidenced by counts and electron micrographs, which show improved adhesion of the cells on the surface of stainless steel at the end of 240 h incubation as compared to the polypropylene surface.

In studies conducted by Boari et al. (2009), that consisted of evaluating *S. aureus* biofilm formation on stainless steel using milk as substrate and different growing conditions, biofilm formation by *S. aureus* was observed by scanning electron micrographs in all conditions tested, revealing the adhesion ability of this bacterium mainly to the stainless steel surface, which was also observed in electron micrographs of the present study. In a review by Chmielewski and Frank (2003), it is shown that a layer of organic matter on the surface can promote and facilitate bacterial adhesion. Moreover,

Table 3. Number of *S. aureus* sessile cells (Log CFU.cm^{-2}) quantified on of AISI 304 stainless steel and polypropylene surfaces at 240 h of biofilm formation after treatment with the control sanitizing solution and the essential oil-based sanitizing solutions.

Sanitizing agents	Surfaces and exposure times			
	Stainless steel (log CFU.cm^{-2})		Polypropylene (log CFU.cm^{-2})	
	5 minutes	10 minutes	5 minutes	10 minutes
Control	7.09±0.06^{dB}	6.68±0.27$^{c\alpha}$	6.32±0.02^{dA}	6.23±0.03$^{c\alpha}$
S. aromaticum	5.25±0.50^{cB}	4.23±0.36$^{b\alpha}$	4.58±0.71^{cA}	4.27±0.57$^{b\alpha}$
T. vulgaris	3.89±0.62^{bA}	0±0.00$^{a\alpha}$	3.47±0.01^{bA}	0±0.00$^{a\alpha}$
Combination	0±0.00^{aA}	0±0.00$^{a\alpha}$	0±0.00^{aA}	0±0.00$^{a\alpha}$

*Results expressed as mean sessile cell counts (Log CFU.cm^{-2}). Means followed by different lower case Latin letters in the same column differ by Tukey test at 5% probability, means followed by different uppercase Latin letters on the same line differ by Tukey test at 5% probability; means followed by different Greek letters on the same line differ by Tukey test at 5% probability.

Table 4. Reduction of the number of *S. aureus* cells (log CFU.cm^{-2}) and in percentage, quantified on the AISI 304 stainless steel and polypropylene surfaces, at 240 h of biofilm formation after treatment with the control sanitizing solution and essential oil-based sanitizing solutions.

Sanitizing agents	Surfaces and exposure time			
	Stainless steel (log CFU.cm^{-2}) - %		Polypropylene (log CFU.cm^{-2}) - %	
	5 min	10 min	5 min	10 min
Control	0.29–3.9%^{aA}	0.70–9.4%$^{a\alpha}$	0.26–3.9%^{aA}	0.35–5.3%$^{a\alpha}$
S. aromaticum	2.13–28.8%^{bA}	3.15–42.6%$^{b\beta}$	2.00–30.3%^{bA}	2.31-35.1%$^{b\alpha}$
T. vulgaris	3.49–47.2%^{cA}	7.38–100%$^{c\beta}$	3.11-47.2%^{cA}	6.58–100%$^{c\alpha}$
Combination	7.38–100%^{dB}	7.38–100%$^{c\beta}$	6.58–100%^{dA}	6.58–100%$^{c\alpha}$

*Values were obtained from subtracting the number of adhered cells without any sanitizing treatment of stainless steel (7.38 Log CFU.cm^{-2}) and polypropylene coupons (6.58 Log CFU.cm^{-2}), at 240 h of biofilm formation after treatment with the control sanitizing solution and the essential oil-based sanitizing solutions. Means followed by different lowercase Latin letters in the same column differ by Tukey test at 5% probability, means followed by different uppercase Latin letters on the same line differ by the Tukey test at 5% probability; means followed by different Greek letters in the same line differ by the Tukey test at 5% probability.

these authors state that the time of contact between cells and surfaces also influence the bacterial adhesion. The irreversible cell adhesion to surfaces occurs between 20 min and a maximum of 4 h of contact. After this period, the removal of these cells requires the application of physical force, chemicals or heat. In this present study, it is possible to observe that the bacterial cells have obtained adhesion to the stainless steel and polypropylene surfaces from 48 h and increasing, to a small extent, up to 240 h.

The probability of cells remaining irreversibly attached after sanitation procedures is high and corresponds to one of the main reasons for the formation of biofilms on surfaces that come into contact with food, becoming a constant source of contamination.

Table 3 presents the counts of sessile cells adhered to the surfaces of the AISI 304 stainless steel and polypropylene coupons after treatment with the control sanitizing solution and the essential oil-based sanitizing solutions. Table 4 shows the reduction percentage of the number of sessile cells after treatment with the sanitizing solutions.

The effectiveness of the sanitizing solutions containing essential oils can be observed by the counts obtained after treatment of coupons on both surfaces and the reduction percentage of these cells. A significant difference in the counts and reduction percentage of adhered sessile cells can be noted among the different treatments. All sanitizing solutions based on essential oils showed more superior antimicrobial activity than the control sanitizing solution.

The effectiveness of the sanitizing solutions based on *S. aromaticum*, *T. vulgaris*, and their combination differ significantly from each other, their combination being the most effective to reduce the number of sessile cells adhered to the surfaces. It can be observed that the five-minute exposure of the coupons containing the biofilm to the sanitizing solution based on the combination of oils was effective in promoting non-recovery of viable cells that adhered to both surfaces.

The sanitizing solution based on *T. vulgaris* was more effective as compared to *S. aromaticum*. This solution allowed the non-recovery of viable cells after exposure for 10 min to both surfaces. The sanitizing solution based on the essential oil of *S. aromaticum* was less effective, presenting a reduction in the number of sessile cells, but after 10 min of stainless steel and polypropylene coupon exposure to this solution, viable cells were still recovered.

As *S. aureus* is Gram positive, it is concluded that the cell wall does not serve as a barrier to the entrance of such antibacterial compounds through the cytoplasmic membrane. Since the cell wall of these bacteria is permeable, usually it does not restrict the penetration of these sanitizing agents (Schaffer and Messner, 2005).

The difference between the performance of the sanitizing solutions within each phase of biofilm formation analyzed can be attributed to environmental and growth factors that are related to the concentration and nature of the chemical constituents, such as composition, functional groups and the structural configuration of the essential oil components (Chang et al., 2001).

The effect of the essential oil on the target microorganism was considerably reduced when applied in the food model (as compared to *in vitro* studies). The application of essential oils for the control of pathogens and spoilage bacteria requires the evaluation of their effectiveness in food products or models that roughly simulate the composition of foods. Generally, the efficiency of some additives and natural antimicrobial agents can be reduced by certain components of foods. If higher concentrations of essential oils are generally required when added to food to maintain product safety, undesirable flavor and sensory changes may occur (Gutierrez et al., 2009). Researchers who have evaluated the effect of essential oil added to meat reported undesirable sensory changes caused by essential oil treatment in food samples (Govaris et al., 2010).

Brugnera (2011) evaluated the antibacterial effect of *O. vulgare* and *S. officinalis* against the growth and production of enterotoxin A by *S. aureus* inoculated in creamy ricotta, as well as the sonsorial acceptance of the ricottas with these spices added. As for the sensorial aspects, there was a higher preference for the ricottas with low spice concentrations.

The effects of colonization of surfaces where food is processed can result in various problems, because of an economic or public health nature. On the economic front spoilage bacteria can contaminate food by changing its characteristics and resulting in economic losses. The risk to public health is the most serious problem, because the biofilm can transport pathogenic microorganisms and be a source of chronic contamination (Ribeiro-Furtini, 2005).

This study led to the description of the sanitizing solutions essential oils of *S. aromaticum* and *T. vulgaris* and their combination on biofilm formed by *S. aureus* (ATCC 25923) on AISI 304 stainless steel and polypropylene surfaces. All solutions showed potential antibacterial sanitizers, being effective in reducing bacterial biofilms on these surfaces. The solution containing the combination of essential oils was more efficient by reducing 7.38 and 6.58 Log $CFU.cm^{-2}$ cells that adhered on the surfaces of AISI 304 stainless steel and polypropylene respectively, after 5 min of contact.

It was also observed that the total reduction in the number of surface-adhered cells presented by the disinfectant solution based on the combination of *S. aromaticum* and *T. vulgaris* essential oils at 240 h of biofilm formation (Table 4) emphasizes the synergistic action of the essential oils utilized. The term synergism can be defined as increase in the activity of compounds or factors when applied together, as compared to their individual activity (Ceylan and Fung, 2004). The study on synergism resulting from the combination of essential oils of different plant species was carried out *in vitro*, presenting promising results (Al-Bayati, 2008). However, no report has been found on the synergistic action of a combination of essential oils against surface-adhered bacteria.

S. aureus and *L. monocytogenes* are Gram positive bacteria, which can facilitate the action of the oils; in other words, there is high incorporation of the additive into the cell wall (Harpaz et al., 2003). In a study using the same test conducted *in vitro*, Dorman and Deans (2000) used essential oils of clove, oregano, geranium and pepper to evaluate their activity on 25 species of Gram positive and Gram negative bacteria. The authors observed that Gram positive bacteria were more susceptible to the essential oils studied than the Gram negative bacteria.

In conclusion, our findings suggested that *S. aromaticum* and *T. vulgaris* essential oils are new alternatives to sanitize industrial stainless steel surfaces contaminated by *S. aureus*. Their synergistic effect must not be ignored, as it can enhance the individual antibacterial activity of these compounds.

Conflict of Interests

The authors have not declared any conflict of interests.

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In vitro susceptibilities of the clinical isolate of *Entamoeba histolytica* to *Euphorbia hirta* (Euphorbiaceae) aqueous extract and fractions

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***Euphorbia hirta* (*Euphorbiaceae*) has been used widely in traditional medicine as a treatment against infectious pathogens. This medicinal plant is also well known for its diverse biological activities. The present study aimed to evaluate the susceptibilities of the clinical isolates of *Entamoeba histolytica* to *E. hirta* aqueous extract, methanol fraction, methylene chloride fraction, and hexane fraction. The clinical isolates of *E. histolytica* grown on polyxenic medium were treated with *E. hirta* aqueous extract (AE), methanol fraction (MF), methylene chloride fraction (CH_2Cl_2 F), and hexane fraction (HF). Metronidazole (MTZ) was used as the reference drug. Furthermore, the effects of the extract as well as the fractions on the activity of *E.* histolytica ribonuclease (RNase), aldolase, acid and alkaline phosphatases (ACP and ALP) were evaluated. The methanol fraction of *E. hirta* inhibits significantly the clinical isolate of *E. histolytica* growth with the IC_{50} of 67.18 ± 7.40 µg/ml after 72 h of incubation but remains lowers compared to metronidazole (IC_{50}<10 µg/ml). The aqueous extract and methylene chloride showed moderate activities, whereas no amoebicidal activity was found associated to the hexane fraction. The enzymes activity assay showed that the inhibitory effect of the methanol fraction against *E. histolytica* RNase, aldolase acid and alkaline phosphatases activities were comparable to that of metronidazole and significantly higher than those of aqueous extract and methylene chloride fraction. According to the above mentioned results, the methanol fraction of *E. hirta* exhibits antiamoebic activity and inhibition of enzymes involved in the metabolism or survival of *E. histolytica*.**

Key words: *Entamoeba histolytica*; *Euphorbia hirta*; Antiamoebic activity.

INTRODUCTION

Amoebiasis is caused by a protozoan parasite, *Entamoeba histolytica* with or without clinical symptoms and it is the third leading cause of death from parasitic diseases after malaria and schistosomiasis(WHO, 1997). This infection remains a major health problem in developing countries and its prevalence varies between countries and between regions with different socio-economic conditions (Jackson, 2000). Sometimes, it may reach 50% of the

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population in regions with poor sanitary conditions (Caballero et al., 1994). The most effective and commonly used drug for treatment of this intestinal protozoan infection is metronidazole (MTZ). However, this drug has been reported to have unpleasant side effects such as metallic taste, headache, dry mouth, and to a lesser extent nausea, glossitis, urticaria, pruritus, and dark colored urine. Carcinogenic, teratogenic, and embryogenic effects have also been documented (Upcroft et al., 1999; Upcroft and Upcroft, 2001) in addition to the fact that it may lower both cell mediated and humoral immune responses in drugs recipients (Saxena et al., 1985). Therefore, immunocompromised recipients of MTZ including those with AIDS (full form) would constitute a high risk. According to these observations above, the search of alternative anti-amoebic compounds with high activity, and low toxicity is still necessary. Segment of the world's population relies on traditional remedies to treat a plethora of diseases. Medicinal herbs constitute an indispensable part of traditional medicine practised all over the world due to the cost, easy access, and ancestral experiences (Martini-Bertolo, 1980).*Euphorbia hirta* belongs to the genus Euphorbia and the family of Euphorbiaceae. It is a common herb in the pan-tropic and partly subtropic areas worldwide, including China, India, Philippines, Australia, Africa, Malaysia, and so on(Huang et al., 2012). *E. hirta* is an important medicinal herb with various pharmacological behaviours. The flavonol glycosides afzelin, quercitin and myricitrin, isolated from *E. hirta* showed inhibition of the proliferation of *Plasmodium falcifarum* at different concentrations (Jackson, 2000). The leaves, flowers, stems, and root extract of the plant exhibited antimicrobial activity against *E. coli*, *C. albicans*, *S. aureus*, and *P. mirabilis* (Mohammad et al., 2010). The antidiarrheal effect of the *E. hirta* herb decoction was studied in mice. It demonstrated an activity in models of diarrhea induced by castor oil, arachidonic acid, and prostaglandin E 2. Quercitrin, a flavonoid isolated from this crude drug contributed to the antidiarrheal activity at a dose of 50 mg/kg, against castor oil and prostaglandin E2-induced diarrhea in mice (Galvez et al., 1993).The *in vitro* and *in vivo* immunomodulatory properties of *E. hirta* is reported elsewhere (Ramesh and Vijaya, 2010). The finding has been proven through macrophage activity testing, carbon clearance test, and mast cell de-granulation assay. The aqueous extract of the leaves of *E. hirta* Linn could serve as an immunostimulant on the experiment of thepathogen-infected *Cyprinuscarpio* Linn. (Cyprinidae). The antiinflammatory activity of the chemicals in *E. hirta* showed that the flavonoids quercitrin (converted to quercetin in the alimentary canal) and myricitrin, as well as the sterols 24-methylenecycloartenol and sitosterol, exert noteworthy and dose dependent anti-inflammatory activity. Triterpene beta-amyrin also seems to exert a similar anti-inflammatory activity (Ekpo and Pretorius, 2007).The crude and polyphenolic extracts of *E. hirta* exhibited antiamoebic potential (Tona et al., 2000). However, the activity of the fractions obtained from this plant have not yet been tested against *E. histolytica*. Therefore, the present study was planned to investigate the *in vitro* susceptibilities of the clinical isolates of *E. histolytica* to *E. hirta* (euphorbiaceae) aqueous extract and fractions.

MATERIALS AND METHODS

Biological materials

Plant material

The aerial part of *E. hirta* (Euphorbiaceae) collected in Yaounde (Cameroon) on April 2011 during morning time was used in the present study.

Microorganisms

Clinical isolates of *E. histolytica* trophozoites from stool sample of Indian patients suffering from amoebiasis collected at the Department of Medical Parasitology of the Postgraduate Institute of Medical Education and Research (PGIMER) of Chandigarh, India were used for polyxenic cultivation.

Euphorbia hirta extract and fractions preparation

The aerial (leaves and stems) part of *E. hirta* was harvested, washed in chlorinated water and dried at room temperature (Moundipa et al., 2005). Dried materials were reduced in powder form and the extract was obtained by decoction of 200 g of the powder in 1000 ml of distilled water for 3 h. Decoction obtained was concentrated in the oven at 50°C for 72 h and the concentrated product constituted the aqueous extract. The extraction yield was calculated according to the following formula:

$$\text{Extraction yield (\%)} = \frac{mas\,sof extractobtained}{massof powderintroduced} \times 100$$

Fractionation of the aqueous extract was performed by using different polarity based solvent from hexane (nonpolar) to methanol (polar) (Zubair et al., 2011). To obtain the hexane fraction (HF), 200 g of the above aqueous extract was macerated in 1000 ml of pure hexane until exhaustion of the solvent. Resulting solution was concentrated using a BüchiRotavapor R-210/R-215 with the temperature of 40°C. The solid material obtained after a total evaporation of the solvent was conserved and constituted our hexane fraction. Then, methylenechloride fraction (CH_2Cl_2 F) was obtained by subjecting the residue to a second maceration in the same volume of pure methylene chloride followed by the concentration as described previously. The residue obtained from the second step was extracted with1000 ml of pure methanol as described above to obtain the methanol fraction (MF). Crude aqueous extract and fractions obtained were subjected to *in vitro* assays for the determination of theirantiamoebic potential.

E. histolytica cultivation

Polyxenic cultivation

Biphasic medium of Boeck and Drbohlav (Parija and Rao, 1995) that involves solid phase (ringer's solution + egg) and liquid phase (lock's solution containing nutrients) was used for *E. histolytica* poloyxenic cultivation. Before inoculation, complete media were pre

incubated at 37°C for 30 min to 1 h and 1000 µl of diarrheal stool sample containing viable trophozoïtes of *E. histolytica* were introduced in each tube. The tubes were incubated at 37°C and the *E. histolytica* growth verified after every 48 or 72 h. Then, the tubes were removed from the incubator and shacked to detach parasites from the solid phase and left for 5 min then the supernatant was decanted to obtain the subculture. The pellet containing the parasites was introduced in a tube containing pre incubated new medium as previously described (Moundipa et al., 2005).

Amoebicidal effect of the *E. hirta* extract and fractions assays

E. hirta aqueous extract (AE) and its fractions (AE, MF, CH_2Cl_2 F) were prepared using sterile DMSO (Sigma-Aldrich, and culture medium leading to concentrations of 200, 20, 2 and 0.2 mg/ml respectively). Each mixture was filtered with sterile syringe filters (Ø 22 µm) and aliquots were prepared from these stock solutions. Parasites grown were harvested at midlog phase at the concentration of 10^7cells/ml of culture by counting using the haemocytometer (Neubauer,Hausser Scientific) and inoculated in tubes containing new 5 ml media in which 25 µl of plant materials were added. MTZ was used as a standard drug and was tested at 0.1, 1, 5 and 10 µg/ml. AE was tested at the concentration of 50, 100, 200 and 400 µg/ml; whereas all the different fractions were tested at 25, 50,100 and 200 µg/ml. One control tube was used in which parasites were incubated on culture medium containing 0.5% DMSO without any drug. Each testing concentration was made in triplicate and the experiment was repeated three times for each compound. All the tested tubes were incubated at 37°C as described by Chitravanshi et al., 1992 and the viability was evaluated by trypan blue method after 24, 48 and 72 h.

Amoebicidal activity was evaluated using the method described by Bansal (1987). In 1.5 ml micro centrifuge tube, 25 µl of parasite suspension and 225 µl of 0.4% trypan blue solution prepared in 0.9% NaCl was introduced. The mixture was homogenized and 10 µl of this mixture was used for cells counting. The chamber was covered with cover slip and the viable (bright) cells as well as the dead (blue) cells were counted at 40X on a light microscope. The concentration of the cell has been calculated using the following formula:

$$N= (n\times d)/v$$

Where, N= concentration of viable cells/ml; n= number of the viable cells counted in the chamber, d= dilution factor and v= the volume of the chamber (0.1 µl)

The percentages of inhibition were calculated also using the formula below and IC_{50} were determined using the software Graphpad Prism 3.0

$$\text{Percentage inhibition (\%)} = (N_C-N_T)/N_C \times 100$$

N_C = Number of viable cells in the control tube and N_T= Number of viable cells in testing tube.

Effect of *E. hirta* extract and fractions on some enzymes of *E. histolytica*

The effect of extract, fractions and metronidazole on some enzyme activities of polyxenically cultivated clinical isolate of *E. histolytica* was studied as described (Sohni et al., 1995).

Amoeba from different tubes of each type of culture were pooled and washed five times with cold phosphate-buffered saline then centrifuged at /min for 10 min. The sediment was suspended in cold phosphate-buffered saline to yield a concentration of 10^6cells/ml following which the suspension was homogenized in cold 0.25 M sucrose solution for 3 h at 4°C. The homogenate was then centrifuged in cold and opalescent supernatant was used as the crude enzyme extract. The amount of total protein in the crude enzyme extract was determined according to the protein colorimetric method assay described by Bradford (1976). All the assays compounds (extract, fractions and metronidazole) were tested at the concentration of 100, 200, 400, and 800 µg/ml. Each concentration was made in triplicate and the experiments were repeated three times.

RNase activity assay

A micro centrifuge tube with a capacity of 1.5 ml was used for preparing the reaction mixture of 1.25 ml containing 150 µM of phosphate buffer (pH 7.6), 1.25 mg of yeast RNA, 0.5 ml of enzyme extract and the testing drug (Sparh and Hollingworth, 1961). The mixture was incubated at 37°C for 1 h. The reaction was stopped by the addition of 0.25 ml of uranyl acetate reagent. The suspension was mixed and chilled for 30 min to precipitate undigested RNA. The precipitate was centrifuged and the absorbance was read at 260 nm against blanks. An enzyme unit was defined as the amount of enzyme which gives an increase in absorbance of 0.1.

Aldolase activity assay

Aldolase activity was assayed according to the method describe by Sibley and Lehninger (1949) with some modifications. In a 1.5 ml micro centrifuge tube, a reaction mixture of 1.25 ml containing 0.5 ml of Tris buffer (pH 8.6), 0.125 ml of 0.05 M fructose-1,6-diphosphate, 0.125 ml of 0.0035 M hydrazine sulphate solution in 100 µM EDTA (pH 7.5) 0.25 ml of enzyme extract and the testing drugs was introduced. The mixture was incubated at 37°C for 30 min. The reaction was stopped by addition of 10% trichloroacetic acid (TCA) and the absorbance of supernatant was read after at 240 nm against blanks. An enzyme unit was defined as the amount of enzyme which gives an increase in absorbance of 1.00.

Acid and alkaline phosphatase (ACP and ALP) activity assay

Aldolase activity was assayed according to the method described by Ashrafi et al.(1969). For the ALP analysis, a reaction mixture containing 50 µl of enzyme extract, 100 µl of substrate p-nitrophenylphosphate (PNP) 1% in 0.1 M glycine/NaOH buffer pH 9, as well as the tested compounds were prepared in a micro plate of 96 wells and the final volumes were adjusted to 250 µl. The reaction mixtures for the ACP activity assay contained 50 µl of enzyme extract, 100 µl of 1% substrate PNP in citrate buffer pH 4, as well as the tested compounds were prepared in a micro plate of 96 wells and the final volumes were adjusted to 250 µl. Both ACP and ALP mixtures were incubated at 37°C for 30 min. The reaction was stopped by addition of 100 µl 0.1 N NaOH and the absorbance was read at 405 nm against blanks. An enzyme unit was defined as the amount of enzyme which catalyses the formation of 1µmol of p-nitrophenolate ion.

The specific activities were calculated for all the enzymes assayed according and the IC_{50} of the inhibition were determined using the software Graphpad Prism 5.0.

Statistical analysis

The tests were performed in triplicate and all data are presented as mean ± SD (standard deviation) values. Statistical analysis was performed using GraphPadPrism and student's t-test was used to determine P-values for the differences observed between test compounds and control. Results were considered significantly different when $P \leq 0.05$.

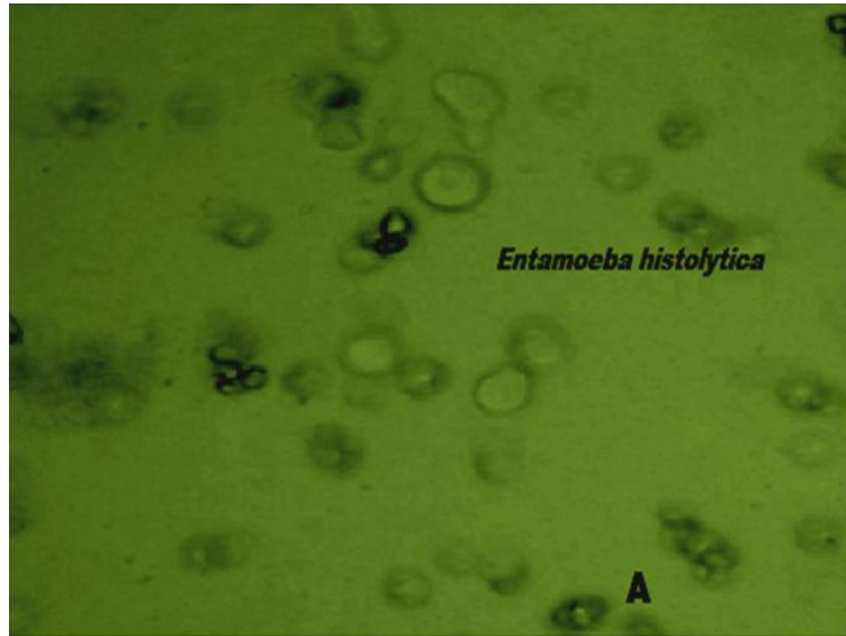

Figure 1. *Entamoeba histolytica* growing on polyxenic Boeck and Drbohlav medium (Pechangou January 30th 2013).

RESULTS

Amoebicidal effect of the *E. hirta* aqueous extract and fractions against polyxenic culture of clinical isolates of *E. histolytica*

The clinical isolates of *E. histolytica* grown maintained on biphasic medium of Boeck and Drbohlav (Figure 1) were incubated with different plant extract and fractions. MTZ and *E. hirta* aqueous extract, methanol fraction, methylene chloride fraction exhibited amoebicidal effects that were concentration dependant (Figure 2). In contrast no amoebicidal activity was found to be associated with HF. The IC_{50} values of MTZ and *E. hirta* AE, MF, and CH_2Cl_2 F were respectively about 4.30, 145.95, 67.18, and 194.04 µg/ml after 72 h of incubation. *E. hirta* MF exhibited higher amoebicidal effect than AE and CH_2Cl_2 F but, remained lower as compared to reference drug MTZ activity (Table 1).

Effect of *E. hirta* extract and fractions on some enzymes of *E. histolytica*

The results of the experiments on the effect of metronidazole and *E. hirta* AE, MF, and CH_2Cl_2 F on enzymes of *E. histolytica* by mean of specific activities is presented in Figure 3. Specific activity was defined as the enzyme unit per milligram of protein. The specific activity units of compound containing reactions were converted into a percentage basis by correlating with those of controls, the inhibitory effect of MF is significantly higher than those of AE and CH_2Cl_2 F, and comparable to that of metronidazole for all the enzymes assayed. The higher inhibition percentage observed with MF was on ACP activity (73.0435 ± 2.30%) at the concentration of 800 µg/ml. The lower percentage observed with the same fraction was on ALP activity (52.2 ± 1.42 %) (Table 2).

DISCUSSION

Clinical isolates of *E. histolytica* were collected from patients with intestinal amoebiasis and cultivated on polyxenic Boeck and Drbohlav medium. Figure 1 shows the presence of *E. histolytica* strain in culture. For the antiamoebic assays HM1:IMSS strain cultivated in axenic culture is mainly used. However in this study, clinical isolates cultivated in polyxenic culture are used as it is well documented that *E. histolytica* is more virulent in association with suitable bacterial cells (Bracha and Mirelman, 1984; Wittner and Rosenbaum, 1970).

It is apparent from the data presented in Figures 2 and 3 that MF of *E. hirta* is more efficient than CH_2Cl_2 F and AE. After 48 h of incubation, the amoebicidal effect of the AE decreasesd. This may be attributed to the resistance of *E. histolytica* against the extract. The same observation was made by Moundipa et al. (2005) with the same extract. The antiamoebic effect of polyphenolic extract of *E. hirta* has been elucidated by Tona et al. (2000). The polyphenols are the major compounds found in the aerial part of *E. hirta* including phenols, flavonoids and alkaloids (Huang et al., 2012) which are

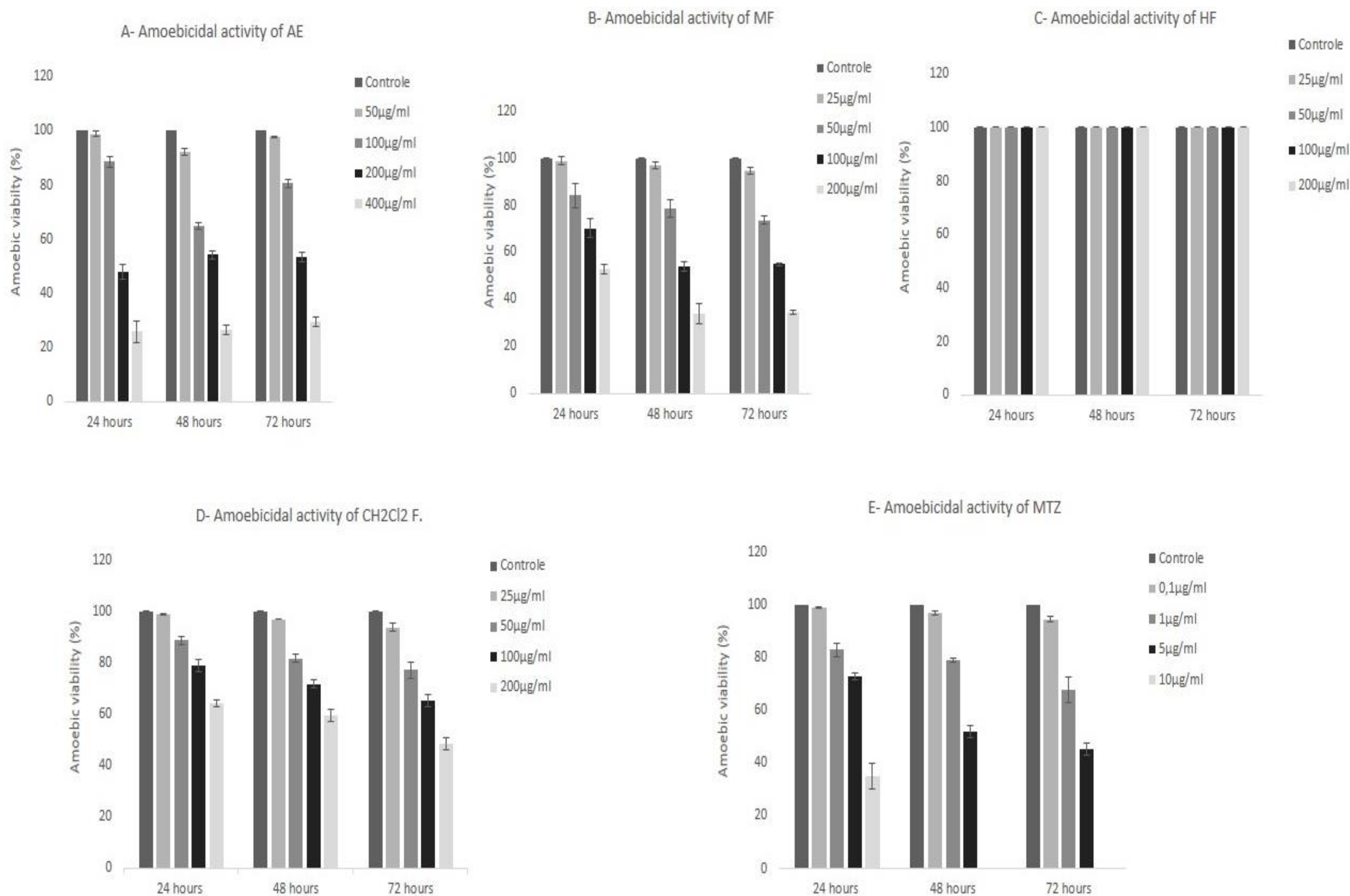

Figure 2. *In vitro* Amoebicidal effect of (A) aqueous extract, (B) methanol fraction, (C) Hexane fraction,(D) methylene chloride fraction of *E. hirta* and (E) metronidazol after 24, 48 and 72 h of incubation. These results are the average of three experiments by mean of standard deviation, repeated three times and compared to the controle (DMSO).

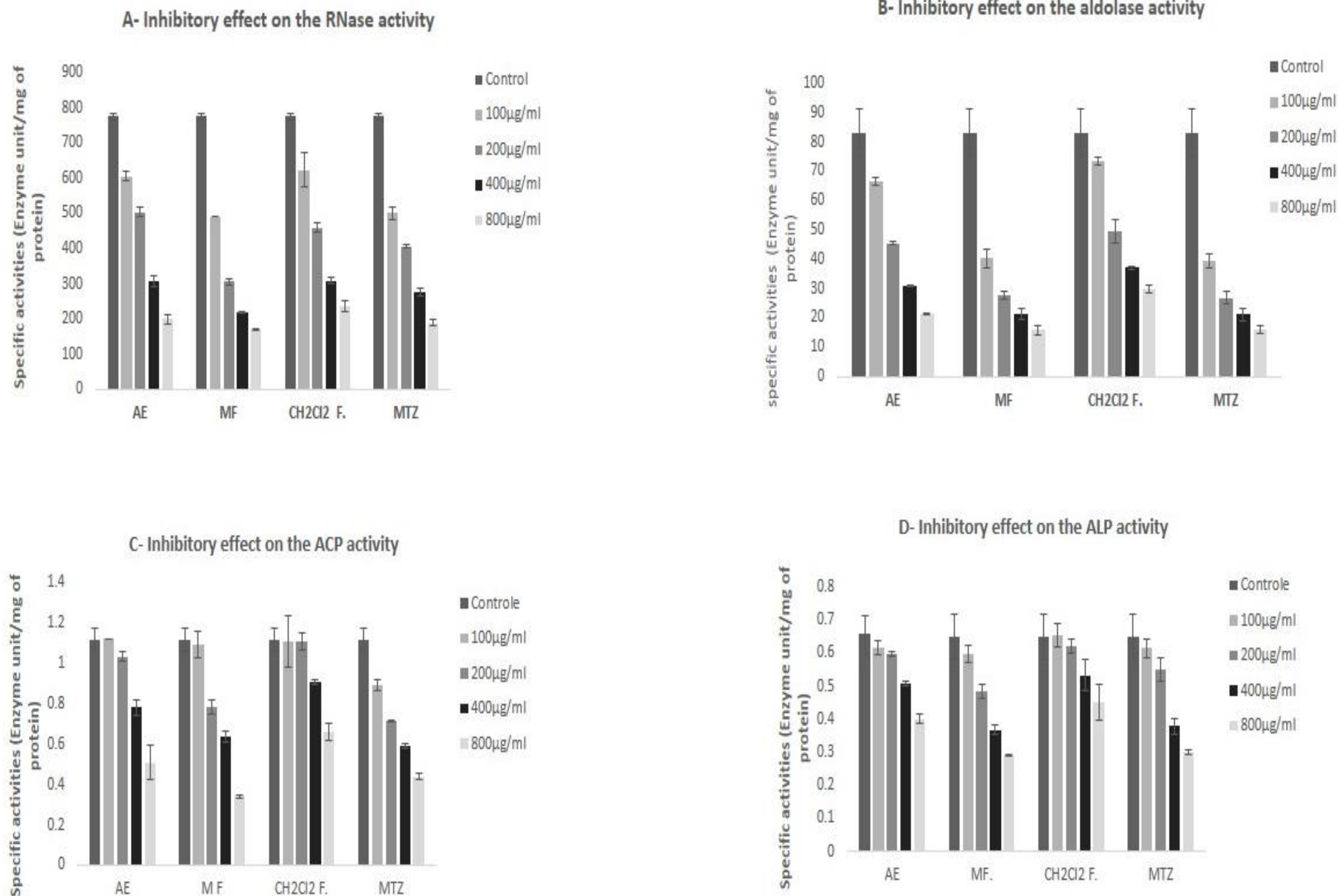

Figure 3. *In vitro* inhibitory effect of*E. hirta* extract/fractions and metronidazole on the activity of (A) RNase, (B) aldolase, (C) acid phosphatase (ACP), and (D) alkaline phosphatase (ALP). These results are the average of three experiments by mean of standard deviation, repeated three times and compared to the control.

Table 1. IC_{50} of the Amoebicidal effect of the aqueous extract, methanol fraction, methylene chloride fraction of *E. hirta* and metronidazole after 24, 48 and 72 h of incubation.

Compounds	IC_{50} (mean ± SD) (µg/ml)		
	24 h	48 h	72 h
AE	170.45 ± 15.91	103.9 ± 3.3	145.95 ± 8.98
MF	93.13 ± 9.06[a]	83.85 ± 4.37 [a]	67.18 ± 7.40 [a]
CH2Cl2 F	262.07 ± 9.01	238.22 ± 13.38	194.04 ± 7.81
HF	N	N	N
MTZ	8.33 ± 0.43[b]	5.12 ± 0.13 [b]	4.30 ± 0.48 [b]

a= significant difference between methanol fraction and other extract/fractions; b= significant difference between extract or fraction compared to metronidazole (n=3; p≤0.05). N= mean test non performed.

Table 2. IC50 values of the metronidazole and *E. hirta* aqueous extract and fractions on *E. histolytica* enzymes activity.

Compounds	IC 50 (mean ± SD) (µg/ml)			
	Aldolase	RNase	ACP	ALP
MTZ	463.71 ± 42.84[a]	658.32 ± 29.6 [a]	391.29 ± 17.83[a]	652.52 ± 11.51[a]
AE	569.50 ± 13.02	829.93 ± 10.04	783.58 ± 10.57	1081.01 ± 50.42
MF	400.19 ± 14.24[b]	639.93 ± 30.25 [b]	394.42 ± 1.59[b]	707.76 ± 14;79[b]
CH_2CL_2	816.54 ± 36.85	848.84 ± 37.18	922.49 ± 22.25	1300.77 ± 35.7

a= significant differencebetween extract or fraction compared to metronidazole; b= significant differencebetween methanol fraction and other extract/fractions (n=3; p≤0.05).

particularly polar compounds, thus found in greater amount in MF than CH_2Cl_2 F. It can also be noted that MTZ exhibited a significantly greater amoebicidal effect as compared to extract and fractions. This can be attributed to the crude nature of the extract and fractions. The antiamoebic activities of *E. hirta* studied previously (Moundipa et al., 2005, Tona et al., 2000) were focussed only on the amoebicidal effect based on the viability evaluation of amoeba in culture. However, in this study we also investigated the effect of *E. hirta* extracts on important bio-molecules of *E. histolytica* which are essential for its survival.

Several mechanisms for the pathogenesis of *E. histolytica* by which the parasite can engage in tissue damage are available. These mechanisms include secretion of enzymes and cell free cytotoxins, contact dependant cytolysis and phagocytosis (Sohni, et al., 1995). Some of the secreted enzymes which have been investigated in the present study are believed to play an important role in the virulence and survival of the parasite. The nucleopolymerases play an important role in the metabolism of all living cells. Amoebas which were fed with cholesterol have been shown to increase in lysosomal level of DNase and RNase activities (Narain, 1979). It is also well documented previously that cholesterol increases the virulence of *E. histolytica (*Meerovitch and Ghadirian, 1978).

There is significantly higher level of ACP than ALP in trophozoites of *E. histolytica* and axenically growth amoeba exhibit increased level of ACP activity (Sohni et al., 1995). ACP may play an important role in the utili-zation of phagocytised food materials. It is also demon-strated that ACP gene expression increases during invasion and cells lesions by *E. histolytica* suggesting that this enzyme plays an important role during tissue invasion by the pathogenic amoeba (Fernandes et al., 2014).

In the present study, clinical isolates of amoeba grown in poly-xenic medium exhibit also a higher level of ACP than ALP. It is apparent from the Figure 3 that MF of *E. hirta* has inhibitory effect non significantly different from that of MTZ for all the enzyme activities studied. CH_2Cl_2 F and AE have inhibitory effects lower than those of MF.

However further studies of MF of the aerial part of *E. hirta* are suggested, mainly to confirm the finding in axenic culture of *E. histolytica* and its effect on the activity and expression of the cysteine proteinase which is the main compound involved in virulence of *E. histolytica*. In future *in vivo* studies can be carried out to confirm the antiamoebic activity of *E. hirta* extracts, so that these can be used for therapeutics.

Conclusion

Methanol fraction of the aerial part of *E. hirta* exhibited higher antiamoebic activity than methylene chloride fractions and aqueous extract against clinical isolate of *E. histolytica*. The extract and fractions also had varying

degree of inhibition on enzymes of *E. histolytica* which are thought to play a role in its survival and virulence. The present finding justifies the use of *E. hirta* aerial part in the traditional medicine for the treatment of dysentery.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Biodegradation of cassava root sieviate with enzymes extracted from isolated fungi

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This study was carried out to investigate changes in proximate and chemical components of cassava root sieviates (CRS) treated with extracted enzymes obtained from (a) *Aspergillus niger (*An*)*; (b) *Rhizopus stolonifer (*Rs*);* (c) *Trichoderma viridae (*Tv*)* and (d) *Mucor mucedo (*Mm*)* applied on autoclaved CRS at 250 ml/kg. Another treatment had a commercial enzyme Roxazyme G2G (RG2G) as the degrading agent. At the end of the seventh day after enzyme application, crude protein, ash and metabolizable energy content increased but crude fibre, pectin, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), hemicellulose and cellulose content decreased. The highest value of metabolizable energy (ME), ash and crude protein were: 2807.81 kcal/kg, 17.32 and 18.32 g/100g dry matter, respectively obtained when enzymes obtained from *A. niger, T. viridae and A. niger* were added respectively. Results of levels of sugars (mg/mL) in undegraded CRS showed that glucose level increased by 72.4, 67.2, 53.8, 50.1 and 32.5% when enzyme preparations from *A. niger, T. viridae, R. stolonifer, M. mucedo* and Roxazyme G2G were applied on CRS respectively. Results obtained indicate that the use of enzymes from the above named fungi defiberised the CRS and hence promoted better bioavailability of the hidden nutrients in it.

Key words: Cassava root sieviate, enzymes, biodegradation, nutrient enhancement.

INTRODUCTION

Feed insecurity describes a situation whereby there is shortage of feed ingredients in quantity or in quality and this is aggravated by the ever widening demand for conventional feed sources in developing countries. No doubt, there is continuous need for animal protein and low availability of it is the chief factor known for the occurrence of child/infant morbidity, kwashiorkor, poor level of productivity and reduction of life span. Agromisa (2006) revealed that the state of consumption of meat and other animal protein in Nigeria is estimated at about 8 g per caput per day and this is about 27g less than the 35 g per caput minimum requirements recommended by the Food Agriculture Organization (FAO, 2003). Besides, in Nigeria, Olerede (2005) noted that poultry constitute over 90% of the current national livestock population and are of appreciable economic and social value to the investors and consumers. Large scale egg production is one of the ways to ensure better availability of animal protein. Regmi (2007)

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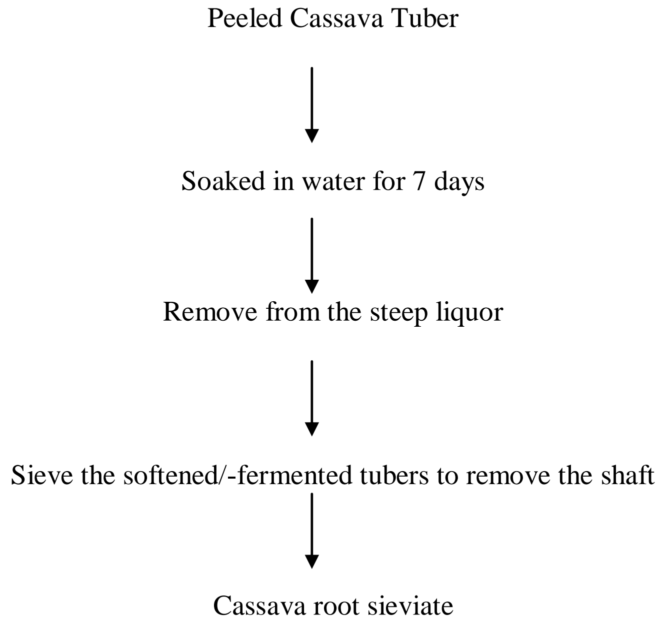

Figure 1. Flow chart for cassava roots sieviate production.

observed the unprecedented population growth that has occurred in the last half of the century has created an additional demand for meat and general food in developing countries. The prices of conventional feed ingredients keep rising steadily because of the competition between man and livestock and their increasing populations. The cost of feeds is as high as 70 to 75% of the total cost of production (Oluremi et al., 2007). In an attempt to overcome this challenge, several studies have been carried out on the possibility of using agro industrial by products and crop residues. Therefore, of late, agro industrial by-products are receiving attention. Meanwhile, agro industrial by-products (AIBs) that have their source from plants have cell walls which contain a variety of polysaccharides, the distribution of which varies within primary and secondary cell wall and between mono and dicotyledonous plants. The polymers are interlinked by covalent linkages or via non-carbohydrate compounds. The non-starch polysaccharides (NSPs) comprise 700-900 g/kg of the plant cell wall with the remaining being lignin, protein, fatty acids and waxes. Plant cell wall NSP is a diverse group of molecules with varying degree of water solubility, size and structure which may influence the rheological pro-perties of the gastro intestinal content (Taibipour and Kermanshahi, 2004; Yineth and Mario, 2014). AIBs constitute those parts of crops that are left after removal of value giving components. These residues still contain considerable amount of energy and protein which may be present as intracellular compounds (Huang et al., 2008). AIBs represent potential valuable and renewable resources which find application in various areas that include use as animal feed. AIBs have been successfully incorporated into poultry diets at various levels in developing countries with resultant effect on reduction of cost of feeding. The use alleviates the exi-sting critical situation of inadequate feed supply. Nume-rous successful studies on the supplementation of agro-industrial by-products with enzymes have been reviewed by several authors (Kelly-Yong et al., 2005; Khajavi et al., 2005). Lignin has been recognized as the chief barrier to monogastric digestion of cell wall structural polysaccha-rides (Kumar et al., 2009) and removal of lignin by chemical treatment enhances fibre digestibility (Oms-Oliu et al*.,* 2009). Lignin offers the recalcitrant and adamant pos-ture to fibre by the strength of its chemical nature though lignin may not be solely responsible for the variation in digestibility that is in AIBs (Pandey and Nagveni, 2007; Bachtar, 2005). In Nigeria, large quantities of CRS are produced by the cassava tuber processing industries (Aderemi, 2000). The aim of this study was to investigate possibility of defiberising the polymers that are interlinked by covalent linkages in the CRS and study the changes in proximate and sugars levels components of cassava root sieviates treated with extracted enzymes obtained from *Aspergillus niger (An)*, *Rhizopus stolonifer (Rs), Trichoderma viridae (Tv)* and *Mucor mucedo (Mm)* and to possibly recommend the most effective biodegrader of CRS among the four fungi and the commercial enzyme.

MATERIALS AND METHODS

The CRS used for this study was obtained from a cassava processing centre in Ibadan, Nigeria. Figure 1 shows the flow chart for cassava roots sieviate production. It was milled with a 0.84 mm sieve and autoclaving was done at 121°C for 15 min.

Sources of fungi used

R. stolonifer and *A. niger* were obtained by exposing a piece of moist bread and a dough made from cassava flour to the air for 24 h and then covered for 3 days. After the third day, growth of the fungi appeared on the bread and cassava dough. Pure *T. viride* was obtained from the Department of Microbiology, University of Ibadan.

Isolation of fungi

A sterile wire loop was used to collect the spores and the mycelia of the actively growing fungi. The spores and mycelia were then inoculated aseptically on the centre of sterile potatoes dextrose agar (PDA) plates. The spores and the mycelia were aseptically spread on the PDA plates using the sterile wire loop in a sterilized chamber. The inoculated plates were then incubated at 34°C in a Gallenkamp incubator. The PDA plates were examined for growth after 48 h. It was observed that there was mix culture of fungi. Spores from each fungus was aseptically isolated and subcultured on fresh sterile PDA and reincubated for 48 h. Pure culture was then obtained and the mycelium from this was put on slant of sterile PDA. The characterization of the obtained *A. niger* and *R. stolonifer* was known by the use of manual of Barnett and Hunter (1992).

Enzyme production procedure

Enzymes were produced by extraction from cassava root sieviate.

Table 1. Proximate analysis of undegraded and degraded cassava sieviates (g/100g DM).

Parameters	Control (undegraded)	CRS+An	CRS+Tv	CRS+Rs	CRS+Mm	CRS+RG	SEM	P-value
Crude protein	9.63^{e}	16.32^{a}	14.38^{c}	15.32^{b}	14.27^{c}	11.50^{d}	2.23	0.0033
Crude fibre	10.98^{a}	4.82^{d}	5.52^{d}	7.42^{c}	6.28^{cd}	8.73^{b}	0.85	0.0024
Ether extract	2.01	2.27	2.09	2.05	2.21	2.24	0.62	0.0830
Ash	9.63^{d}	17.47^{b}	15.32^{a}	14.76^{b}	11.21^{c}	11.52^{c}	2.85	0.0052
Nitrogen free extract	68.75^{a}	60.62^{d}	60.69^{d}	57.45^{e}	64.03^{c}	66.01^{b}	2.62	0.0033
Pectin	11.24^{a}	3.86^{d}	4.21^{c}	3.52^{d}	4.26^{c}	8.21^{b}	0.52	0.0014
Hemicellulose	20.52^{a}	15.70^{d}	14.46^{d}	17.92^{c}	13.71^{e}	18.22^{b}	2.01	0.0041
Cellulose	22.71^{a}	15.70^{c}	14.46^{c}	18.55^{b}	17.26^{b}	19.85^{a}	1.10	0.0032
Metabolizable energy (kcal/kg)	2219.55^{d}	2807.81^{a}	2676.14^{b}	2293.72^{a}	2728.31^{a}	2465.30^{c}	11.85	0.0045

Means with different superscripts along the same row are significantly different (P<0.05).An=Aspergillus niger, Tv=Trichoderma viride, Rs=Rhizopus stolonifer, Mm=Mucor mucedo, RG=Roxazyme G2G.

The fungi used were *A. niger, R. stolonifer* and *T. viridae*. A commercial exogenous enzymes *(*Roxazyme G2G) that contained cellulase (endo-1, 4-(β-glucanase), β- glucanase (endo-1, 3 (4)-β-glucanase) and xylanase (endo-1, 4- β xylanase) was also used. Every 50 g of the substrate was moistened with 20 mL of the requisite basal medium (KNO_3, 5.0 mg; KH_2PO_4, 2.0 g; $MgSO_4.7H_2O$, 0.5 g; Tryptone, 0.5 g; $FeSO_4.4H_2O$, 3.5mg; $C_6H_5NO_2$, 0.5 mg; $C_{12}H_{17}ClN_4OS$, 0.05 mg and $C_{10}H_{16}N_2O_3S$. Biotin, 0.05 mg per litre of distilled H_2O) and then 1.0 ml of an aqueous spore's suspension of each isolate was added. The conical flasks were covered with sterilized cotton wool and kept in the incubator at 330C for seven days. After the growth of the fungi, the contents of each flask were mixed with 100 mL of the requisite buffer (Phosphate; pH of 7.2) and then filtered through double layered muslin clothe. The filtrate in the flask was in a chilled environment (4°C) to prevent denaturation of the enzymes. It was centrifuged at 3000 rpm for 15 min by using the centrifuge manufactured by Measuring and Scientific Equipment, MSE (UK) Ltd named MSE Cellsep 6/720R centrifuge. The supernatant was collected and taken as raw enzymes (Onilude and Oso, 1999). Raw enzymes were concentrated 5-fold by means of a Vacuum Rotator Evaporator at 3000 rpm. The concentrated enzymes were dialyzed using phosphate buffer of pH 7.2 (0.1M). The *in vitro* dry matter enzymic degradation (IVDMED) was carried out in three replicates per treatment. Ground and sterilized CRS was obtained from the mentioned fungi. The enzymes were directly applied on CRS at 250 ml/kg and allowed to stay for seven days. Mouths of conical flasks that contained the CRS and enzymes were plugged with sterilized cotton wool to prevent contamination. This was carried out in a sterilized environment. At the end of the seventh day, samples were oven dried at 70°C for 16 h to stop further actions of enzymes. This was tagged biodegraded CRS. Soluble sugars were determined spectrophotomerically using the methods of Association of Official Analytical Chemists (1995).

Chemical and statistical analyses

After drying, all the samples were milled with a 0.84 mm sieve and the following analyses were carried out: crude protein, crude fibre and ether extract, using Association of Official Analytical Chemists, AOAC (1995) method while acid detergent fibre, neutral detergent fibre, cellulose and acid detergent lignin were determined using the method of Van Soest and Robertson (1991). The absorbencies were read from the spectrophotometer at the specific wavelength 105°C for 8 h. Hemicellulose was estimated as the difference for each sugar. Dry matter was determined by drying the samples at between neutral detergent fibre and acid detergent fibre. Crude protein was determined as Kjeldahl nitrogen x 6.25. metabolizable energy (ME) of degraded and undegraded GNP samples was determined with the use of Pauzenga (1985) method: ME = 37 x CP% + 81.8 x fat% + 35.5 x NFE%. Data collected were subjected to analysis of variance (SAS, 1999). Significant differences between means were determined using the Duncan multiple range test (SAS, 1999).

RESULTS AND DISCUSSION

Results of the enzymic biodegradation of cassava root sieviate (CRS) as shown in proximate and detergent fibre composition are presented in Table 1. Enzymes increased the crude protein content of the CRS by 37.14, 40.99, 32.61, 32.52 and 16.26% with *A. niger*, *R. stolonifer, M. mucedo, T. viride* and Roxazyme G2G respectively. Values were significantly different (P<0.05) among fungi. Increase in the crude proteins value of the degraded AIBs was partly due to the ability of the enzymes to increase the bioavailability of the protein hitherto encapsulated by the cell walls. Fungal enzymes have the potential of improving not only the NSPs but also the crude proteins as well as other dietary components such as ash and fatty acids (Liu and Baidoo, 2005). Crude fibre content in the CRS was also significantly (P<0.05) reduced. *A. niger* caused the highest reduction in crude fibre level by reducing it from 10.98 g/100 g to 4.82 g/100 g which represented a 56.10% reduction and this was followed by changes orchestrated by *T. viridae* (49.73%). The cellulose content in the degraded CRS decreased by 36.3, 30.9, 24.0, 18.3 and 12.6% with *T. viride, A. niger, M. mucedo, R. stolonifer* and Roxazyme G2G, respectively. This confirms the reports by other authors (Iyayi and Aderolu, 2004; Iyayi and Losel, 2001; Yoshinori et al., 2014). Iyayi and Aderolu (2004) reported reduction in crude fibre content of brewer dried grain, maize offal and wheat offal when *A. niger, A. flavus* and *Penicillium* sp. were used for their biodegradation.

Table 2. Levels of soluble sugars (mg/mL) in undegraded and biodegraded cassava root sieviate.

Soluble sugars	Undegraded CRS	CRS+Tv	CRS+Mm	CRS+Rs	CRS+An	CRS+RG	SEM	P-value
Glucose	257.34 [e]	784.20 [b]	516.06 [c]	556.40 [c]	932.22 [a]	381.43 [d]	0.41	0.0014
Fructose	89.0 [d]	218.03 [b]	203.20 [b]	137.04 [c]	221.30 [a]	228.44 [a]	0.22	0.0022
Galactose	140.0 [d]	840.0 [a]	230.0[c]	760.0 [a b]	620.0 [b]	200.5 [c]	0.05	0.0016
Sucrose	55.0 [d]	226.20 [b]	209.05 [b]	218.21 [b]	314.54[a]	178.70 [c]	0.03	0.0020

Means with different superscripts along the same row are significantly different (P<0.05). An = *Aspergillus niger*, Tv = *Trichoderma viride*, Rs = *Rhizopus stolonifer*, Mm = *Mucor mucedo*, RG = Roxazyme G2G.

Table 3. Mineral composition of undegraded and degraded cassava root sieviates mg/kg (PPM).

Minerals	Control undegraded	CRS+An	CRS+Tv	CRS+Rs	CRS+Mn	CRS+RG	SEM
Sodium	0.04[c]	0.06[a]	0.06[a]	0.05[b]	0.05[b]	0.05[b]	0.001
Potassium	0.49[d]	0.64[a]	0.51[c]	0.56[b]	0.63[a]	0.55[b]	0.004
Manganese	6.04[c]	6.86[a]	6.84[a]	6.83[a]	6.81[a]	6.64[b]	0.21
Calcium	0.03[c]	0.05[b]	0.05[b]	0.06[a]	0.06[a]	0.04[c]	0.008
Magnesium	5.60	5.61	5.61	5.62	5.61	5.60	0.02
Phosphorus	31.21[c]	32.86[a]	32.84[a]	31.22[b]	32.25[a]	31.41[b]	1.22
Copper	5.43	5.44	5.44	5.43	5.44	5.43	0.21
Chromium	3.94[d]	4.50[b]	4.62[a]	4.63[a]	4.52[b]	4.01[c]	0.16
Iron	22.83[c]	22.87[ab]	22.94[a]	22.94[a]	22.70[b]	22.78[b]	1.95
Zinc	12.86	12.87	12.87	12.87	12.86	12.86	1.14

Means with different superscripts along the same row are significantly different (P<0.05). An = *Aspergillus niger*, Tv = *Trichoderma viride*, Rs = *Rhizopus stolonifer*, Mm = *Mucor mucedo*, RG = Roxazyme G2G.

Crude fibre in the above mentioned AIBs were significantly (P<0.05) reduced by all the fungi until after the fourteenth day. *A. niger* consistently caused the highest reduction in crude fibre in all the AIBs followed by *A. flavus* and *Penicillium* sp. The use of enzyme has predominantly been related to the hydrolysis of fibre or non-starch polysaccharides fractions in the AIBs. Glucans chains of cellulose are held together in an organized manner by inter and intra molecular hydrogen which renders the carbohydrates and other nutrients insoluble and resistant to enzymic hydrolysis. The structure and properties of β-glucans are described as polymer of glucose with a β-1, 4 linked backbones and β-1, 3 side linkages (Atik et al., 2006; Ezieshi and Olomu, 2004). Apart from the fact that β-glucans and the insoluble NSPs arabinoxylans are also found in crude fibre and they are in endosperm cell walls. Crude fibres were hydrolyzed by the synergetic action of xylanase and glucanases (Dare et al., 2010; Hughes et al., 2000). Table 3 shows mineral composition of undegraded and degraded cassava root sieviates. It reveals improvement in the potassium, phosphorus, calcium, iron, chromium and manganese content after enzymic degradation of CRS. The highest value (32.86 mg/kg) for phosphorus was found in the *A. niger* degraded sample. Possibly, enzyme phytase was part of the cocktail of enzymes produced by the used fungi and this must have assisted in the liberation of the phytate bound phosphorus. Phytate, like oxalates and tannins, is an organic compound (myo-inositol hexaphosphate) which occurs in all plants and these mineral binding factors possess anti-nutritional properties because they are potential chelators of minerals and, thus, their presence in any AIBs will strongly have negative effect on the digestibility of such materials (Sofia et al., 2014; Hannah et al., 2014). From the results, biodegradation of CRS by the fungi did enhance the bioavailability of the mineral elements (Na, K, Mg Ca, P and Fe). The results of sugar fractions (glucose, fructose, galactose and sucrose) in the undegraded and degraded CRS are shown in Table 2. Glucose was the highest produced sugar (glucose > fructose > sucrose > galactose). The highest glucose yield was in the CRS degraded with *A. niger* with a value of 932.22 g/mL as compared to 381.43 g/mL in the RG2G degraded CRS. The enzymes increased the metabolic energy content of the CRS by 21.0, 18.7, 17.1, 10.0 and 3.2% with *A. niger*, *M. mucedo, T. viride,* Roxazyme G2G and *R. stolonifer*, respectively. The values were significantly different (P<0.05) among the fungi. The increase in the sugar content

and the metabolizable energy value of the degraded CRS was partly due to the ability of the enzymes to disrupt the cell wall structure (Ates et al., 2008; Gunal and Yasar, 2004). AIBs are known to have low ME values and they often have a high content of growth inhibiting, viscous, water-soluble, non starch polysaccharides (WNSPs). According to Oluremi et al. (2007), the monogastrics are unable to release the intrinsic energy portion of the AIBs as the energy remains latent in the fibrillian complex which renders resistance to the endogenous enzymes. In addition, Martins et al. (2000) observed a continuous increase in sugar production in the substrates until after the fourteenth day of fungi on the substrates when there was gradual reduction in sugar availability in the substrates. There were significant (P<0.05) increase in sugar production when *A. niger, A. flavus and Penicillium* species were inoculated on brewer dried grain, maize offal and wheat offal. This study shows that with fungal biomass increase, the nutrients in the substrate medium were quickly used up. Beyond fourteenth days, the fungi started using up the products of breakdown of the NSPs, hence, the observed reduction in the sugar level. There was increase in the ME because there was increase in the soluble sugars availability. Soluble sugars are the assimilable forms of carbohydrates needed for energy production. However, fungal enzymes added to the AIB were able to break the cell walls. Hence, the degraded CRS had higher values of sugars than the undegraded one and this shows the effects of the enzymes on the CRS.

Conclusion

This study leads makes us believe that the treatment of cassava root sieviate with extracted enzymes from *A. niger, M. mucedo, R. stolonifer, T. viride* and Roxazyme G2G may improve the crude protein, metabolizable energy, sugars and mineral and reduce the fibre fractions. Furthermore, the increase in crude protein and meta-bolizable energy of the degraded CRS due to enzymic degradation made the CRS beneficial thereby increasing its nutritive value and solving the problem of environmental pollution. The application of this technique helps in the conversion of AIBs into animal feeds thereby improving their preservation and utilization by livestock animals. Ultimately, this will have direct positive effect on availability of animal protein. Of the four fungi and the commercial Roxazyme G2G, enzymes from *A. niger* appeared to be the most suitable for the biodegradation of cassava root sieviate as it succeeded in effecting the highest improvement in terms of crude protein, crude fibre, ash, sugar and metabolizing energy. Therefore, the use of *A. niger* in enhancement of CRS as animal feed ingredient is preferred to *T. viride, M. mucedo, R. stolonifer* and Roxazyme G2G.

Conflict of Interests

The authors have not declared any conflict of interests.

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