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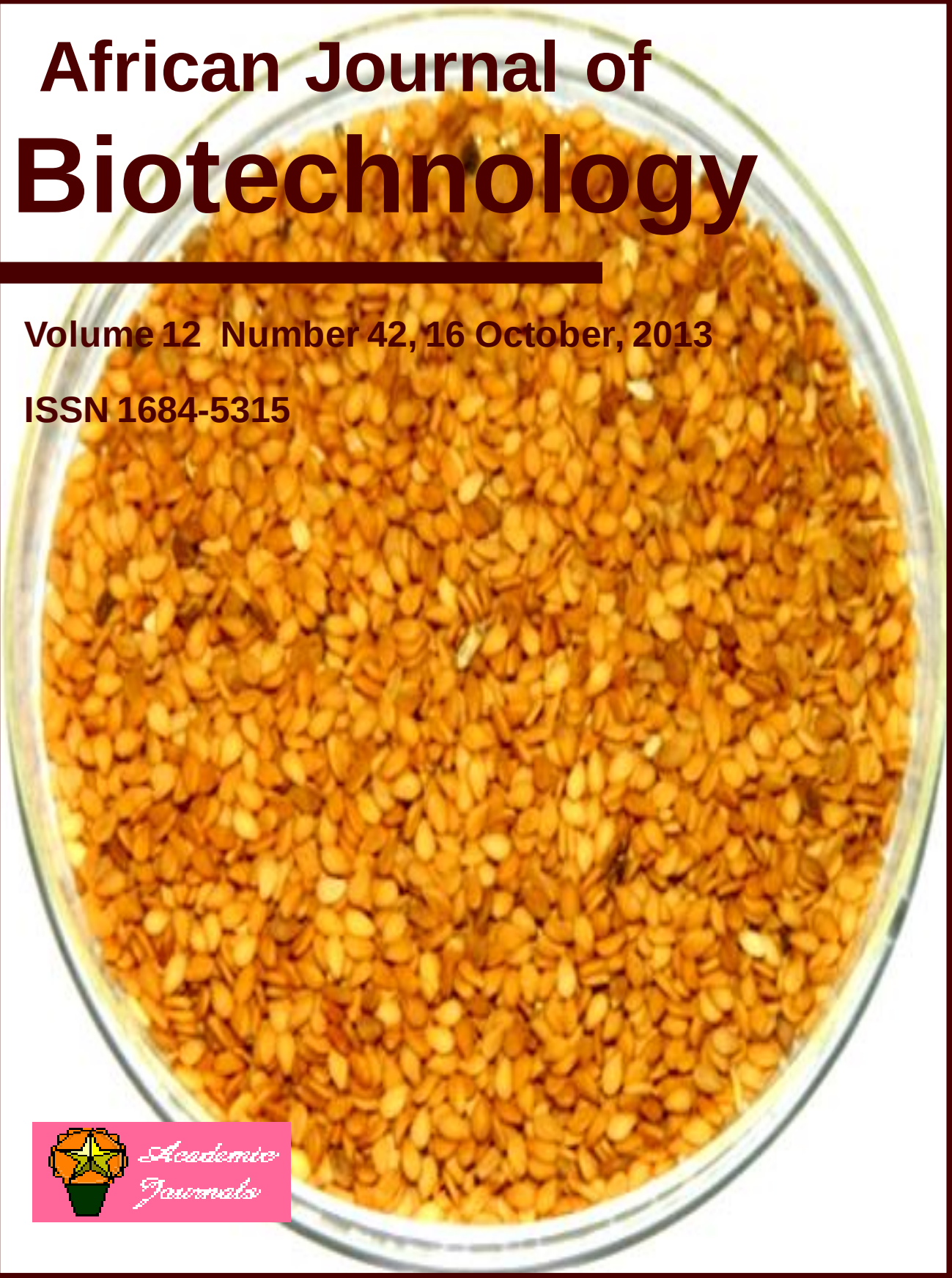

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: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

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Full Length Research Paper

Associated technologies ensures complete loop mediated isothermal amplification platform for pathogen diagnosis

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Loop Mediated Isothermal Amplification (LAMP) assay could be a useful adjunct diagnostic assay along with the conventional methods that would preclude the requirement of continuous maintenance of pure cultures. Moreover, LAMP assay is simple, rapid, specific and sensitive for the detection of pathogens. Having developed and validated LAMP method for the detection of an isolated pathogen, *Escherichia coli* O157:H7, an attempt was made to progress the LAMP platform to realistic point of care for resource-poor endemic areas. Reaction time of the LAMP method was only 1 h and also, the amplification products of O157, which had the corresponding target genes, turned green by visual inspection when added with Calcein/Sybr green. However, sample preparation and lyophilized master mix preparation before LAMP assay as well as developing a closed detection system for detection of LAMP amplified products remained a quest. Hence, the current study was conducted to develop a lyophilized LAMP master mix for easy platform to take LAMP to realistic point of care and Dot-Elisa based 'lateral flow dipstick' that could ease the detection of LAMP products in a closed environment. Sample preparation is another associated technology that is yet-to-be developed.

Key words: Loop mediated isothermal amplification assay, polymerase chain reaction, lyophilized master mix, and closed amplification system.

INTRODUCTION

Escherichia coli O157:H7 is an enterohemorrhagic strain of the bacterium *E. coli* and a cause of food-borne illness. Most illness has been associated with eating undercooked, contaminated ground beef, drinking unpasteurized milk, swimming in or drinking contaminated water and eating contaminated vegetables. Young children and females had an increased risk of Hemolytic Uremic Syndrome (HUS) after Shiga toxin producing *E. coli* (STEC) O157 infection. With or without HUS, elderly persons had the highest pro-portion of deaths associated with STEC O157 infection. These data support recommendations for aggressive sup-portive care of young children and the elderly early during illness due to STEC O157 (Gould et al., 2009). Haemo-lytic uremic syndrome is characterized by three features: acute renal failure, microangiopathic haemolytic anaemia and thrombocytopenia (Levin et al., 1984). *E. coli* O157:H7 was the causative agent of many out-breaks worldwide. Outbreaks of HC caused by VTEC occurred in different areas of USA in 1982 and since then outbreaks and spo-radic cases have been reported in several other coun-tries including Canada, Britain and Japan (Johnson et al.,

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1983; Pai et al., 1984). Incidence of sporadic cases of hemorrhagic colitis due to *E*. *coli* O157:H7 may be higher than suspected (Chik et al., 1984). An epidemiological outbreak of *E*. *coli* O157:H7 can be expected at any point of time and hence a constant study has to be conducted throughout the world for the early diagnosis of such pathogens.

Major advance in diagnostic testing includes PCR for viruses, culture methods for bacteria and microscopy for parasites. Moreover, many tests that form the backbone of the "modern" microbiology laboratory are based on very old and expertise-intensive technologies such as ELISA for pathogenic antigens or its antibodies. Pressing needs include more rapid tests without sacrificing sensitivity, value-added tests, and point-of-care tests for both high- and low-resource settings. In recent years, research has been focused on alternative methods to improve the diagnosis of pathogens. One such method is loop media-ted isothermal amplification (LAMP) that amplifies a tar-get DNA under isothermal conditions, since discovered by Tsugunori et al. (2000). LAMP is a novel method which amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. Unique characteris-tics of LAMP includes: 1) Amplifies a target DNA under isothermal conditions; 2) relies on autocycling strand dis-placement DNA synthesis performed by using the *Bst* DNA polymerase large fragment; 3) less expensive, rapidity (results within 1 h), low reaction temperature (60 to 65°C), high specificity for the target and sensitivity; 4) requires only a regular laboratory water or heat block to carry out the reaction; 5) the end product can be visua-lized by naked eyes.

Application of LAMP under field conditions has been limited, partly due to the infancy of the technologies associated with LAMP, such as field-based template preparation methods and product detection formats (Njiru, 2012). In this viewpoint, an attempt was made for the development of lyophilized LAMP master mix and lateral flow dipstick for detection of LAMP products. However, field-based sample template preparation method is yet-to-be developed for LAMP application in resource-poor endemic areas. Considering the state of art status of LAMP technique that has achieved its advancement for the diagnosis of various viruses worldwide, the current study emphasizes on its application on a bacterial pathogen and can be extended towards parasitic and fungal pathogens in future.

MATERIALS AND METHODS

Preliminary work

An *E*. *coli* O157:H7 strain isolated from ground beef, India was used and stx1 gene, a potent virulence gene, was chosen as target gene. Selected gene was confirmed by conventional PCR. Oligonucleotide primer sequences used for PCR amplification of stx1 gene was derived from a study conducted by EL-Jakee et al. (2009) in Egypt, namely F1: 5'ACA CTG GAT GAT CTC AGT GG 3' and R2: 5'GGG ATA TCT TAG CTC TGT GAG AGC TCG CGG3' flanking a size of about 614 bp were used. Amplification was performed in Thermo-cycler for 32 cycles after initial denaturation for 3 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72°C for 1 min 20 s. The primer extension was extended to 7 min at 72°C in the final cycle. The PCR amplified products were detected by electrophoresis on 1.5% agarose gel in 1X TAE buffer at 80 v for 1 h (Sambrook et al., 1989). After PCR ampli-fication, PCR gel bands were cut, purified and sequencing of stx1 gene was done. The stx1 gene was cloned in *E*. *coli* BL21 using pET 32a vector, since sample is not available in surplus and also, for sensitivity check to be done with serially diluted plasmid DNA.

LAMP primer designing, optimization and sensitivity check

In earlier studies, primers for LAMP test were designed by targeting the antigen coding rfbE of EHEC O157:H7, the Shiga-like toxin stx2 and the fliC encoding gene of H7 flagella antigen (Zhu et al., 2009; Deguo et al., 2009). But the present study targeted stx1 gene of EHEC O157:H7. The reaction condition and reaction system of LAMP were optimized. Primer Explorer V3/4 software is specifically for designing the primer sets for LAMP method (Qi-Wei et al., 2012). One primer set contains 4 primers, forward inner primer (FIP), F3, back-ward inner primer (BIP) and B3. F1, F2, F3 are about 20 bp long sequences selected from the target gene B1, B2, B3 are about 20 bp long sequences selected from the complementary strand. F1c and F1, B1 and B1c are complementary regions. This software can also design the loop primers, LF and LB. LAMP primers were successfully designed using Primer Explore V4 software for stx1 gene of STEC and tabulated in Table 1. FIP and BIP alone are biotin labeled for lateral flow dipstick development in later stages. Once primers are ready, the concentration of $MgSO_4$, temperature and time points were optimized as 4 mM, 62°C and 60 min respectively, until a ladder-like pattern observed in gel run. The end product visualized by naked eyes using SYBR green I (Saleh et al., 2008) or Calcein.

Conventional PCR made for the dilutions of plasmid DNA extractions to check the sensitivity of PCR (Note: Before making the dilutions, purity of plasmid DNA was checked using nanodrop spectrophotometer and obtained as 1.9 under 260/280 absorbance; hence, serially diluted for 8 times until 10^{-8} dilution is obtained). Sensitivity of LAMP was compared with that of conventional PCR.

Lyophilized master mix and lateral flow dipstick preparation

The lyophilized isothermal master mix is an optimized master mix designed to simplify the preparation of an isothermal amplification assay. It is prepared corresponding to the optimised conditions of LAMP assay for the detection of *E*. *coli* O157:H7. Hence, a master reaction mix set up for 20 reactions were prepared and lyophilized and stored at -20°C. Since FIP and BIP primers were labeled with biotin, LAMP products must possess proportional biotin concentration as that of the product. Hence, lateral flow dipstick was developed involving the principle of Dot-Elisa, where LAMP products are considered equi-valent of the antigens against which anti-biotin antibody were added, secondary antibody conjugated with Horseradish peroxidase (anti-species anti-mouse HRP) added, then chromogenic substrate (DAB) added to observe for colored product as dot in positive controls. No dots obtained indicated absence of biotin labeled amplified DNA products and hence, interpreted as negatives. Positive controls were able to produce clear dots indicating the presence of biotin in the

Table 1. Primers used in LAMP for stx1 gene of STEC.

Primer	5' Pos	3' Pos	Length	Sequence
F3	44	61	18	GAACTGGGGAAGGTTGAG
B3	229	246	18	CACGGACTCTTCCATCTG
FIP			44	TCCCAGAATTGCATTAATGCTTCC-GTCCTGCCTGATTATCATGG
BIP			47	AGCGTGGCATTAATACTGAATTGT-ACATAGAAGGAAACTCATCAGAT
F2	66	85	20	GTCCTGCCTGATTATCATGG
F1c	120	143	24	TCCCAGAATTGCATTAATGCTTCC
B2	200	222	23	ACATAGAAGGAAACTCATCAGAT
B1c	144	167	24	AGCGTGGCATTAATACTGAATTGT
LF	86	110	25	TCTTCCTACATGAACAGAGTCTTGT
LB	168	188	21	CATCATCATGCATCGCGAGTT

Table 2. Lyophilized LAMP master reaction mix components.

Component	Volume (500 µl for 20 reactions)
F3 and B3 primers	20 µl each
FIP and BIP primers	40 µl each
LF and LR primers	20 µl each
dNTP (10 mM/ml)	70 µl
Betaine	60 µl
Bst DNA polymerase large fragment	20 µl
Thermopol buffer	50 µl
$MgSo_4$	80 mM

LAMP amplified product as expected whereas negative controls had no biotin and hence, clear dots were not formed.

RESULTS AND DISCUSSION

Conventional PCR and LAMP amplification

Conventional PCR amplification confirmed the presence of 614 bp sized stx1 gene product when run on agarose gel electrophoresis. Figure 1 shows the gel band of PCR gene amplification of stx1 under UV illumination. Figure 2 represents the pattern of LAMP products observed after gel run. This ladder-like pattern as shown in this figure is unique for this technique and easily detectable as positive or negative control under gel documentation. Figure 3 depicts analytical sensitivity of LAMP compared with conventional PCR. It was observed that for both PCR as well as LAMP technique, sensitivity was up-to 10^{-2} dilution that is, amplification was possible up-to 10^{-2} dilution of plasmid DNA and could not be amplified beyond this dilution level.

Lyophilised LAMP master mix and lateral-flow dipstick development

Optimised conditions of LAMP assay for the detection of *E. coli* O157:H7 contained F3 and B3 primers 1 µl each (10 pmoles each); FIP and BIP primers 2 µl each (40 pmoles each); LF and LR primers 1 µl each (20 pmoles each); dNTP (10 mM/ml) 3.5 µl (1.4 mmol/L); betaine 3 µl (0.8 M/L); Bst DNA polymerase large fragment 1 µl (12 U/µl); thermopol buffer 2.5 µl and $MgSO_4$ 4 mM. Hence, a master reaction mix set up for 20 reactions were prepared as shown in Table 2 and lyophilized. This master mix can be taken directly to the point of care for the addition of sample directly and has an advantage of shelf life of more than a week at -20°C. When LAMP amplified products were subjected to lateral flow dipstick assay, they were able to produce clear dots indicating the presence of biotin and hence the amplified DNA products were interpreted as positive. This assay can be further developed by coating anti-biotin antibody in latex particles (beads) and hence developing a latex agglutination kit which can give immediate results by the principle of latex agglutination of particles. Figure 4 shows the Dot-Elisa representing positive control and negative control along with the test, performed in blotting paper that can be applicable for developing lateral flow dipsticks for detection of LAMP products.

It is also to be noted that the more the concentration of product, the more the intensity of dot produced. Even though the results of Dot-Elisa were obtained consistently with repeated tests in frequent intervals of time, detection

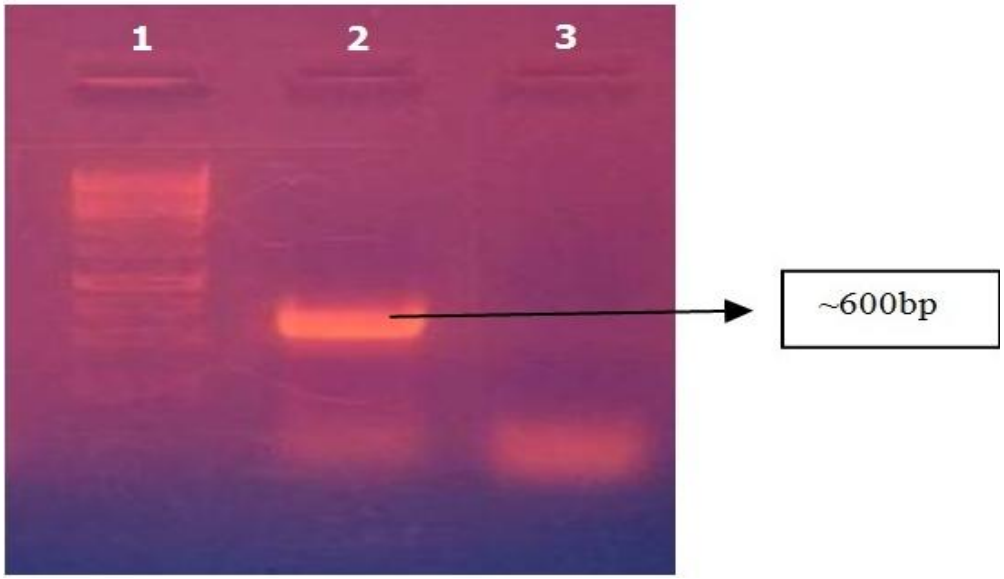

Figure 1. Agarose gel electrophoresis of amplified stx1 gene from the DNA extracted from *E. coli* O157:H7 (Lane 1: 1 Kb DNA Ladder; Lane 2: amplified product of stx1 gene; Lane 3: negative control).

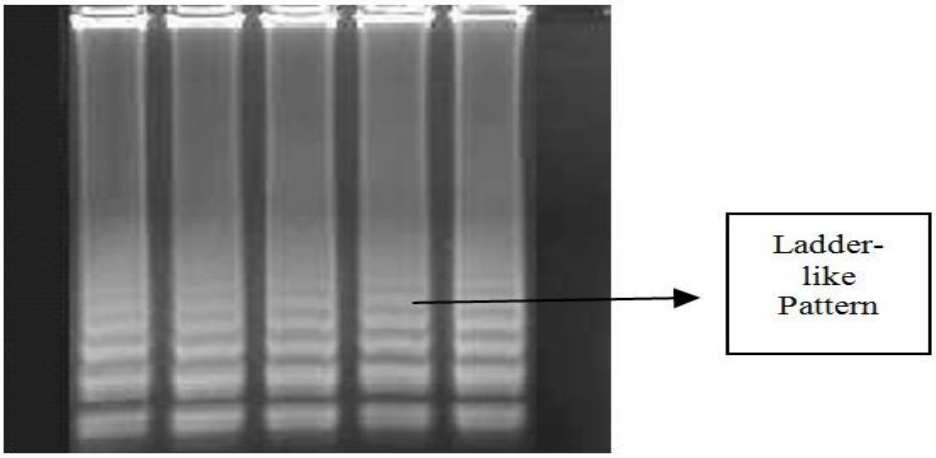

Figure 2. Ladder-like pattern developed by LAMP products (unique pattern for LAMP amplified products) when run on electrophoresis gel.

systems such as dipstick assays can be avoided in case of naked eye detection system of LAMP results using Calcein/SYBR green confirms the diagnosis.

Conclusion

LAMP from research lab to clinical diagnosis: it is suggested here that the technologies associated with LAMP can be considered and developed as part of a LAMP platform, rather than developing them as separate entities. To achieve these levels in resource-poor areas, specimen processing methods, production of lyophilized kits, and a closed amplification and detection system need to be developed, which will facilitate the provision of a same-day testing strategy in even the most remote rural health facilities. Hence, this should be followed by rigorous evaluation of test performance to determine feasibility and acceptability under field conditions. However, detection systems such as dipstick assays can be avoided in case of resource unavailability, provided naked eye detection system of LAMP results is sufficiently achieved. Considering the state of art status of LAMP technique that has achieved its advancement for the diagnosis of various viruses worldwide, current study emphasizes on

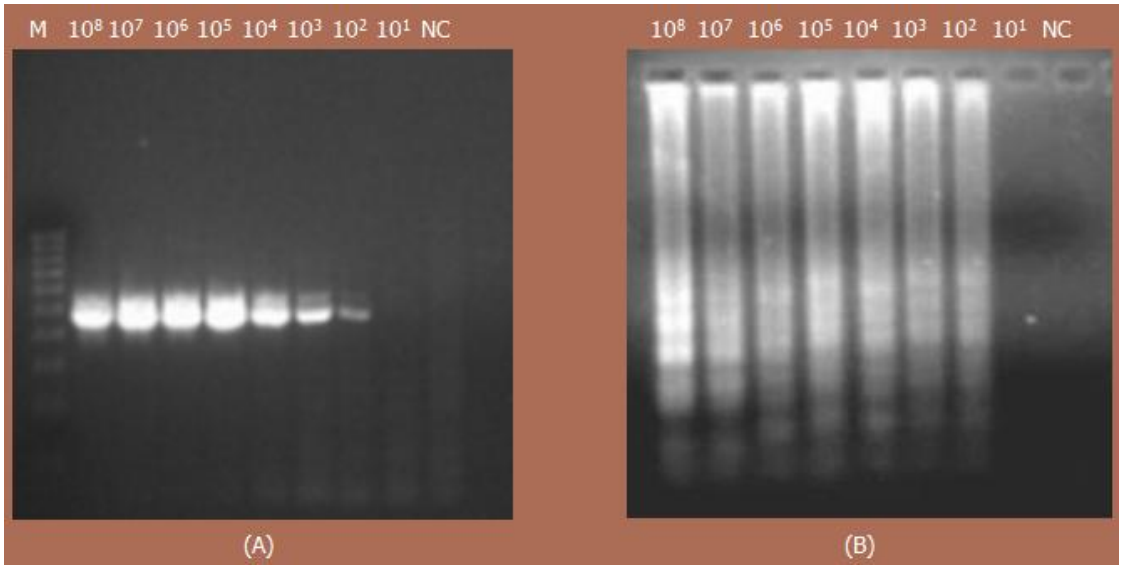

Figure 3. Analytical sensitivity of LAMP compared with conventional PCR. Gene amplification: RT-PCR with dilutions 10^8 to 10^1, NC (A) and LAMP with dilutions 10^8 to 10^1, NC (B).

Figure 4. Dot-Elisa representing positive (P), negative (N) and test (T).

its application on a bacterial pathogen and can be extended towards parasitic and fungal pathogens in future.

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African Journal of Biotechnology

Full Length Research Paper

Random amplified polymorphic DNA (RAPD) markers reveal genetic diversity in bael (*Aegle marmelos* Correa) genotypes of Andaman Islands, India

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The present study evaluated genetic variability of superior bael genotypes collected from different parts of Andaman Islands, India using fruit characters and random amplified polymorphic DNA (RAPD) markers. Genomic DNA extracted from leaf material using cetyl trimethyl ammonium bromide (CTAB) method was subjected to polymerase chain reaction (PCR) with 12 polymorphic primers. A total of 476 polymorphic loci were identified with mean value of 39.66 bands per primer and 63.99% polymorphism. Application of unweighted pair group method using arithmetic average cluster analysis generated three genotypic groups. 'Bael-5' and 'Bael-8' were most similar genotypes whereas 'Bael-7' and 'Bael-1' were extreme divergent. The clusters based on molecular data were not in agreement with the morphological traits in most of the cases as low level of correlation was observed between the classification methods based on fruit characteristics and RAPD markers. The bael genotypes were found to have considerable genetic variability, demonstrating the importance of RAPD markers to analyse each genotype in a collection in order to efficiently maintain the germplasm collection for genetic improvement of bael.

Key words: Aegle, Bael, random amplified polymorphic DNA (RAPD) and genetic diversity.

INTRODUCTION

Aegle marmelos Correa, the bael, belongs to the Rutiaceae family and indigenous to India. It is one of the most important underutilized fruits of India, Burma and Ceylon (Srivastava et al., 1998). The bael fruit pulp contains important bioactive compounds such as carotenoids, phenolics, alkaloids, pectins, tannins, coumarins, flavonoids and terpenoids. It has several ethnomedicinal applications and source of traditional medicines such as anti-diarrhea and anti-dysentery (Brijesh et al., 2009), hepatoprotective activity (Rajasekaran et al., 2009) and antifertility effect (Chauhan et al., 2008). The leaves are also used in several traditional formulations used in diabetes and ophthalmic disorder.

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Abbreviations: RAPD, Random amplified polymorphic DNA; **CTAB,** cetyl trimethyl ammonium bromide; **EST,** expressed sequence tags; **SCAR,** sequence-characterized amplification regions; **SSR,** simple sequence repeats; **SNP,** single nucleotide polymorphism.

Table 1. The studied bael genotypes and their place of collection and fruit quality traits.

Genotype	Place of collection	Fruit weight (kg)	Fruit length (cm)	Fruit width (cm)	Shell thickness (mm)	Wt. of shell (g)	No. of seeds	Wt. of seeds (g)
Bael-1	North Andaman	1.80±0.12	26.00±0.58	8.67±0.33	2.87±0.09	429.67±5.78	131.33±5.93	27.33±1.20
Bael-2	North Andaman	1.06±0.08	13.67±1.15	7.38±0.39	3.20±0.06	244.38±16.27	126.46±5.95	24.71±1.10
Bael-3	Middle Andaman	1.10±0.06	16.00±0.58	9.00±0.58	3.47±0.15	265.33±5.7	130.67±2.96	24.67±0.33
Bael-4	Middle Andaman	1.16±0.09	15.00±0.58	10.33±0.33	2.90±0.12	231.33±5.93	126.67±9.28	26.00±1.53
Bael-5	Middle Andaman	1.00±0.012	12.00±1.15	7.67±0.33	3.07±0.09	230.00±7.64	115.33±7.31	22.33±1.20
Bael-6	Middle Andaman	0.93±0.09	11.33±1.2	5.33±0.33	3.37±0.03	228.00±4.36	112.00±1.53	22.33±0.33
Bael-7	Middle Andaman	1.20±0.06	13.00±0.58	6.00±0.58	3.60±0.01	240.33±5.78	120.00±2.89	21.33±0.88
Bael-8	Middle Andaman	0.56±0.03	8.00±0.5	5.33±0.88	3.20±0.05	162.33±6.23	86.00±0.58	17.67±0.33
Mean		0.73±0.06	8.00±0.58	6.67±0.33	3.10±0.05	168.00±10.12	189.67±5.78	36.00±1.15
LSD @ 5%		0.25	2.31	14.99	0.24	19.94	16.00	2.93

Due to the long historic cultivation of bael in India, synonymies and homonymies can be observed among genotypes cultivated in different regions. Thus, diversity of bael germplasm based on morphological characteristics of the fruit has been performed by Nath et al. (2003) and Rai and Misra (2005). However, due to the effects of environmental factors on these attributes, their use can be ambiguous. Therefore, markers independent from the environment are necessary for reliable identification and discrimination of genotypes and cultivars (Sarkhosh et al., 2006). DNA based molecular markers are independent from environmental interaction, developmental stage, unlimited in number and show high level of polymorphism (Sarkosh et al., 2006).

Further, pre-conservation analysis of potential germplasm using DNA markers reduces duplicity in germplasm (Singh et al., 2012). These markers are also used for various purposes in horticultural crops such as, construction of genetic linkage map (Venkateswarlu et al., 2006), phylogenetic (Kafkas and Perl-Treves, 2001) and genetic diversity (Zamani et al., 2007).

Out of the different marker systems, random amplified polymorphic DNA (RAPD) marker system is an easy and informative molecular marker requiring no previous information of DNA sequences and its efficacy had been justified to be the same as other molecular markers in studies on genetic diversity (Zamani et al., 2010). The information about the nature and magnitude of genetic variability as well as relatedness among bael genotypes would be helpful in formulating an effective breeding programme.

The Andaman Islands are situated in Bay of Bengal and 1200 km away from mainland India. This region is rich in diversity of different horticultural crops (Abraham et al., 2008) including bael. However, none of the studies has been carried out on bael diversity from these islands. Therefore, the aim of the present study was to analyse the genetic variability and relatedness among the superior genotypes of bael collected from Andaman Islands, India.

MATERIALS AND METHODS

Plant materials

During survey of Andaman Islands in 2011-2012, great diversity in bael was observed in different villages. From this diversity, a total of eight representative bael genotypes were collected from different habitats in Andaman Islands. These genotypes represented maximum diversity of bael in islands (Table 1). From these, young healthy leaves were collected in plastic bags packed in ice for transport to the laboratory. The leaf samples were washed three times in sterile distilled water, frozen in liquid nitrogen and stored in the freezer at −20°C for further analysis. Five fruits from each genotype were collected for recording morphological characters (Table 1) previously reported to be important in bael evaluation (Nath et al., 2003) and means of these traits were used for statistical analysis.

DNA extraction

Leaves from each accession were macerated separately and genomic DNA was extracted according to the cetyl trimethyl ammonium bromide (CTAB) method suggested by Saghai-Maroof et al. (1984)] with minor modification. The purity and quantity of genomic DNA was determined spectrophotometrically and confirmed by comparative analysis with the intensity of known concentrations of unrestricted lambda DNA after electrophoresis in 0.8% agarose gel and staining with ethidium bromide. Following quantification, a portion of the DNA sample was diluted in TE buffer to final concentration of 10, 20, 30 and 40 ng per µl for subsequent RAPD analysis. The remaining DNA stock samples were stored in the freezer at −20°C.

Amplification reactions

In order to select the most polymorphic primers for RAPD analysis, DNA samples from the genotypes Bael-1 and Bael-2 were amplified using 44 primers. Elevated levels of polymorphism were detected with 12 primers and these 12 primers were employed in the subsequent reactions. PCR were carried out in a thermocycler (G-

Table 2. List of selected informative RAPD primers, their sequence and some information about generated bands in this study.

DNA marker	Marker sequence (5' to 3')	Range of amplicon size (bp)	Total No of bands	Average No of bands across genotypes	PIC value
OPA-02	TGCCGAGCTG	200-1200	57	6.33	0.26
OPN-03	GGTACTCCCC	200-1250	45	4.09	0.18
OPN-12	CACAGACACC	100-1000	71	5.92	0.25
OPM-05	GGGAACGTGT	300-1300	70	4.67	0.2
OPM-06	CTGGGCAACT	300-1500	80	5.33	0.22
OPX-17	GACACGGACC	200-1200	75	5.77	0.24
OPM-12	GGGACGTTGG	300-750	40	4.00	0.17
OPM-15	GACCTACCAC	200-600	32	4.57	0.2
OPM-20	AGGTCTTGGG	300-1500	71	5.07	0.21
OPB-1	GTTTCGCTCC	300-1400	70	5.38	0.22
OPA-08	GTGACGTAGG	300-1600	60	5.45	0.23
OPA-1	CAGGCCCTTC	200-1500	62	5.64	0.23

Storm, USA). The reaction mixtures (20 µl) were comprised in 1X buffer solutions, 1.0 mM $MgCl_2$, 1x PCR buffer, 1.75 mM $MgCl_2$, 200 mM dNTPs, 0.5 mM of a single decamer primer and 1 U Taq DNA polymerase (Genaxy Scientific Ltd., India). The reactions were performed in a thermocycler (G-Storm, USA) involved an initial denaturation step of 4 min at 94°C, followed by 35 cycles comprising of denaturation for 1 min at 92°C for 1 min, annealing for 1 min at 37°C, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Amplicons were separated by electrophoresis on 1.5% agarose (Genaxy Scientific Ltd., India) gel in TE buffer at 80 v for 3 h. Gels were treated with 0.5 µl/ ml ethidium bromide for 30 min and bands were visualized and photographed under UV light by a gel documentation system (UVP, Bio Doc. Co., USA).

Data processing

The number of polymorphic bands generated by each primer was determined initial visual examination of the gel photographs, taking into account the resolution and degree of amplification. Only bands of medium and strong intensity were included in the subsequent analysis. For RAPD analysis, presence of band was scored 1 whereas the absence of the band was scored 0. The scored RAPD markers were converted onto a binomial (0/1) matrix. Based on the matrices, the genetic similarities between bael genotypes were determined using Jaccard's coefficients and dendogram was constructed by applying unweighted pair group method with arithmetic average (UPGMA) clustering based on Jacard's similarity index bands. These computations were performed using the programme NTSYS-pc software ver. 2.11 (Rohlf, 2000).

RESULTS

Mean values of the studied fruit characteristics showed considerable variations between genotypes for all characters. Significant differences were recorded between genotypes for all characteristics based on analysis of variance (Table 1). In preliminary experiments of RAPD reproducibility, it was revealed that the concentraton of template DNA was crucial to obtain the maximum number of reproducible bands. Varying the concentration of template DNA (10, 20, 30 and 40 ng) revealed that 20 ng resulted to the maximum number of reproducible bands, and therefore 20 ng was used in all subsequent PCR reactions. Out of 44 RAPD primers, 12 primers which produced good and reproducible polymorphic bands among the 8 bael genotypes were used for further analysis. Polymerase chain reaction of bael genomic DNA using 12 selected polymorphic primers generated a total of 733 amplified bands. Among them, 476 bands were polymorphic in nature. The highest number of polymorphism was observed with primer OPA-01. The size of amplified fragments ranged between 250 and 1600 bp for all primers (Table 2). Comparatively low polymorphic information content (0.17 to 0.26) was shown by selected polymorphic primers. Average number of bands across genotypes were found maximum in primer OPA-02 (6.33) while minimum in primer OPM-12 (4.00). Results of similarity matrix showed the moderate degree of genetic similarity coefficients ranged from 0.49 to 0.72. The lowest similarity (0.49) was between genotypes Bael-7 and Bael-1 which were the most divergent while the highest similarity (0.72) was found between the genotypes 'Bael-5' and 'Bael-8' (Table 3).

A dendogram was constructed from values of similarity coefficients generated from RAPD data. According to the dendrogram (Figure 1), the genotypes were divided into two major genotypic groups at a 0.54 similarity coefficient, containing 7 and 1 genotypes, respectively, based on unweighted pair group method using arithmetic average cluster analysis. The genotype Bael-7 placed in a distinct cluster while other cluster sub divided in to 2 sub-clusters. Sub-cluster 'a' consisted of two genotypes, 'Bael-1' and 'Bael-2', where these genotypes separated from each other at 0.63 similarity coefficients. The 'sub-cluster b' comprised of five genotypes, 'Bael-4', 'Bael-6', 'Bael-5', 'Bael-8' and 'Bael-3'. Within sub-cluster 'b', genotypes 'Bael-5' and 'Bael-8' placed very closely with 0.72 similarity coefficients. In this study, the genetic

Table 3. Genetic similarity matrix based on RAPD data among 8 bael genotypes.

	Bael-1	Bael-2	Bael-3	Bael-4	Bael-5	Bael-6	Bael-7	Bael-8
Bael-1	1.00							
Bael-2	0.63	1.00						
Bael-3	0.52	0.61	1.00					
Bael-4	0.63	0.61	0.65	1.00				
Bael-5	0.59	0.61	0.66	0.62	1.00			
Bael-6	0.63	0.53	0.51	0.67	0.59	1.00		
Bael-7	0.49	0.53	0.56	0.55	0.53	0.56	1.00	
Bael-8	0.61	0.64	0.66	0.63	0.72	0.60	0.64	1.00

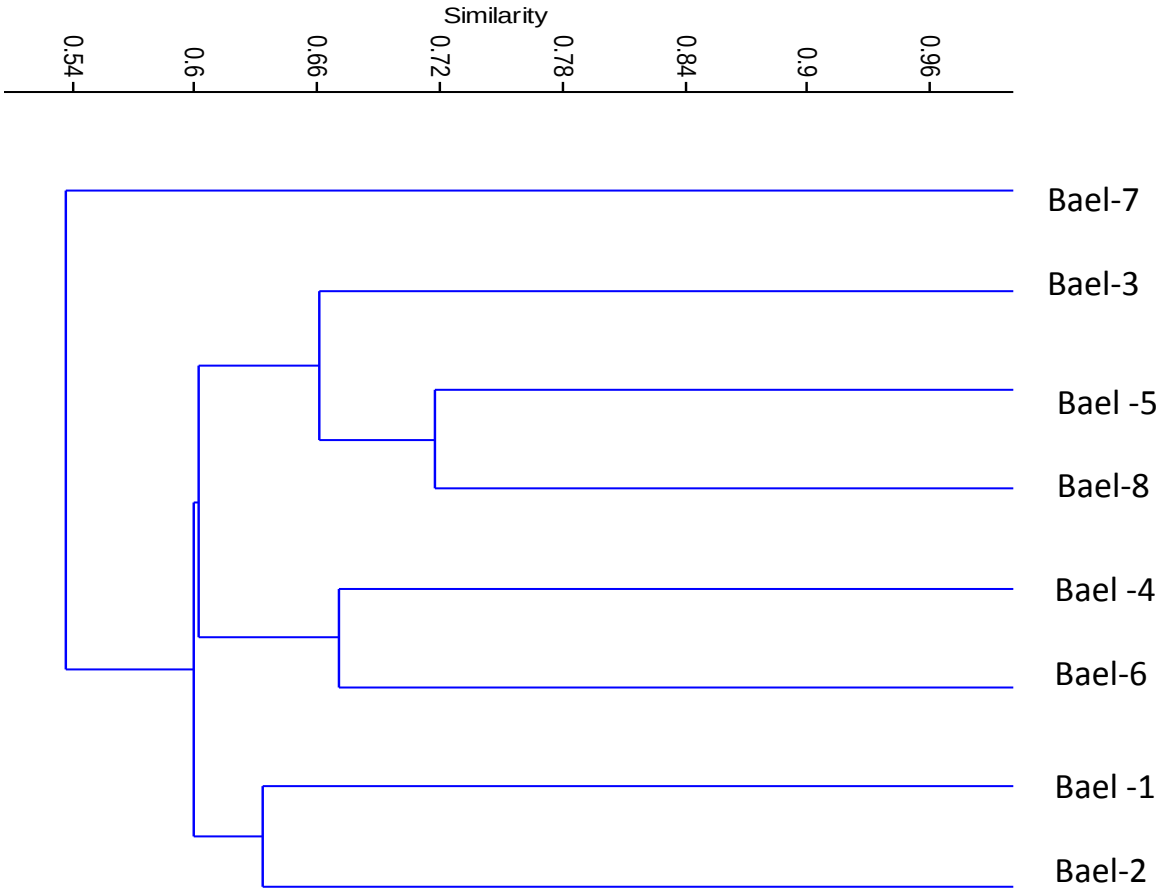

Figure 1. The UPGMA dendogram depicted by NTsys software based on RAPD data derived from similarity coefficients, showing the relationship of bale genotypes.

diversity results from RAPD markers differ from the morphological diversity. The cluster analysis of morphological characteristics, 'Bael-2' was the most dissimilar genotypes while in molecular analysis showed 'Bael-7' as the most divergent genotype. The high level similarity detected between 'Bael-5' and 'Bael-6' in the morphological analysis was not corroborated by the RAPD analysis that showed a large genetic distance between them.

DISCUSSION

Characteristics showing greater quantity range had higher coefficient of variation (CV), meaning a higher selection possibility for those characteristics in improvement programme of bael. Fingerprinting and assessment of genetic variability among accessions is of interest in terms of genetic resource conservation but also for practical application of breeding. Phenotypic des-

criptor can identify accessions but are environmentally affected. They do not allow the quantification of the genotypic similarity between accessions as do genetic distances from DNA polymorphism. The simplicity of laboratory assay for RAPD markers makes them an attractive method for obtaining intraspecifc distinctions. This technique is already used for cultivar identification and genetic variability analysis of several underutilized fruit crops like loquat (Badenes et al., 2004), tamarind (Diallo et al., 2007) and for protecting plant patents (Baird et al., 1996), although some question regarding reliability of RAPDs have been raised. In this study, a set of RAPDs was used for distinguishing the superior genotypes of bael. The comparatively higher percentage of polymorphic bands detected in the present study clearly indicated that RAPD fragments are moderately polymorphic and particularly informative in the estimation of genetic relationship of bael genotypes. According to Faleiro et al. (2009), the number and percentage of polymorphism in RAPD fragments depends on the number and variability of the genotypes and/or accessions analysed. Comparatively high amplitude of the genetic similarity coefficient established in the present study confirms the occurrence of considerable genetic variability among bael genotypes. However, variation was higher than that reported by Rajwana et al. (2008) for 25 cultivars of mango (range 0.69-0.89) but lower than that reported by Karihaloo et al. (2003) for mango cultivars (0.32 -0.75). The highest genetic similarity coefficient of 0.72 between the 'Bael-5' and 'Bael-8' might be due to their same place of origin (North Andaman) or occurrence of an intense gene flow between these genotypes.

The genotypes 'Bael-1' and 'Bael-7' showed the lowest similarity coefficient, which could be due to their differences in fruit characteristics and genetic back ground. But, the molecular diversity was not in agreement with the most of the morphological diversity as also reported by Singh et al. (2012) in *Colocasia esculenta*. These findings support the view that morphopomological characteristics are not the only reliable tools in estimating genetic relationships among large and diverse groups of genotypes/cultivars and should be used mainly for discrimination. However, genetic diversity of some *Rosa* genotypes which were analyzed by RAPD, amplified fragment length polymorphism (AFLP) and morphological characteristics showed a significant correlation among the different marker systems (Wen et al., 2004).

Dif-ferences in results obtained in grouping by using RAPD markers and grouping by phenotaxonomical characters were also reported in strawberry and banana (Garcia et al., 2002; Uma et al., 2004). This can be related to many reasons; one is the effects of grouping by phenol-taxonomical characters were also reported in different climatic conditions on morphological traits, which do not influence RAPD markers (Kumar, 1999; Gupta and Rustgi, 2004). The fragments of genome amplified with RAPD primers may not be part of codons for morphological characteristics (Sarkhosh et al., 2009). It should also be noted that post-transcriptional modifycations and non-nuclear inheritance of some characterristics can cause the lack of fitting of morphological markers with molecular markers (Gupta and Rustgi, 2004). Most of the developed DNA markers are based on the genomic DNA and therefore could belong to either the transcribed regions or the non-transcribed regions of the genome. These markers derived from any region of the genome have also been described as random DNA markers (Sarkosh et al., 2006).

However, during the recent past, research emphasis shifted towards the development of molecular markers from the transcribed region of the genome. The availability of a large number of cDNA clones in a variety of plant systems, and also accumulation of a large number of expressed sequence tags (EST) have made this possible. So far, not a single report has been published on genetic diversity analysis of bael using advanced molecular marker systems viz. sequence-characterized amplification regions (SCAR), simple sequence repeats (SSR), single nucleotide polymorphism (SNP) and EST due to lack of genetic information in bael. Therefore, future research programme should be concentrated to develop advanced marker systems for genetic diversity analysis and identification of candidate marker linked desirable traits and genes.

Conclusions

RAPD analysis has been shown to be useful technique for providing information concerning the degree of polymorphism and diversity of bael as well as for characterizing the germplasm. Findings of this experiment indicate that identification and collection of genotypes from various locations of the country / province, which was mainly based on morphological characteristics, may have encountered the mismatches and mistakes. This emphasizes the importance of characterization both at molecular and morphological level for efficient maintenance and exploitation of precious germplasm and to determine groups of high genetic similarity and dissimilarity, which is the key for establishing breeding strategies in genetic improvement programme of bael.

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Full Length Research Paper

Genetics of seed coat color in sesame (*Sesamum indicum* L.)

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Seed coat colour of sesame is commercially an important trait. Developing white seeded varieties with long lasting luster has received momentous attention in most of the major sesame producing countries including India. The present investigation centered on the genetic control of seed coat colour in sesame. No genetic nomenclature is available in sesame to describe seed coat colour. This is the first attempt to designate genes engendering specific seed coat colour. The findings are based on five different crosses with seed coat colour belonging to white, beige and various shades of brown colour. In general, tetragenic model corroborated with the colour combinations. The colour beige seemed to be fixable as well as suppressive over other colors. In white seeded seeds, several genes co-existed and those genes produced colored segregants in F_2 generation. Two loci, *Gr* and *I,* regulated suppression or intensification of pigment production. Two major genes *V* and *B* were accountable for basic colour production. The recessive allele *'b'* tightly linked with *'r',* resulted into beige colour which suppressed the effect either of the loci *V* and *B*. The study of inheritance pattern of seed coat would aid to evolve varieties with specific desired seed coat color.

Key words: Genes, inheritance, seed coat colour, nomenclature and *Sesamum indicum* L.

INTRODUCTION

Sesame, an ancient oilseed crop, is termed as a 'queen of oilseeds' by Bedigian and Harlan (1986) as it is rich in polyunsaturated fatty acid, proteins, vitamins, niacin, minerals and lignans (Budowski and Markley, 1951; Moazzami and Kamal-Eldin, 2006; Nakimi 1995; Yamashita et al., 1992) and is popularly used as a food and medicine (Mochizuki et al., 2010; Liao et al., 2010; Jan et al., 2009; Jan et.al. 2011; Coulman et al., 2009). The seed coat color of healthy sesame seed ranges from black to white through different intermediates like, dark brown, brown, light brown, beige, and cream. White sesame seeds, have higher oil, protein and moisture ratios as compared to black seeded sesame (Kanu, 2011). Seed coat color in sesame seems to be associated with seed biochemical properties, antioxidant content and activity, level of disease resistance among sesame accessions, in addition to being a marker of evolution within the *Sesamum* genus (Budowski and Markley, 1951; Nakimi, 1995; Kanu, 2011; Shahidi et al., 2006; El-Bramawy et al., 2008; Zhang et al., 2012, 2013). Pathak and Dixit (1992) reported that the preference for colour in sesame differs from region to region. In spite of ameliorating demand of white seeded sesame, high yielding white seeded sesame varieties with good oil content are quite limited in India. The lack of knowledge about genetic complexity of the trait might be responsible for less

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progress in evolving varieties of desired colour (Tiwari and Campbell, 1996).

The physical appearance of seed colour is a key marketing factor and acceptability of sesame type varies greatly with cultural preference. Japanese prefer black seeded sesame and Sudanese favor white seeded ones (Hossain et al., 2010). In particular, a larger seed size, coupled with a light-colored seed coat like white often command price premiums in a market-dependent manner (Graham et al., 2001). Darkening or segregation of desirable color in the seed coat due to genetic recombination results in a less-marketable product (Cassells and Caddick, 2000). In Eastern India, white seeded sesame is sold at a price at least 30% higher than that of brown seeded or black seeded varieties because of consumer's preference and greater culinary utility (Chakraborty et al., 1984).

Considerable strides over the last few years at the National and International level have resulted into up-grading agronomic traits including yield in sesame. But the issue of inheritance of seed coat colour remained unattended. All earlier researchers in sesame, outlined seed coat colour to be under digenic control with several confusing segregants beyond plausible explanation (Nohara et al., 1933; Gutierrez et al., 1994; Baydar and Turgut, 2000; Falusi, 2007). Recently, Zhang et al. (2013), using a high-density linkage map analyzed the genetic segregation and quantitative trait loci (QTL) for sesame seed coat color and showed that two major genes with additive-dominant-epistatic effects along with polygenes were responsible for controlling the seed coat color trait. Keeping in view, complicated picture of inheritance, the present investigation was undertaken to unravel genetics of seed coat colour under different genetic back ground.

MATERIALS AND METHODS

Seed colour classification and evaluation

Genotypes belonging to different seed coat colour were selected as experimental materials (Table 1 and Figures 1-6). Seed coat colour accession number was assigned for each colour according to norms of the International Plant Genetic Resources Institute (IPGRI and NBPGR, 2004). Seeds were classified phenotypically as soon as possible after harvest to minimize misclassification resulting from seed darkening, immaturity, weathering, or disease.

Sesame, a self-pollinating crop with epipetalous flowers has the advantage of easy emasculation and pollination. To carry out crossing program flowers were hand-emasculated the previous afternoon and covered with waxy pollination bags. Next day cross pollination was made by dusting the pollen grains of the desired flower directly onto the stigmas of emasculated flowers. Total 41 crossings were done among the parental materials for inheritance study. The F_1 plants were self-pollinated to produce the F_2. The parents, F_1, reciprocals, F_2 and F_3 progenies were grown in the Agricultural experimental station, Calcutta University at Baruipur, West Bengal, India (22.35°N, 88.44°E.). Normal recommended agronomical practices were followed for cultivation. The selfed seeds of individual plants were harvested and scored for seed colour to determine the inheritance pattern of the trait. Out of 41 crosses, 5 crosses are being reported here: OSC-593 X Amrit, Rama X VRI-1, Tillotama X NIC-8316, Tillotama X Gujarat Til-2, Uma X TKG-352.

RESULTS AND DISCUSSION

The F_1 seeds of any cross involving two parents and their reciprocals were similar in colour to that of the maternal parent of the specific cross and hence depicted seed colour was under control of maternal tissue, Wilson and Hudson (1979), earlier reported similar findings. Chen and Heneen, (1992) were of the opinion that interaction between maternal and embryonic component determined seed coat colour in Brassica. Van-Deynze and Pauls (1994) reported more conclusively that seed coat colour albeit, is primarily determined by maternal genotype, but genetic constitution of parents finally determine expression of character.

The genetic control of seed colour in the investigated crosses seemed to fit the tetragenic ratio. In sesame, nomenclature is unavailable to describe the genes controlling seed coat colour. This is the first attempt where genes of seed coat colour have been designated. The investigation confirmed that four genes namely *Gr, B[R], V and I* were involved in the determination of seed coat colour. The recessive genes, *gr* and *i* prevented production of colour pigment that is, they acted as inhibitors. The white seeded sesame transpired when both the locus *Gr* and *I* were in recessive homozygous state. The locus *I* in dominant state was responsible for engendering more intensified colour.

Seed coat colour would be black when one dominant allele was present at these three loci namely *Gr, V* and *R*, with either dominant or recessive gene at *I* locus Dominant *I* gene produced bright black, whereas, recessive *i* generated dull black. In general, colored seeds were dominant over non-colour seed. Coloured light brown was dominant over white seeded coat colour. The gene, *B,* responsible for colour production was very closely linked to *R* locus and did not produce any recombinant. The gene products of *I* and *[BR]* produced brown colour which was dominant over white colour. Interestingly, the dominance relations of the four genes were in the order *[BR], I, V, Gr,* where the presence of colour was due to dominant genotype in each case. Most cultivated sesame varieties homozygous for a dominant form of the *I* gene resulting in varieties with colored seed coat.

On the basis of above description, the total explanation of genetic ratio for colour inheritance was attempted.

Cross 1: OSC-593 X Amrit

The parent OSC-593(♀), a white seeded variety was crossed to Amrit (♂), a light brown seeded variety, the F_1 progenies engendered white seeded hybrid (F_1) with colour like that of OSC-593 and the F_2 progenies segregated into brown, light brown and white seeded plants in 9:6:1 ratio (Table 2). The reciprocal cross involving Amrit as female and OSC-593 as male parent produced F_1 progenies of light brown colour.

Table 1. Seed coat colour and parentage of the genotypes used in this study.

Genotype	Parentage	Source	Seed coat colour	Score for Seed-coat colour
GT-2	Gujarat Til-1x TC-25	ICAR Sesame Project, Jabalpur	White	1
OSC-593	Not available	ICAR Sesame Project, Jabalpur	White	1
RAMA	Selection from 'Khosla' local	Calcutta University	Medium brown	5
UMA	Mutant of Kanak	ICAR Sesame Project, Jabalpur	Beige	3
NIC-8316	Indigenous collection	ICAR Sesame Project, Jabalpur	Beige	3
TILLOTAMA	Selection from local germplasml Jinardi Ducca-2	Calcutta University	Medium brown	5
VRI-1	pureline selection from thirukattupalli local of TN	Calcutta University	Dark brown	6
Amrit	Selection from XU-2 X Krishna	ICAR Sesame Project, Jabalpur	Light brown	4
TKG-352	Not available	ICAR Sesame Project, Jabalpur	White	1

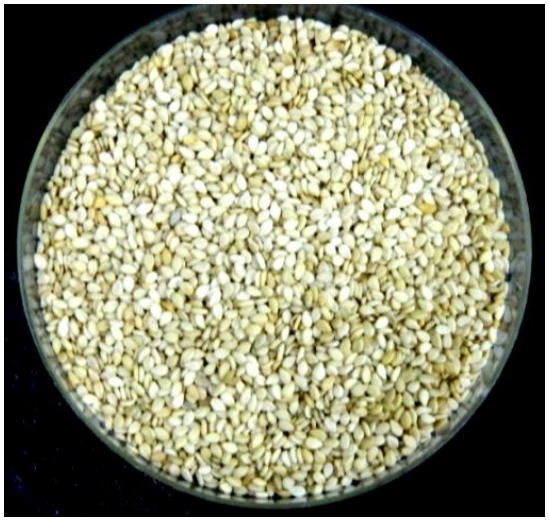

Figure 1. White colored seed, code-1.

Figure 3. Light brown colored seed, code-4.

Figure 2. Beige colored seed, code-3.

Figure 4. Medium brown colored seed, code-5.

Figure 5. Dark brown colored seed, code-6.

Figure 6. Bright black coloured seed, code-12.

Cross 2: Rama X VRI-1

The 2nd cross combination was involved with Rama, a medium brown seeded variety, as a female parent and VRI-1, a dark brown seeded variety, as a male parent. The hybrid (F_1) seed coat colour corroborated with female parent that is, Rama and reciprocal cross between varieties VRI-1 and Rama confirmed similar earlier observation of maternal tissue inheritance (Table 3). A segregation pattern of 9:3:3:1 ratio belonging to black, dark brown, medium brown and light brown seeded plant respectively, was found in F_2 generation. The segregation nature confirmed simple digenic control but the present explanation is not based on two genes only.

Cross 3: Tilottama X NIC-8316

A popular variety, Tilottama characterized by medium brown seed coat, when was hybridized with a beige colored seed, NIC-8316, the F_1 progenies were like to that of female parent (Table 4). The reciprocal cross also affirmed similar findings. The F_2 progenies produced three groups of different seed coat colour namely brown, light brown and beige colored seed coat having 9:6:1 ratio, respectively. Surprisingly, such ratio was obtained only when the variety Tillotama was acted as a female parent. The F_2 progenies of the reciprocal cross did not exhibit further segregation and only beige colored seeds were obtained. In other words, beige colored seeds were fixable in nature. The recessive gene, *bb,* in association with tightly linked gene, *rr,* generated beige colour and an inhibitory effect over other genes was responsible to produce beige colour of fixable nature. It is a unique observation of this inheritance study.

Cross 4: Tilottama X Gujarat Til-2

The medium brown seed coated variety Tilottama, when combined with Gujarat Til-2, a white seeded variety, generated F_1 progenies, belonging to medium brown colored seed. The reciprocal cross of Gujarat Til-2, as female, and Tilottama, as male, engendered F_1 seeds, like that of maternal tissue (Table 5). The F_2 progenies segregated into 15 brown: 1 white colored seeded plants.

Cross 5: Uma X TKG-352

The cross combination consisted of variety Uma, a beige colored type of seed and TKG-352, a white seeded variety. The beige colored variety, Uma, when was hybridized with TKG-352, all F_1 progenies expressed seed coat colour like that of Uma (Table 6). The F_2 progenies, on the other hand, did not exhibit further segregation and produced only beige colored seeds. Interestingly, the beige colored seed did not segregate further after F_2. The reciprocal cross combination that is, TKG-352 X Uma produced F_1 progenies having white seeded sesame.

Since, the seed coats consisted of maternal tissue; seed coat colour is determined by the genotype of the plant in which the seeds develop, rather than by the genotype of the embryo within that seed. The ovule is the progenitor of the seed and embryonic development proceeds within the protective maternal tissue of the ovule, which turns into the seed coat encircling the developing embryo along with endosperm (West and Harada, 1993). Thus, testa is a maternally determined tissue, whilst the endosperm and the embryo development are the result of fertilization (Albert et al., 1997). A maternal effect on seed

Table 2. Observed segregation ratio of plants in different generation of cross OSC-593 X Amrit.

Possible genotype of light brown-seeded parents	Number of lines	Generation	Seed color			Ratio	χ^2	P value
			Brown	Light brown	White			
grgr[B_R_]vvii/ grgr[B_r_]vvii/ grgr[bbrr]vvii	90	P1	-	-	90			
GrGr[bbrr]vvII/ GrGr[bbR_]VVii/GrGr[B_R_]vvii	90	P2	90	-	-			
F_1	94	F_1	-	-	94			
F_1 (Reciprocal)	92	RF_1	-	92	-			
F_2	210	F_2	120	74	16	9:6:1	0.94	5.991

Table 3. Observed segregation ratio of plants in different generation of cross Rama X VRI-1.

Possible genotype of light brown-seeded parents	Number of lines	Generation	Seed color				Ratio	χ^2	P value
			Black	Dark Brown	Medium Brown	Light Brown			
GrGr[B_R_]vvII/ GrGr[b_R_]V_II	90	P1	-	-	90	-			
GrGr[B_r_]VVii/ GrGr[B_r_]VVII	90	P2	-	90	-	-			
F_1	81	F_1	-	-	81	-			
F_1 (Reciprocal)	78	RF_1	-	78	-	-			
F_2	232	F_2	132	39	45	16	9:3:3:1	0.68	7.815

Table 4. Observed segregation ratio of plants in different generation of cross Tilottama X NIC-8316.

Possible genotype of light brown-seeded parents	Number of lines	Generation	Seed color			Ratio	χ^2	P value
			Medium Brown	light Brown	Beige			
GrGr[B_R_]vvII/ GrGr[b_R_]V_II	90	P1	90	-	-			
GrGr[bbrr]vvii	90	P2	-	-	90			
F_1	68	F_1	68	-	-			
F_1 (Reciprocal)	60	RF_1	-	-	60			
F_2	199	F_2	106	78	15	9:6:1	0.99	5.991

Table 5. Observed segregation ratio of plants in different generation of cross Tilottama X Gujarat Til-2.

Possible genotype of light brown-seeded parents	No. of lines	Generation	Seed color		Ratio	χ^2	P value
			Brown	White			
GrGr[B_R_]vvII/ GrGr[b_R_]V_II	90	P1	90	-			
grgr[B_r_]vvii/ grgr[B_R_]vvii/ grgr[bbrr]vvii	90	P2	-	90			
F_1	89	F_1	89	-			
F_1 (Reciprocal)	74	RF_1	-	74			
F_2	237	F_2	218	19	15:1	1.75	3.841

is not surprising since the supply of carbohydrates, nutrients and water provided by the plant to the developing seeds as well as the structure of the seed coat could affect seed development and deposition of reserve (Bewley and Black, 1994). Segregation for seed coat can be determined only by advancing the progenies an additional generation (Liu et al., 2005). Reciprocal crosses was performed in order to confirm the pigment accumulation in maternal tissue. The present study did not deviate from earlier observation of crosses and so, irrespective of the crosses, seed coat colour of F_1 was determined by the seed colour of the female parent.

Table 6. Observed segregation ratio of plants in different generation of cross Uma X TKG-352.

Possible genotype of light brown-seeded parents	Number of lines	Generation	Seed color		Ratio	χ^2	P value
			Beige	White			
GrGr[bbrr]vvii	90	P1	90	-			
grgr[bbrr]vvii/ grgr[B_r_]vvii/ grgr[B_R_]vvii	90	P2	-	90			
F_1	93	F_1	93	-			
F_1 (Reciprocal)	96	RF_1	-	96			
F_2	443	F_2	443	0	-	-	-

It is pertinent to discuss some of the genesis of variation of seed coat colour in crops. Flavonoids including anthocyanin play an important role in emending discernible colour variation (Mol et al., 1998). A similar finding has been reported in seed colour in sweet clover where, pigment of seed coat and the embryo interact for generating seed colour (Gorz. et al., 1975). Seed coat colour variation in bean is broadly controlled by production of flavonoids (Beninger et al., 1998). Modifying genes also interact with colour producing genes to express different colour combination and sometimes make hindrance for a definite conclusion (Beninger and Hosfield, 1999; Beninger et al., 1999). The study carried out by Ono et al. (2006), hinted that the lignan formation was developmentally regulated keeping with same pace with seed development and this also interfered in seed coat colour development. Sesamin, a major shareholder of lignan, is widely distributed in vascular plants, of *Sesamum* spp., but its biosynthesis and physiological roles are yet to be established. The recent findings of Zhang et al. (2013) have further confirmed the presence of two major genes in seed coat colour inheritance, though all seed colors were not included in the study. The present investigation disclosed the involvement of two genes in the production of pigments and two other additional genes as modifier genes. A number of genotypes may give rise to the same phenotypes by following the present genetic model. Bedigian (2010) and Falusi (2007) reported earlier similar kind of observation.

Presently, somewhat unique finding was obtained so far as beige colored seed was concerned, as the colour has inhibitory effect on other colors, whatever the genetic constitution may be. Genetic structure of seed coat colour and identification of candidate gene through QTL or association mapping may not lead to precise definition of genes unless functional genomics studies are also not involved (Zhang et al., 2013). However, Plant breeders would like to get benefit of developing new varieties of commercially important seed coat colour by utilizing the information of genetics and desired goal is expected to be achieved through conventional breeding program.

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Molecular and genetic study of wheat rusts

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***Puccinia triticina, Puccinia graminis* and *Puccinia striiformis* cause leaf, stem and yellow rust, respectively. Wheat rusts can cause losses as high as 70%. The rusts ability to evolve fungicide resistance has resulted in the use of resistant cultivars as the primary method of control. Breeding resistant cultivars is a long process and requires an accurate picture of the current and future pathogen population. Differentiation of wheat rust pathotypes using conventional plant pathology techniques is time consuming, labour intensive and requires the services of a highly skilled and experienced plant pathologist. Modern molecular biology techniques have the potential to aid the conventional techniques and provide fast, accurate same-day results. Microsatellite markers were used to differentiate *P. triticina* and *P. striiformis* pathotypes. Amplified fragment length polymorphisms (AFLP) were used to differentiate stem rust *P. graminis* pathotypes. Phylogenetic trees were created for leaf and stem rust pathotypes. Field isolates of leaf, stem and yellow rust were collected from eleven sites across the Western Cape Province. Microsatellite markers were used to type leaf and yellow rust isolates. AFLP markers could not be used on field isolates due to the presence of plant DNA. Novel alleles found in the Leaf and yellow rusts isolates prevented the assigning of a specific pathotype to each isolate. UVPrt10 (25.2%) and UVPrt9 (21.5%) were the most prevalent leaf rust pathotypes. Only 6E16A+ was identified in the yellow rust isolates. Pathotype incidence was similar to previous studies. The prevalence of multiple pathotypes with a variety of virulence genes in the rust population shows that breeding lines with single major resistance genes will not be effective and breeding programmes should concentrate on lines that exhibit quantitative resistance.**

Key words: Prevalence, microsatellite, amplified fragment length polymorphisms (AFLP), phylogeny, *Puccinia*.

INTRODUCTION

Puccinia triticina Eriks, *Puccinia graminis* f.sp. *tritici* Eriks and Henn and *Puccinia striiformis* Westend f.sp. *tritici* Eriks are the causative agents of leaf, stem and yellow wheat rust, respectively. Wheat rusts are one of the primary biotic restrictors of wheat production globally (Keiper et al., 2006). Rust infections can cause losses as high as 70% (Murray et al., 1998).

The rusts are highly adaptable and can rapidly evolve resistance to control methods such as fungicides (Boshoff et al., 2003). Selection is the most powerful force shaping the genetic diversity of a population (McDonald and Linde, 2002). In wheat, rust selection is caused by the widespread introduction of resistant cultivars tha cause, cause an increase in the frequency of virulent alleles (Harvey et al., 2001).

The effect of selection can be reduced by several methods. Pyramiding several major resistance genes and breeding for durable resistance based on the amassing of additive minor genes through nonspecific pathotype (slow rusting) resistance is a successful strategy. With slow rusting, disease progression is not prevented but rather slowed. The result is intermediate to low levels of disease with all the pathotypes of the particular pathogen (Duvellier et al., 2007; Li et al. 2010). Such quantitative resistance

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cannot be rapidly broken and will only be gradually eroded (McDonald and Linde, 2002).

Rotation of genes, that is, where different major resistance genes are rotated in time and space or cultivars with different combinations of resistance genes are grown; has also been shown to disrupt selection (Zhu et al., 2000; McDonald and Linde, 2002).

In South Africa, rust control has focused on the introduction of resistant cultivars which has been shown to be an effective method of reducing rust infections (Pretorius et al., 2007). However, the use of these resistant cultivars must be carefully managed because if they are introduced and used too widely (effectively creating a monoculture) the rusts will quickly develop new virulences due to the very high selective pressures being placed upon them (McDonald and Linde, 2002) as can be seen with the emergence of race TTKS in East Africa in 1999 (Pretorius et al., 2000; Expert Panel on the Stem Rust outbreak in East Africa, 2005) and its rapid spread and acquisition of new virulences (Terefe et al., 2011; Pretorius et al., 2012). Breeding a new resistant cultivar requires extremely long timeframes; sometimes above of fifteen years can pass between the initial cross made for a cultivar and the first release of commercial seed. For this reason it is necessary to have an accurate picture of the pathotype composition of the current and future rust population. In order to gain this knowledge and make predictions as to the future pathogen population structure, population genetic studies of the wheat rusts have to be conducted.

Conventional plant pathology that is used to differentiate the pathotypes of a pathogen, such as the *Puccinia* spp. Is a complex and time consuming process. They require a highly skilled plant pathologist to ensure accurate results. Modern molecular biology techniques have the potential to complement the conventional techniques and generate accurate results the same day (Brown, 1996; McCartney et al., 2003).

A wide variety of molecular markers have been used in the differentiation of wheat fungal pathogens. These include: microsatellite markers (Enjalbert et al., 2002; Szabo and Kolmer, 2007; Szabo, 2007; Zhong et al., 2009; Kolmer et al., 2013; Kolmer, 2013), restriction fragment length polymorphisms (Chen et al., 1994; Keller et al., 1997a, b; Zhan et al., 1998), Southern Blots (Shan et al., 1998), random amplification of polymorphic differences (Kolmer et al., 1994; Park et al., 2000), amplified fragment length polymorphism (AFLP) (Visser et al., 2009), and high resolution melt analysis of Real-time polymerrase chain reaction (RT-PCR) of the ITS region of the rDNA gene (Schena et al., 2004; Barnes and Szabo, 2007; Liu et al., in press) and whole genome sequencing (Cuomo et al., 2010).

There are several genetic and practical considerations that affect a particular marker systems efficacy in differentiating between the pathotypes of a pathogen (Brown, 1996). Microsatellite markers satisfy most of the conditions as they are highly polymorphic, co-dominant and relatively easy to score. They are also suitable for use in high throughput systems. AFLP markers also meet many of the conditions.

Molecular marker based techniques have demonstrated that they possess the potential to complement conventional plant pathology techniques in the differentiation of fungal pathogens of wheat.

MATERIALS AND METHODS

The following rust pathotypes were used: Stem rust: UVPgt50 (2SA4), UVPgt51 (2SA36), UVPgt52 (2SA100), UVPgt53 (2SA102), UVPgt54 (2SA55), UVPgt55 (2SA88), UVPgt56 (2SA102K) and UVPgt57 (2SA105). Leaf rust: UVPrt2, UVPrt3 (3SA123), UVPrt4, UVPrt5, UVPrt8 (3SA132), UVPrt9 (3SA133), UVPrt10 (3SA126), UVPrt13 (3SA140) and UVPrt19. Yellow rust: 6E16A-, 6E22A- and 7E22A- All the samples were obtained as frozen urediniospores from L. Snyman (University of Stellenbosch). The original source of most of the samples was Prof. Z. Pretorius (University of the Free State).

DNA extractions were done from 40 µg of frozen urediniospores. The CTAB protocol of Liu and Kolmer (1998) was followed. Primers: All primers were used at a concentration of 10 ng/µl and were obtained from IDT. All PCRs were performed in an Applied Biosystems 2720 Thermal Cycler.

Microsatellites

The reaction mix to amplify the leaf and yellow rust microsatellites was as follows: 1 µl 10X Kapa Buffer A (KapaBiosystems), 2 µl 25 mM $MgCl_2$ (KapaBiosystems), 0.8 µl 10 mM Kapa dNTP mix, a volume of primer as specified in Table 1, 0.042 µl 5 U/µl KapaTaq (KapaBiosystems), 1 µg 100 ng/µl DNA and sufficient distilled, autoclaved water (dH_2O) to make the final reaction volume 10 µl. The following PCR programme was used to amplify the microsatellites: 2 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C with a final elongation step of 10 min at 72°C. The reaction products were analysed on an Applied Biosystems 3130xl Genetic Analyser. The electropherograms were analysed using GeneMapper 4.0 (Applied Biosystems).

AFLP

AFLP primers from Visser et al. (2009) were used to differentiate the stem rust pathotypes (Table 2). The AFLP protocol from Honing (2007) was used. The reaction products were analysed on an Applied Biosystems 3130xl Genetic Analyser. The electropherograms were analysed using GeneMapper 4.0 (Applied Biosystems).

Microsatellite data analysis: A matrix that recorded the size of each specific microsatellite fragment for each pathotype was constructed in Excel. Data analysis was performed using Power Marker v3.25 (Liu and Muse, 2005; http://statgen.ncsu.edu/powermarker/). PowerMarker was chosen because of its ability to handle microsatellite data as well as complete all the required calculations with no additional software required. The data was imported into PowerMarker from a text file. The allele frequencies were calculated. Genetic distances were calculated from the frequency data using the CS Cord 1967 distance calculation (Cavalli-Sforza and Edwards, 1967) as it has been shown that this model can produce true tree topology irrespective of the microsatellite mutation model used (Takeszaki and Nei, 1996). Cladograms were generated in PowerMarker using the neighbour-Joining clustering method, as this method is more suited to determine tree topology from CS Cord distance than the unweighted pair group method with arithmetic mean (UPGMA) method (Takeszaki and Nei, 1996). Cladograms

Table 1. Primers used to type leaf and yellow rust field isolates.

Name	Label	Sequence	Volume (µl)	Annealing temperature (°C)
Leaf rust				
PtSSR68-F	NED	5' – GAC TCA GCC CAC TGC TAA CC- 3'	0.250	60
PtSSR68-R		5' – GAT GGC GAC GTA TTT GGT CT- 3'	0.250	60
PtSSR151A-F	VIC	5' – TCA TCG CAC TCC ACT CAG AC- 3'	0.225	60
PtSSR151A-R		5' – ATG CTG CCC AAC CTG CTC- 3'	0.225	60
PtSSR154-F	NED	5' – ACG GTC AAC AGC CAA CTA CC- 3'	0.225	60
PtSSR154-R		5' – CCT CGT CAT CCT GGT TGA GT- 3'	0.225	60
Yellow rust				
RJ22-F	6-FAM	5' - CCC TTC GTC TGT CAT CCG - 3'	0.350	60
RJ22-R		5' - ATC AAG AAG ATT CCT GGG TGA G - 3'	0.350	60
RJ27-F	NED	5' - CGT CCC GAC TAA TCT GGT CC - 3'	0.300	60
RJ27-R		5' - ATG AGT TAG TTT AGA TCA GGT CGA C - 3'	0.300	60

Table 2. Sequences of the AFLP primers used to differentiate the pathotypes of stem rust.

Name	Label	Sequence	Volume (µl)
EcoRI Adapter[a]		5'- CTC GTA GAC TGC GTA CC -3' 3'- CAT CTG ACG CAT GGT TAA -5'	1.00
MseI Adapter[a]		5'- GAC GAT GAG TCC TGA G -3' 3'- TAC TCA GGA CTC AT -5'	1.00
EcoRI Primer+0		5'- GAC TGC GTA CCA ATT C -3'	1.50
EcoRI Primer+1.0	NED	5'- GAC TGC GTA CCA ATT CA -3'	0.25
EcoRI Primer+2.1	VIC	5'- GAC TGC GTA CCA ATT CAA -3'	0.25
EcoRI Primer+2.2	6-FAM	5'- GAC TGC GTA CCA ATT CCC -3'	0.75
EcoRI Primer+2.3	PET	5'- GAC TGC GTA CCA ATT CTG -3'	0.75
MseI Primer+0		5'- GAT GAG TCC TGA GTA A -3'	1.50
MseI Primer+2.1		5'- GAT GAG TCC TGA GTA AAT -3'	1.00
MseI Primer+2.2		5'- GAT GAG TCC TGA GTA AAG -3'	1.00
MseI Primer+3.0		5'- GAT GAG TCC TGA GTA ACA T -3'	1.00

[a]Adapters were obtained as double stranded molecules.

were visualized in TreeView v1.66 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

AFLP data analysis

A binary matrix for each AFLP fragment was constructed in Excel. The data was then analysed in Power Marker 3.25. The allele frequencies were calculated and the genetic distances were determined using the CS Cord 1967 distance calculation. Dendrograms were generated using the neighbour-joining clustering method. The cladograms were visualised in Tree View 1.66.

Field isolates of leaf and yellow rust were collected from 11 sites in the Overberg and Swartland regions of the Western Cape. The field isolates were collected from wheat and triticale commercial cultivars as well as Stellenbosch University Plant Breeding Laboratory advanced breeding lines. DNA was extracted from the isolates using the protocol from Liu and Kolmer (1998) and diluted to 100 ng/µl.

The leaf and yellow rust isolates were typed using microsatellites markers (Table 1) amplified by fluorescently labelled primers (Szabo and Kolmer, 2007; Enjalbert et al., 2002). Amplification of leaf and yellow rust was done by separate multiplex reactions with a reaction mixture as follows: 1.0 µl 10x Kapa Buffer A (KapaBiosystems), 2.0 µl 25 mM $MgCl_2$ (KapaBiosystems), 0.8 µl 10 mM Kapa dNTP mix (KapaBiosystems), a volume of primer as specified in Table 1, 0.042 µl 5 U/µl KapaTaq (KapaBiosystems), 1.0 µl DNA, and sufficient distilled, autoclaved water (dH_2O) to make the final volume 10 µl. The PCR programme used to amplify the microsatellites was 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, concluding with 10 min at 72°C and a soak temperature of 4°C.

RESULTS

Eighteen microsatellite primer pairs (Szabo and Kolmer, 2007) were used for leaf rust. Of the eighteen, eight pairs either did not amplify or were monomorphic across all the pathotypes. The remaining ten primers pairs amplified twenty nine alleles (including six null alleles) across the

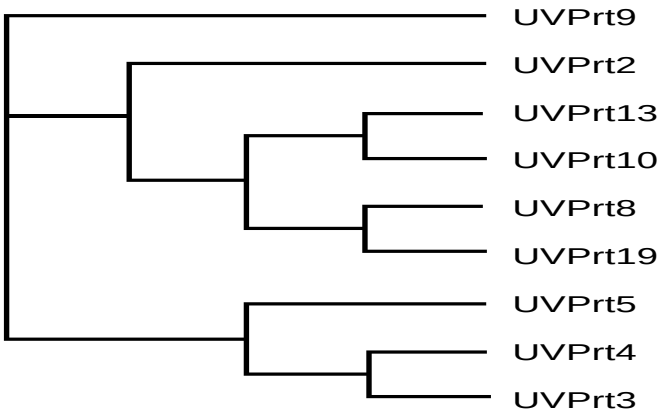

Figure 1. Cladogram of leaf rust pathotypes based on microsatellite data.

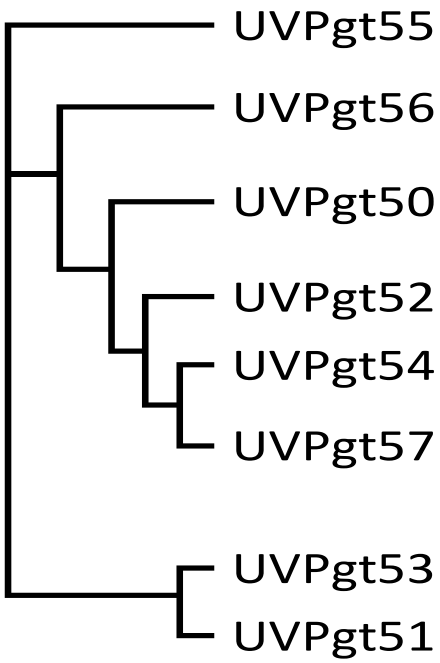

Figure 2. Cladogram of stem rust pathotypes based on AFLP data.

pathotypes. A cladogram was constructed (Figure 1) based on the genetic distances between the pathotypes. The analysis grouped the pathotypes into three main groups with UVPrt 2, UVPrt 13, UVPrt 10, UVPrt 8 and UVPrt 19 together; UVPrt 5, UVPrt 4, UVPrt 3 together and UVPrt 9 alone. It was possible to distinguish between the pathotypes using a subset of the microsatellites, namely PtSSR68, PtSSR151A and PtSSR154.

Stem rust samples had insufficient diversity to allow the pathotypes to be distinguished using only the microsatellites from Szabo (2007) and Zhong et al. (2009).

Twelve different AFLP primer combinations were used, yielding 1926 reproducible fragments. A cladogram was constructed based on the genetic distances between the pathotypes (Figure 2). There were three main groups, UVPgt55 on its own, UVPgt50, UVPgt52, UVPgt54, UVPgt56 and UVPgt57 in the second group and UVPgt51 and UVPgt53 in the third group. It was not possible to distinguish between the pathotypes using only the microsatellite markers as there was insufficient allelic diversity found.

Twelve microsatellite primer pairs were obtained from Enjalbert et al. (2002) for yellow rust. Of the twelve, nine did not amplify or were monomorphic across all the pathotypes. The other three primers (RJ3, RJ22 and RJ27) amplified nine alleles (including three null alleles) across the pathotypes. It was possible to differentiate between the pathotypes using only two markers, RJ22 and RJ27.

Ninety one field isolates of rust were collected from eleven sites in the Swartland and Overberg regions of the Western Cape (Figures 1 and 2). Of these, fifty six were leaf rust, thirty seven were stem rust and three were yellow rust. Many of the isolates were infected by multiple pathotypes and multiple species of wheat rust. Novel alleles were found in the leaf and yellow rust isolates which when combined with the presence of multiple pathotypes and species in some isolates precluded the unambiguous assigning of a specific pathotype or pathotypes to each isolate.

DISCUSSION

Szabo and Kolmer (2007) as well as Kolmer et al., 2013 and Kolmer (2013) found much more diversity at the microsatellite loci tested in their leaf rust samples than was found in this study. These observed differences could be due to only one sample per pathotype being tested or limited diversity in leaf rust in South Africa. The cited studies tested leaf rust isolated from far larger areas than this study which could account for the observed differences. The phylogenetic analysis of the leaf rust pathotypes created three separate groupings: UVPrt9 alone; UVPrt2, UVPrt13, UVPrt10, UVPrt8 and UVPrt19; and UVPrt5, UVPrt4 and UVPrt3. The positioning of UVPrt9 in a separate group to the other pathotypes would imply that it was introduced into South Africa relatively recently, whereas the other two groups appear to have been present in South Africa for some time, allowing differentiation to occur.

Due to the lack of diversity at the tested microsatellite loci, replicating the findings of Visser et al. (2009) and contrary to that of Szabo (2007) and Zhong et al. (2009), it was not possible to distinguish the pathotypes of stem rust. The lack of diversity is probably due to reasons similar to those outlined above for leaf rust. AFLP markers were used, and were found to be highly diverse, again replicating the results of Visser et al. (2009). There was sufficient diversity in the AFLP markers to distinguish each pathotype. This study found significantly more AFLP fragments than Visser et al. (2009) but this can be attribu-

buted to the use of fluorescently labelled primers and the analysis on the automated sequencer, which is much more sensitive than the analysis of poly-acrylamide gels. The phylogenetic analysis of the AFLP data created three separate groups: UVPgt55 alone; UVPgt56, UVPgt50, UVPgt52, UVPgt57 and UVPgt54; and UVPgt53 and UVPgt51 (Figure 2). On an avirulence-virulence level UVPgt55 is very closely related to the East African rust pathotype Ug99 (Pretorius et al., 2007). The positioning of UVPgt55 separately from the other pathotypes and its close pathogenic relationship to Ug99 supports the conclusion that UVPgt55 did not originate in South Africa but was introduced. Visser et al. (2009) used AFLP markers to compare South African stem rust pathotypes to Ug99 and came to the same conclusion. AFLP markers are not suitable for the analysis of field samples as the process of amplifying the DNA is not specific to fungal DNA and will be contaminated by the presence of plant DNA. Individual AFLP markers, specific to each pathotype, could be sequenced and specific primers designed allowing the analysis of field isolates. Our inability to type field isolates of stem rust meant that we could draw no comparisons with the findings of Terefe et al. (2010).

A study by Enjalbert et al. (2002), of ninety six yellow rust isolates from France and China found low levels of diversity at the loci tested. As yellow rust has only beeen recorded in South Africa since 1996 (Pretorius et al., 1997) the expectation was that even lower levels of diversity would be found as South African Yellow rust has not had sufficient time to diversify to the same extent as the yellow rust found internationally. There was sufficient polymorphism in the loci tested (nine alleles across all pathotypes) to allow the pathotypes to be differentiated.

This study found two alleles per locus at the RJ3, RJ17 and RJ21 loci as was found by Enjalbert et al. (2002) and addtionally, seven novel alleles were found, two at the RJ3 locus and one at each of the RJ17, RJ20, RJ22, RJ24 and RJ27 loci. Enjalbert et al. (2002) tested the 6E16 pathotype but do not give the haplotypes for the pathotypes they tested, so it is not possible to compare the South African 6E16 pathotype with the French 6E16 pathotype.

The South African pathotypes are less diverse than the international samples tested by Enjalbert et al. (2002), but this was expected due to the lack of time for differentiation to occur. The novel alleles seems to indicate that the origin of the South African rust pathotypes is not France or China but it is not possible to say this definetively as Enjalbert et al. (2002), do not give the haplotypes of the pathotypes they tested.

Only two primers, RJ22 and RJ27, are required to distinguish between the three yellow rust pathotypes. Phylogenetic analysis of the pathotypes was not done as there are too few pathotypes for meaningful results.

Considerably, more marker diversity was found in the leaf and yellow rust field isolates than had been found in stored samples. However, while these alleles are novel in a South African context, they have already been recorded by Szabo and Kolmer (2007) and Enjalbert et al. (2002). Even with this increase in the number of alleles, South African rust pathotypes still do not display as much diversity as their foreign counterparts. The presence of multiple infections in a single isolate and the presence of novel alleles precluded the definitive assigning of a specific pathotype or pathotypes to each isolate. It also prevented the determination of genotype frequencies which ultimately prevents testing for Hardy-Weinberg equilibrium. Our inability to test for Hardy-Weinberg equilibrium prevents the further use of these markers in population genetic studies until when it is possible to calculate both allele and genotype frequencies.

Leaf rust was found at almost all the eleven localities, this result agrees with the findings of Pretorius et al. (2007), who found leaf rust was widely distributed across the Western Cape (Figures 3 and 4). Yellow rust was confined to only three of the localities (Figures 3 and 4). Yellow rust has a smaller range of optimal growth conditions than leaf rust and the central region of the south Western Cape was the only area in which these conditions occurred during the sampling period.

This study found considerable differences in pathotype prevalence of leaf rust when compared with the findings of previous studies (Figure 5) (Terefe et al., 2009). However, as pathotype prevalence is mostly a function of where pathotypes were collected, the size of the study and the focus of the study (previous studies had excluded triticale and only sampled rust from wheat cultivars), this is not particularly significant. The inability to definitively assign a specific pathotype to every isolate of leaf rust also had an influence on the prevalence of pathotypes. The incidence of pathotypes did not differ greatly when compared with previous studies, indicating that there have been no pathotype shifts and no introductions of new resistance genes. Only three isolates of yellow rust were found and only one pathotype, 6E16A+ was identified making it impossible to draw any conclusions on changes in yellow rust prevalence.

Conclusion

This study has shown that it is possible to conduct molecular marker based studies of wheat rust pathotype prevalence, incidence and distribution. It is possible to distinguish the pathotypes of leaf and yellow rust using only microsatellite markers. It was possible to use these markers to identify which pathotypes are present in field samples, however the presence of novel alleles in the field isolates precluded the assigning of a pathoype (or pathotypes) to each isolate. More work is required to assign these novel alleles to pathotypes.

AFLP markers cannot be used to distinguish field samples because of the presence of plant DNA. It may be possible to convert AFLP markers that are unique to a pathotype to sequence characterised amplified region

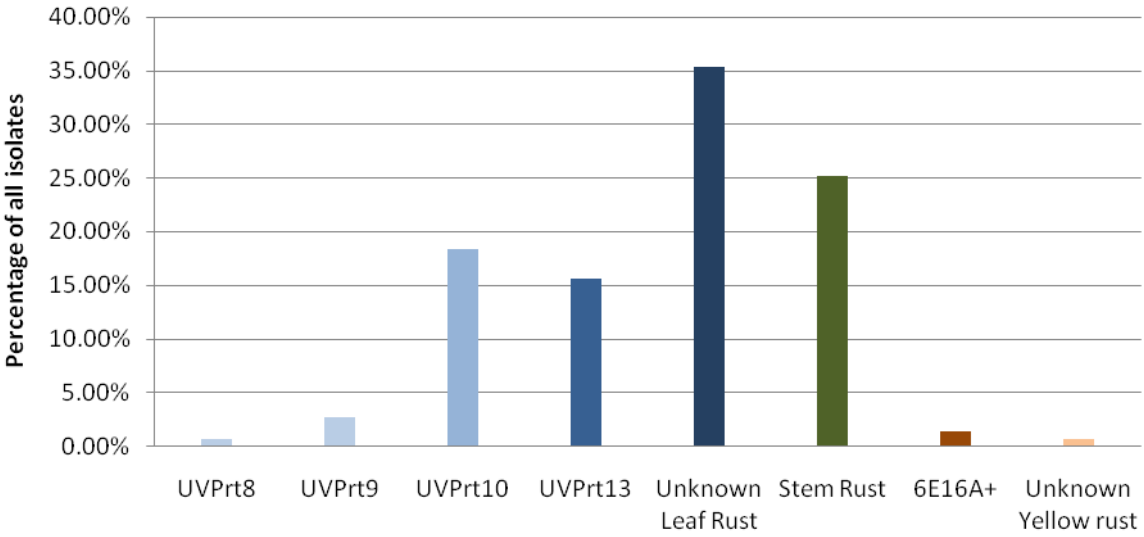

Figure 3. Wheat rust isolates by pathotype.

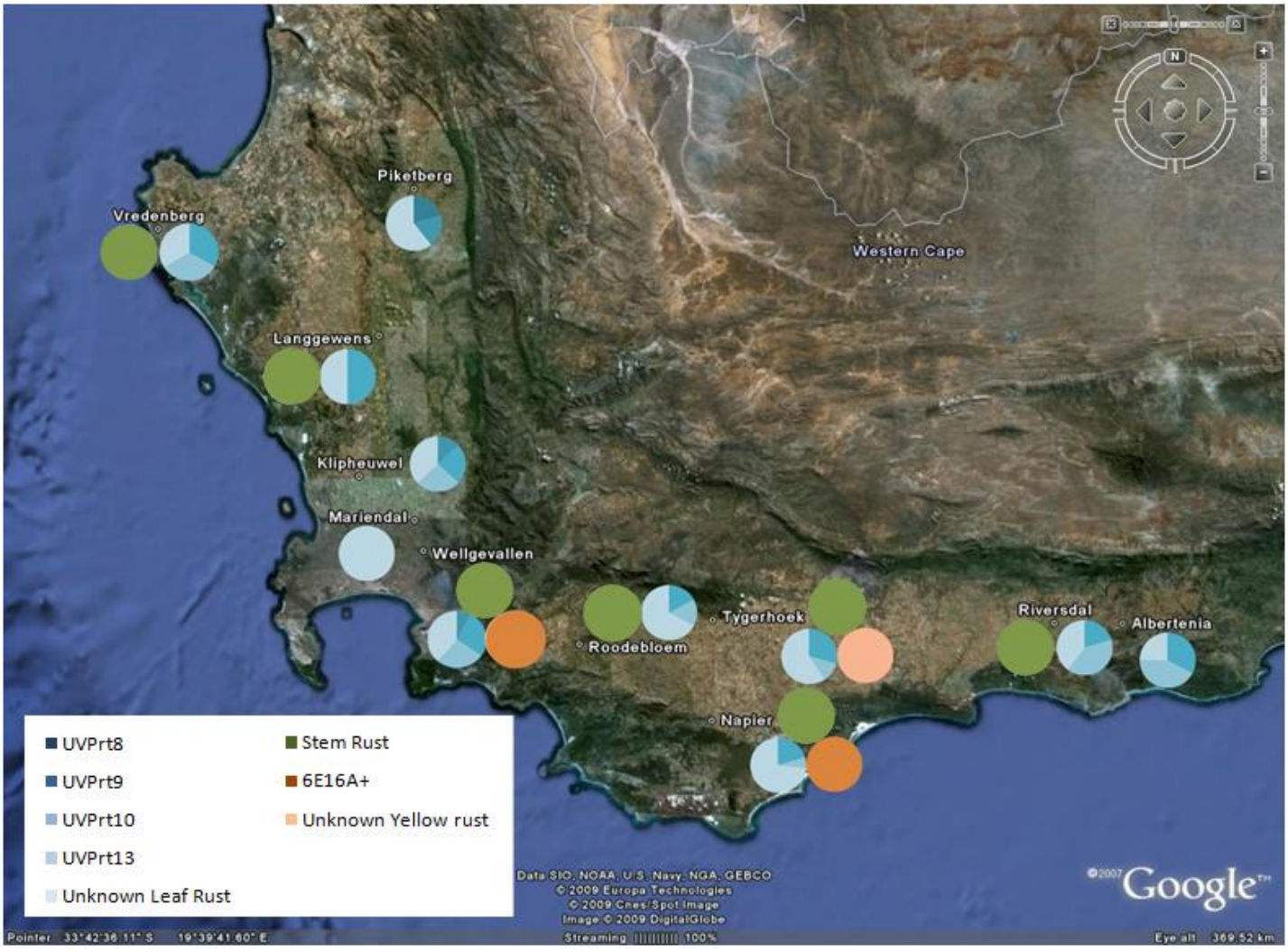

Figure 4. Distribution of wheat rust isolates.

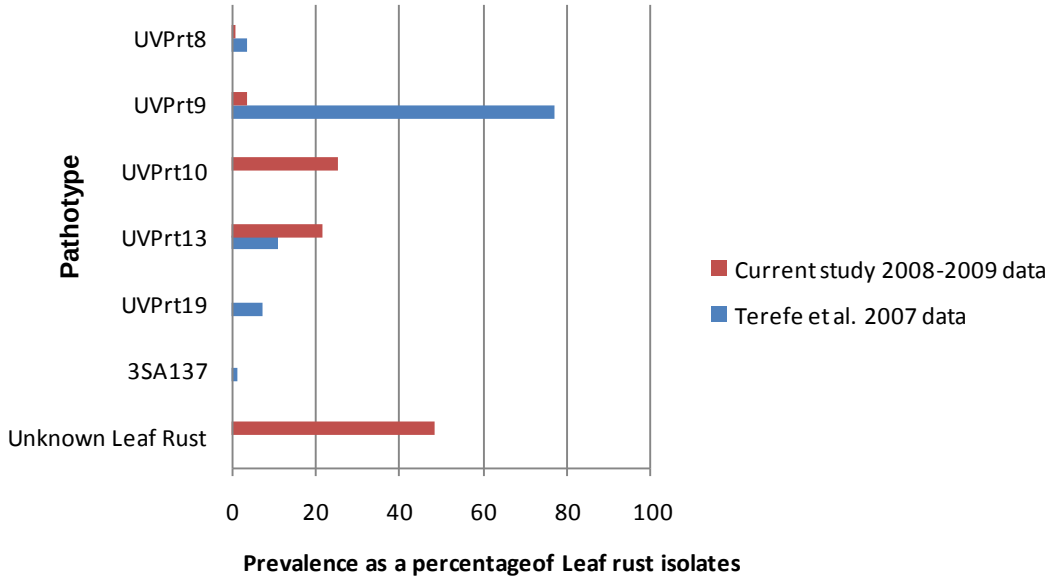

Figure 5. The prevalence of leaf rust isolates found by this study in comparison with the findings of Terefe et al. (2009).

(SCAR) markers. These SCAR markers can then be used to type field isolates. This type of study is not with-out shortcomings, however, multiple infections and novel alleles are confounding factors that will have to be resolved before these techniques can be further developed and used in comprehensive studies of wheat rust diversity and population genetics.

The prevalence of multiple pathotypes with a wide variety of virulence genes in the population shows that breeding for major resistance genes is possibly not the best method as the introduction of cultivars resistant to a single pathotype will simply increase the prevalence of other virulent pathotypes. Rather, breeding programmes should focus on introducing quantitative resistance which provides some resistance to all pathotypes.

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Short Communication

Isolation of NBS-LRR class resistant gene (I2 gene) from tomato cultivar Heamsona

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Three races of *Fusarium oxysporum* f. sp. *lycopersici* race 1, 2 and 3 are identified depending on the avirulence protein or effector protein secreted by fungal pathogen during the host colonization in tomato. These effector proteins are recognized by the host innate immune system based on R gene expressions that are I1, I2 and I3 in tomato for each races. Amongst the three, I2 protein has been cloned and characterized for the incompatibility against race 2 type of the pathogens. In India race 1 type of *F. oxysporum* f. sp. *lycopersici* observed commonly which require presence of I1 gene in tomato plant for the incompatibility reactions but in the present study, I2 gene was partially isolated from the tomato cultivar Heamsona and observed to be resistance against race 1 type of pathogen.

Key words: Fusarium wilt, race, R-gene, resistance, tomato.

INTRODUCTION

Resistance to pathogens is suspected to involve a specific interaction between a resistant plant and a pathogen. These molecules are encoded by resistance (R) genes in plant and each resistant protein typically initiates a defense response in the presence of one pathogen-derived elicitor protein that is known as avirulence (Avr) determinant. The genetic relationship between R and Avr proteins was elegantly stated in the gene for gene hypothesis (Flor, 1971) and this type of plant defense is now described as the plant innate immunity.

Number of polymorphic resistance genes has been identified in the tomato species and each confers resistance against a subset of *Fusarium oxysporum* f.sp. *lycopersici* (Fol) strains. It includes I (for Immunity), I1, I2 and I3 (Huang and Lindhout, 1997). Races of Fol are numbered as per the R gene that is effective against them: the I gene and the (unlinked) I1 gene are effective against race 1 type of *F. oxysporum* f. sp. *lycopersici* that is if tomato plant encompasses it then it will show resistance against race 1 pathotype; similarly race 2 overcomes I and I1, that is it will infect the tomato plant encompassing I and I1 gene and will kill it, but is stopped by I2, while race 3 overcomes I, I1 and I2 but is blocked by I-3 (Rep et al., 2005). So it states that to achieve the resistance against the Fol pathotype or race, the plant must encompass the respective R gene against it.

The I2 locus conferring resistance to race 2 of the soilborne fungus *F. oxysporum* f sp *lycopersici* is the only gene which is characterized in tomato. The selective amplified restriction fragment polymorphism (AFLP) positional cloning strategy was used to identify I2 in the tomato genome (Simons et al., 1998). As the gene responsible for the race 2 resistance has been

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Abbreviations: R, Resistance; **Avr,** avirulence; **AFLP,** amplified restriction fragment polymorphism; **RFLP,** restriction fragment length polymorphism; **NCBI,** National Center for Biotechnology Information.

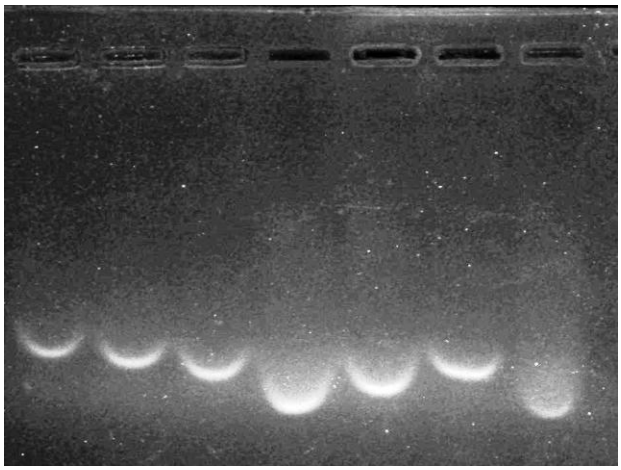

Figure 1. Screening of resistant and suceptible cultivars through I1 primers showed amplicon in all the cultivars.

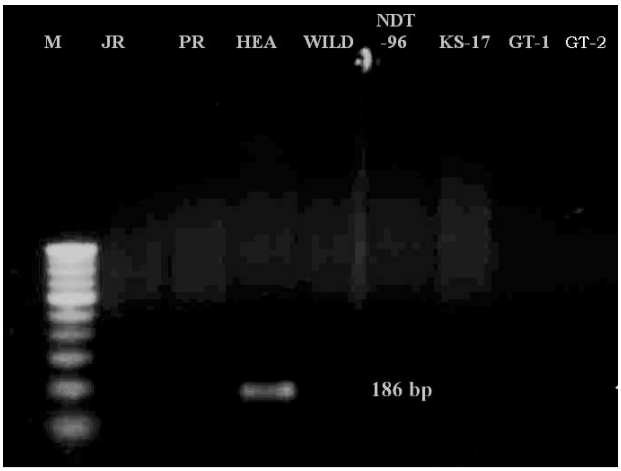

Figure 2. Screening of resistant and suceptible cultivars through I2 LRR primer showed amplicon of size 186 bp in Heamsona cultivar. (M=100bp ladder).

characterized, it will be easy to achieve the resistance in other cultivars by genetic engineering but what is the case with the other races for which the gene has not been charecterized. In this study, we focused on isolation of resistance gene from Indian tomato cultivar to reveal that I2 gene can be employed to achieve resistance against race 1 pathogen.

MATERIALS AND METHODS

Plant material

Heamsona, Gujarat tomato -1 (GT-1), Gujarat tomato-2 (GT-2), Pusa ruby (PR), Junagadh ruby (JR), Wild, NDT-96 and KS-17 cultivars of tomato were cultivated in open field at Main vegetable research station, Anand Agricultural University, Anand, Gujarat, India.

Oligonucleotide design

Two sets of oligonucleotide with varying amplicon size were designed using Primer 3.0 software. First set of primer were constructed for screening of tomato population for presence of I2 gene, within the LRR region of the gene for amplification of twenty two amino acids in two repeats as it is only observed in the active copy of the I2 gene. Forward primer I2 FP 'TCTCACCTCACTTCGCTTCA' and reverse primer I2 RP 'TAGGGCAATCCTGGATGAAC'. TG 194 a restriction fragment length polymorphism (RFLP) probe sequence was used for the screening of I1 gene isolation amongst the tomato population (Sarfatti et al., 1991). Forward primer I1 FP 'TAGGGAGACAGCTTGCATGCCT' and reverse primer I1 RP 'CAAGTTGAAGGATATGAGTATTAT'.

DNA isolation and PCR screening of I1 and I2 gene

Genomic DNA was extracted in bulk from young fresh leaves of tomato cultivars, using the phenol - chloroform method described by Oza et al. (2008) and quantified on a spectrophotometer. Amplification was carried out for the screening of I1 and I2 gene in 12.5µl of reaction mixture, containing 7 µl distilled water, 1.25 µl of 10x assay buffer with 15 mM $MgCl_2$, 2 µl of 100 ng template DNA, 1.5 µl of primer, 1 µl dNTP mix and 0.25 µl Taq DNA polymerase. Polymerase chain reaction (PCR) was performed in a thermal cycler. The PCR profile starts with initial denaturation at 93°C for 5 s, followed by 30 cycles of denaturation at 93°C for 30 s., annealing as per the primers for 2 min, extension at 68°C for 4 min and finally extension at 72°C for 5 min. The products were size-separated on a 0.8% agarose gel in 1×TAE (Tris acetic acid EDTA) buffer at 50 V, stained with ethidium bromide (1 $\mu g \cdot ml^{-1}$) and visualized on a GelDoc.

RESULTS AND DISCUSSION

PCR based screening of I1 and I2 gene

In the present study, the marker TG-194 linked to the resistance gene I1 on chromosome 7 was used to determine the presence or absence of the I1 gene in the eight varieties selected because the gene I1 is not characterized. DNA fingerprint of eight different varieties developed with TG-194 probe (Figure 1) could not show the polymorphism, indicating that it is not linked to resistance and cannot be used further for the identification of resistant varieties in breeding programs. Figure 2 shows the fingerprint pattern of eight varieties with primer pairs designed specifically from the LRR region of the I2 gene. An amplicon of 186 bp could be obtained only in Heamsona tomato cultivar which was reported by Parmar et al. (2011) as a resistant entity while others showed no amplicons.

Sequencing and identification of gene

Sequencing of the isolated gene (I2) was done with specific

primer at Xcelaris, Laboratory, Ahmedabad. Subsequently the identification of sequence was done using bioinformatics tool. Searches of the National Center for Biotechnology Information (NCBI) genbank database using BLAST algorithm showed 100% similarity with the I2 active gene copy. The sequence has been deposited in NCBI database (Parmar et al., 2009).

Conclusion

The present investigation has shown application of I2 gene for the development of resistance against race 1 pathotype, which results into resistance only if I1 gene is available in the tomato cultivars. Positive response of I2 gene against race 1 pathotype suggest that both I1 and I2 gene are having some similarity in terms of their expression against avirulence genes, it may be the case that both have identical recognition site for the avirulence gene but it is to be disclosed which can be possible only after its characterization. Present study has showed strategies that can be employed for the eradication of the wilt disease especially when the R gene responsible for the resistance against such pathotype is not characterized.

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Full Length Research Paper

Nutritional composition, vitamins, minerals and toxic heavy metals analysis of *Trianthema portulacastrum* L., a wild edible plant from Peshawar, Khyber Pakhtunkhwa, Pakistan

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This study aimed at analyzing the nutritional potential of a wild edible plant, *Trianthema portulacastrum* L. widely used in Peshawar, Khyber Pakhtunkhwa, Pakistan. The nutritional composition, vitamins, minerals and heavy elements were analyzed following the standard methods of Association of Official Analytical Chemists (AOAC). The nutritional assessment included determination of moisture, ash, lipid, fiber, protein, carbohydrate and energy. Among the nutrient values, fiber was found to be the highest (430.0 mg/g), followed by ash (348.0 mg/g), total protein (91.9 mg/g), moisture (80.0 mg/g), carbohydrate (30.2 mg/g) and total lipid (20.0 mg/g). The vitamins analyzed were found to have greater value for riboflavin (2.02 mg/g), than retinol (0.81 mg/g). Among the macro minerals, potassium was present in high concentration (51.6 mg/g) than sodium (44.0 mg/g). The trace elements were assessed using atomic absorption spectrophotometer (AAS) and their decreasing order was Fe>Zn>Mn>Ni>Cu. Two toxic metals, Pb and Cd were present in very minute quantities of 0.08 and 0.0006 mg/g, respectively. The results suggest that *T. portulacastrum* L. is a good source of fiber, proteins, riboflavin, potassium, sodium and iron.

Key words: Peshawar, Association of Official Analytical Chemists (AOAC), *Trianthema portulacastrum* L., nutritional composition, vitamins, minerals.

INTRODUCTION

Nutrition is the most important fundamental needs of humans, for development, productivity and mental health. In developing world, the high rate of population growth, cause shortage of fertile land, leading to hunger and mal-nutrition problems (Pelletier et al., 1995). Plants have been handled by human societies for food purposes, since time immemorial. It is true that today, human plant food is more than 85 to 90% based on mainly twelve crops, but it is also fact that in many parts of the world, wild plants have major contribution in daily intake of food (Prescott-Allen and Prescott-Allen, 1990; Scherrer et al., 2005; Bussmann et al., 2006; Bussmann and Sharon, 2006; Kunwar et al., 2006; Cavender, 2006; Pieroni et al., 2007). Green leafy vegetables are well known for their

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Table 1. Description of the selected sample plant analyzed.

Vernacular names		Scientific Classification	
English	Horse purslane	Kingdom	*Plantae*
Urdu	Narma	Subkingdom	Tracheobionta
Pujabi	It sit	Super division	Spermatophyta
Hindi	Santhi, Patharchata	Division	*Magnoliophyta*
Sanskrit	Chiratika	Class	*Magnoliopsida*
Marathi	Pundharighentuli	Subclass	*Caryophyllidae*
Telugu	Ambatimaadu	Order	*Caryophyllales*
Arabic	Zaleya pentandra	Family	*Aizoaceae*
Indonesian	Subang-subang	Genus	*Trianthema*
Thai	Phak bia hin	Species	*Trianthema portulacastrum* L.

nutritional importance as rich sources of proteins, vitamins like ascorbic acid, carotene, folic acid, riboflavin, and minerals like calcium, iron and phosphorus (Kris-Etherton et al., 1988; NRC, 1996; Osler et al., 2001; Sheela et al., 2004). Green wild edible plants are commonly found in countries with varied climates. Many research studies have shown several wild species fit for human consumption due to good proximate and nutritional values. Even in some modern cultures today, people consume wild plants as a normal food source, for obtaining good amounts of several nutrients and it is widely accepted that leafy green vegetables are significant nutritional sources of minerals (Grau et al., 1989; Kuhnlein, 1990; Khan et al., 2013a).

Around the world, many workers have reported the nutritional compositional evaluation and functional properties of various types of edible wild plants used in the developing countries (Lockeett et al., 2000; Akindahunsi and Salawu, 2005; Edeoga, 2006; Hassan and Umar, 2006; Ekop, 2007; Adnan et al., 2010; Mohan and Kalidass, 2010; Hussain et al., 2010; Gafar and Itodo, 2011; Vishwakarma et al., 2011; Valvi and Rathod, 2011; Naryan et al., 2011; Seal, 2011). Since Pakistan came into being, numerous wild vegetables are used as food in both urban and rural areas. The researchers have investigated several wild plants of Pakistan, for nutritional composition (Khattak et al., 2006; Imran et al., 2007; Qureshi and Bhatti, 2009; Hussain et al., 2009; Marwat et al., 2010; Jan et al., 2011; Khan et al., 2011, 2013b). The database of the nutrient and chemical compositions of these plant foods is still incomplete as many plants are still not investigated properly and thus much work is still needed to be done. In Peshawar Pakistan, many of the local wild vegetable materials are used as food without any nutritional studies.

In order to contribute to the growing body of knowledge and to narrow the growing gap of this subject, the present study was designed on wild edible plant, *Trianthema portulacastrum* L. commonly used as food by the people in Peshawar, Khyber Pakhtunkhwa, Pakistan, to analyze for proximate and nutrient analysis, vitamins, minerals, trace and toxic trace metals, using standard AOAC methods.

MATERIALS AND METHODS

Collection of samples

The plant materials were collected from the provincial capital city, Peshawar, as wild grown plants in agriculture fields. Ten samples were collected, at three times from different fields, during July to November 2011, and each sample was studied in triplicate, thus making a total of 90 samples analyzed. Standard methodology was used for collection of plant samples (Humphry, 1993). Specific samples were obtained with the aid of interpreters and field guides. Genus and species was identified by plant taxonomist, by comparison with herbarium reference materials. The voucher specimen was preserved in Department of Plant Sciences, KUST, Kohat, for future references. The samples were shed dried, pulverized and stored in an airtight container. Details of each plant species, in respect of their scientific, family and local names, part used and status are elaborated in Table 1 (Shah et al., 2006; ENVIS, 2012).

Nutritional composition

The content of moisture was measured by keeping the sample in oven (HB-502M Hanbaek Co, Korea) at 100 to 110°C for overnight and then cooling in desiccator to constant weight. The loss in weight was regarded as a measure of moisture. For determination of ash, the sample was heated in furnace (F6010, Branstead Thermolyne Co., Dubuque, IA, USA), at 550°C, until white or grayish white ash was obtained. Weight of the ash was noted directly. Crude fiber was determined by treating the sample with 1.25% H_2SO_4, 1.25% NaOH and then 1% HNO_3, filtered and washed with hot water after each step. The residue obtained was dried in oven at 130°C and ashed at 550°C in furnace. The loss in weight on ignition was expressed as content of crude fiber (AOAC, 2000). Total lipid was extracted from the sample with petroleum ether (60 to 80°C) in a Soxhelt apparatus for about 6 to 8 h. The

Table 2. Nutritional composition of the sample plant *Trianthema portulacastrum* L. analyzed.

Nutritional parameter	Quantity (mg/g±SD)
Moisture	80.0[e] ± 2.2
Ash	348.0[g] ± 6.2
Total lipid	20.0[c] ± 0.6
Saponifiable lipid	11.2[b] ± 0.8
Non saponifiable lipid	8.8[a] ± 0.2
Total protein	91.9[f] ± 3.2
Fiber	430.0[h] ± 8.2
Carbohydrate	30.2[d] ± 1.2
Energy (kcal/ 100 g)	76.01

[a-h] Values are mean ± standard deviations of three (n = 3) measurements. Different superscript letters within same columns are significantly different ($p < 0.05$).

Table 3. Vitamins constituents of the sample plant *Trianthema portulacastrum* L. analyzed.

Vitamin	Quantity (mg/g ± SD)
Vitamin A	0.81[a] ±0.2
Vitamin B_2	2.02[b]±0.3

[a-b]Values are mean ± standard deviations of three (n = 3) measurements. Different superscript letters within columns are significantly different ($p < 0.05$).

Table 4. Minerals and toxic heavy metals of the sample plant *Trianthema portulacastrum* L. analyzed.

Mineral	Quantity (mg/g ± SD)
Macro-mineral	
Sodium	44.00[f] ± 1.4
Potassium	51.60[g] ± 5.2
Micro-mineral	
Zinc	0.20[d] ± 0.02
Copper	0.02[c] ± 0.1
Iron	6.44[e] ± 0.4
Manganese	0.04[c] ± 0.01
Nickel	0.03[c] ± 0.006
Toxic heavy metal	
Lead	0.08[b] ± 0.01
Cadmium	0.0006[a] ± 0.0001

[a-g] Values are mean ± standard deviations of three (n = 3) measurements. Different superscript letters within columns are significantly different ($p < 0.05$).

residual solvent was evaporated in a pre-weighed beaker and increase in weight of beaker gave total lipid (AOAC, 2000). Total lipid content was fractionated into saponfiable and non-saponfiable lipids by the saponification of total lipid followed by extraction of non-saponifiable fraction with petroleum ether, 40 to 60°C (AOCS, 1993).

Nitrogen content in the sample was estimated by using micro Kjeldahl method and crude protein was calculated by multiplying the evaluated nitrogen by 6.25. The value of total carbohydrate was given by: 100- (percentage of ash + percentage of total lipid + percentage of protein + percentage of crude fibre) (AOAC, 2000). The calorific value was calculated by multiplying the values of total carbohydrate, lipid and protein by the factors 4, 9 and 4 respectively, taking the sum of the products and expressing the result in kilocalories (Guil-Guerrero et al., 1998).

Vitamins analysis

Vitamin A was estimated by extraction with ethanol and then mixing with petroleum ether. The amount in extract was determined by UV-Visible Spectrophotometer (Hitachi U-2000, Japan) at 450 nm using Fikselova (2008). For riboflavin (vitamin B_2), the ethanol extract was added to potassium permanganate and H_2O_2 and allowed to stand over hot water. Then 40% sodium sulphate was added and the absorbance was measured at 510 nm by spectrophotometer (James, 1995).

Minerals and toxic heavy metals analysis

Two macro minerals, sodium and potassium were estimated by using flame photometer (Model 410 Corning, Germany). Standard solution of each was used for calibration of the instrument before analysis (AOAC, 2000). The micro minerals including Zn, Cu, Ni, Mn, and Fe along with toxic heavy metals Pb and Cd were determined by wet digestion of the sample followed by analysis using atomic absorption spectrophotometer (A-Analyst 700 Perkin Elmer/ USA) equipped with standard burner, air acetylene flame and hollow cathode lamps as radiation source (Indrayan et al., 2005).

Statistical analysis

Data were reported as mean ± standard deviation of triplicate measurements. Significant differences ($p < 0.05$) within means were analyzed by analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test in the SPSS Statistics Software Version 20 (IBM, New York, USA).

RESULTS AND DISCUSSION

The proximate and nutritional contents are mentioned in Table 2, vitamins constituents in Table 3, while minerals and toxic heavy metals are given in Table 4. The results are discussed in comparison with published literature and biological applications under the following four headings.

Nutritional composition

Moisture is considered as a good source of water and it is necessary that 20% of the total water consumption must come from food moisture (FNB, 2005). The average

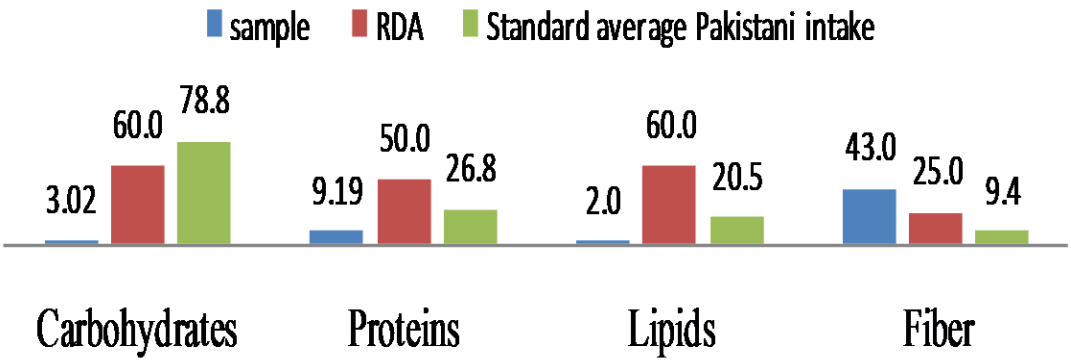

Figure 1. Nutritional composition in comparison with RDA and average nutrient intakes in Pakistan.

moisture content of *T. portulacastrum* L. studied was 8.0% (Table 2), very low compared to other wild edible plants such as *Amanthus viridus*, *Chenopodium murale*, *Nastrium officinale* and *Scandex pectin-veneris* with 88.90, 89.50, 90.54 and 81.31% respectively (Imran et al., 2007). However, it was very close to some common leafy vegetables such as *Xanthosem sagittifolum* (13.17%) and *Adensonia digitata* (9.5%) (Ladan et al., 1996). Ash content is the index of mineral contents in plants such as calcium, sodium, potassium, nickel and zinc. The ash content of the sample studied was 34.8% (Table 2), which showed that the leaves were rich in minerals. The value obtained was higher compared to 1.8% reported in sweet potato leaves, and 5% in *Tribulus terristris* leaves, 1.85% in *A. viridus* leaves, 2.70% in *C. murale* leaves, 1.77 and 3.10%, in *N. officinale* and *S. pectenveneris* leaves respectively (Imran et al., 2007). The crude protein content of the sample was found to be 9.19% (Table 2). This value was higher compared to 2.11% in *A. viridus*, 2.98% in *C. murale* leaves, 2.76% in *N. officinale*, 6.30% in water spinach and 6.40% in *Momordica foecide* leaves (Imran et al., 2007). But lower than 11.29% in balsam apple leaves, 24.85% in sweet potato leaves, *Piper guineeses* and *Talinum triangulare* with values of 29.78 and 31.00%, respectively (Akindahunsi and Salawu, 2005).

Among the reported wild edible plants of the same family, sample leaves contain higher crude protein value. According to the WHO recommended dietary allowance (RDA) of protein, for children, adult male and adult female is 28, 63 and 50 g respectively (Akindahunsi and Salawu, 2005), while in Pakistan the average protein intake is 43.4g/day (Figure 1) (NNSP, 2011). As the plant protein is also considered as biological value, so for 100 g of *T. portulacastrum* L. provide 9.19 g of proteins indicate that the sample plant is a good source of daily proteins. Lipid in food is considered as a chief source of storage form of energy, essential fatty acids and fat soluble vitamins and precursors of vitamins. The sample plant contained 2.0% crude lipid (Table 2). It is lower than 11% in water spinach leaves, 12% in *Senna obtusfolia*, 11% in *Amaranthus caudatus* leaves, 28.2% in *Centilla asiatica* leaves, 29% in *Bahunian purpurea* leaves and 60% in *Amaranthus hybridus* but higher than 0.47% in *Amaranthus viridus*, 0.54% in *C. murale* and 0.63% in *S. pectenveneris* leaves (Imran et al., 2007). The crude fiber content of sample leaves was 43% which is higher compared to 7.20% in sweet potato leaves, 13% in *Tribubus terristris* leaves, 29.0% in balsam apple leaves, 1.93% in *A. viridus*, 3.82% in *S. pectenveneris* leaves (Akindahunsi and Salawu, 2005).

Dietary fiber helps to reduce serum cholesterol level, risk of coronary heart diseases, colon and breast cancer and hypertension. The high level of fiber in diet can cause intestinal irritation, lower digestibility, difficult absorption of minerals found in plant and overall decrease nutrient utilization (Imran et al., 2007). The carbohydrate content of sample leaves was 3.02%, considerably low when compared to other wild edible plants such as *A. caudatus* leaves (61.03%), 55.67% in *T. terristris*, 54.2% in water spinach leaves, 75% in sweet potato leaves, 82.8% in *Corchorus triden* leaves. But closely similar when compared to *A. viridus*, *C. murale*, *Nastritium officinale* and *S. pectenveneris* leaves that is 4.74, 3.41, 3.38 and 7.32% respectively (Imran et al., 2007). Carbohydrates are principal and indispensible source of energy. The RDA for carbohydrates is 130 g (FAO, 1998), while in Pakistan 349 g of carbohydrate intake is reported (Figure 1) (Ministry of Health and Nutrition, 1994). Due to carbohydrates content sample, plant can be a good food source. The 100 g of *T. portulacastrum* L. provide 76.01 kcal of energy. This reveals that the sample plant can contribute meaningfully to the daily energy requirement of a person.

The caloric value of the sample plant was high compared to *A. viridus* (31.63 kCal), *S. pectenveneris*

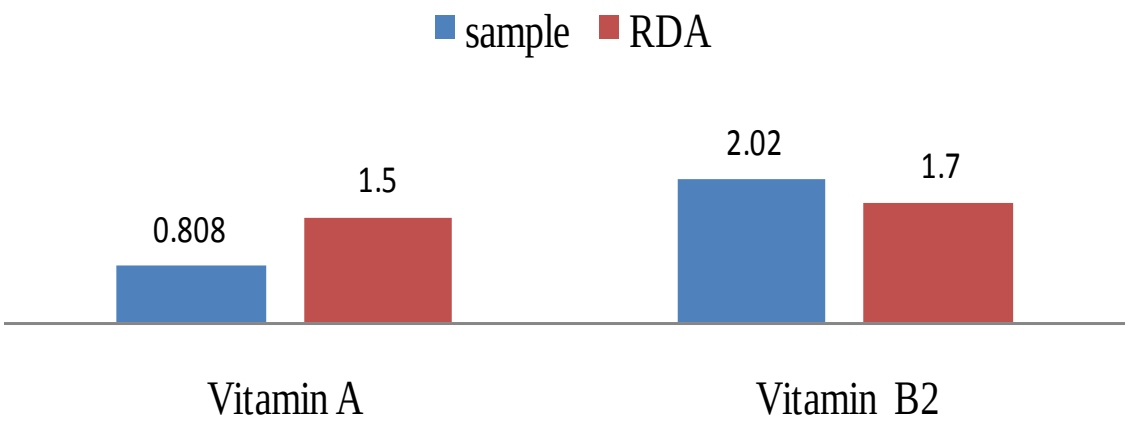

Figure 2. Vitamins content in comparison with RDA.

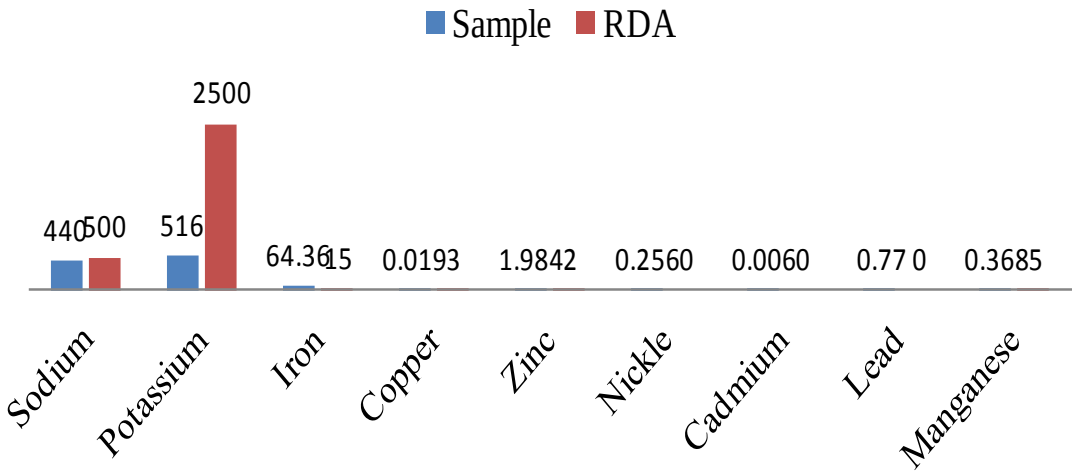

Figure 3. Sodium and potassium contents in comparison with RDA.

(50.23 kcal) (Imran et al., 2007), but lower when compared to *A. caudatus* 326.7 Kcal, *Discorea bulbifera* 304.7 kcal and 333.1 kcal of *Ficus bengalensis* (Imran et al., 2007).

Vitamin analysis

Vitamin A is necessary for vision process and also plays a role in skin mucosa, normal reproductive capabilities and is an important anti oxidant (FAO, 2001). The sample contained 0.081 mg/100 g of vitamin A (Table 3). The RDA for vitamin A is 1.5 mg/100 g (Figure 2). The results show that the sample is not a good source of vitamin A. The sample contained 0.202 mg/100 g of vitamin B_2. The RDA for riboflavin is 1.7 mg. The results show that it is a rich source of riboflavin compared to vitamin A. Riboflavin is present in body as co–enzyme which acts as hydrogen acceptor in amino acid metabolism (FAO, 2001).

Minerals analysis

Sodium maintains fluid volume outside the cell thus normalize the cell functions. The sodium content of the sample was 44 mg/g (Table 3), slightly low compared to reported (50 mg/g) for *Tribus terristis* leaves and very low than 450 mg/kg in *S. obtusfolia* but quite high in comparison to *Asparagus officinalis* (0.184 mg/kg) and *Momordica dioica* (0.151 mg/kg) (Khan et al., 2011). The RDA for sodium is 500 mg for adults (Figure 3). The plant sample leaves can be good source of food for hypertensive patients (FAO, 2001). Potassium content of the sample was 51.60 mg/g (Table 4), high compared to other green leafy vegetables as 64.2 mg/kg found in *Diospyros mespiliformis*, 1.09 mg/kg in *A. officinalis*, 0.825

mg/kg in *M*. *dioicas* and 4.409 mg/kg in *I. astrgaalina* (Khan et al., 2011). The RDA for potassium is 2500 mg for adults (Figure 3), and the sample contributes good percentage to RDA, meaning the good source that can contribute to the diet of hypertensive patients (FAO, 2001).

Micro minerals analysis

The copper content of plant sample is 20 mg/kg (Table 4), which is higher compared to 0.1 mg/kg in *D*. *mespiliformis* and 0.25 mg/kg in *F*. *bengalensis*, 12.8 mg/kg in *T. terristis* leaves and 5.0 mg/kg in *Cassia siamea* leaves (Khan et al., 2011; Gafar and Itodo, 2011). The RDA value for copper is 1 to 3 mg for adult (Figure 3). Copper contributes a role in hemoglobin formation and play a role in iron and energy metabolism (FAO, 2001). The zinc content of sample leaves was found to be 200 mg/kg, higher compared to 0.200 mg/kg in *Diospyros mespiliformis*, 1 mg/kg in *T. terristris* leaves but lower when compared to 68.5 mg/kg in *C*. *siameea* (Khan et al., 2011; Gafar and Itodo, 2011). Zinc plays a vital role in gene expression, regulation of cellular growth and participates as a cofactor of many enzymes. It also plays an important role in motility of sperm during liquation and mating. The RDA of zinc is 12 to 15 mg for adults (FAO, 2001). Iron content of sample was 6.44 mg/g, higher than other vegetables like *C. siamea* 700 mg/kg (Khan et al., 2011). The RDA value of iron is 10 to 15 mg/100 g. This sample is a good source of iron. Iron is required for hemoglobin formation and its deficiency leads to anemia (FAO, 2001). Manganese content of plant sample was 40 mg/kg higher than 9.8 to 38 mg/kg reported in some leafy vegetables and lower than 116 mg/kg in balsam apple leaves (Khan et al., 2011). The RDA for manganese is 2 to 5 mg. The result showed that *T*. *portulacastrum* L. is a good source to provide daily manganese.

Manganese is a co-factor for many enzymes which take part in glucose and amino acid metabolism (FAO, 2001). The amount of nickel present was 30 mg/kg in the sample. This quantity was quite higher when compared to other edible wild plants like *F*. *bengalensis* 1.14 mg/kg (Gafar and Itodo, 2011). Nickel is needed in very small amount to the body. The health benefits of Ni are healthy skin and optimal growth and also take part in iron metabolism. Higher quantity leads to toxicity (Gafar and Itodo, 2011).

Toxic heavy metals analysis

The lead content of sample was 80 mg/kg (Table 4), higher compared to *F*. *bengalensis* (0.25 mg/kg). Lead is toxic and non essential element for human body as it causes rise of blood pressure, kidney damage, miscarriage, subtle abortion, brain damage, decline fertility of men through sperm damage and diminishes learning abilities due to neuron damaging actions (Gafar and Itodo, 2011). Cadmium concentration of *T*. *portulacastrum* L. was 0.6 mg/kg higher compared to *F*. *bengalensis* (0.017 mg/kg) and lower than other usual edible plants. Cadmium is highly toxic for a body and it causes several health hazards, including cell death and cell proliferation (Gafar and Itodo, 2011).

Conclusions

The present study provides an evidence of the potential nutritional value of the selected wild edible plant, *T*. *portulacastrum* L. It showed to have good proximate values of proteins, lipids, carbohydrates, fiber and moisture. The macro minerals (Na and K), micro minerals (Fe, Cu, Ni and Mn), and vitamins (A and B_2) were also present in appreciable quantities. The toxic heavy metals, Pb and Cd were present in very minute quantities and therefore may not pose any threat to health. Though, *T*. *portulacastrum* L., may not provide all nutrients required by man, yet it contains sufficiently good amount of some essential nutrients like vitamin B_1, iron and fiber. These results were making *T*. *portulacastrum* L., a good source of food and therefore could be recommended for edible purposes.

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Full Length Research Paper

Can Bt maize change the spatial distribution of predator *Cycloneda sanguinea* (L.) (Coleoptera: Coccinelidae)?

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Cultivation of Bt crops is an important tactic in integrated pest management. The effect of Bt maize on arthropod predators needs to be investigated because of the important role of these natural enemies in the absence of target pests. The objective of the present study was to generate information on the distribution model of *Cycloneda sanguinea* (L.) (Coleoptera: Coccinelidae) in Bt and non-Bt maize. A sampling field of 2.500 m^2 area, divided into 100 plots, was used in this study. Five plants per plot, totaling 500 plants in each field, were studied. We counted the total number of adults every week, totaling six samples for each field (Bt and non-Bt). The aggregation index (variance/mean ratio, Morisita index, and exponent k of the negative binomial distribution) and Chi-square fit of the observed and expected values to the theoretical frequency distribution (Poisson, binomial, and negative binomial positive) revealed that, in both cultivars, the adults of *C. sanguinea* were distributed according to the random distribution model, which fits the pattern of Poisson distribution.

Key words: Spatial distribution, natural enemy, lady beetle, Poisson.

INTRODUCTION

Since its commercial release in 2008, the use of Bt crops containing *Bacillus thuringiensis* (Berliner) genes for insect resistance is being increasingly adopted by Brazilian farmers (James, 2011), especially as it decreases the need of insecticide application for targeted pest such as *Spodoptera frugiperda* (Brookes and Barfoot, 2008; Mendes et al., 2011). However, the widespread use of this technology may have unknown effects on the non-targeted species as well (Dutton et al., 2002; Sanvido et al., 2009).

Bt crops may introduce changes that can directly influence beneficial insects which come in direct contact of toxins in prey and hosts, called as tri-trophic interactions (Lumbierres et al., 2011; Dutra et al., 2012; Mota et al., 2012). In addition, Bt crops may have indirect effects because of possible changes in the behavioral aspects of natural enemies of pests in insufficient target technology (Zwahlen et al., 2000; Stephens et al., 2012). For example, coccinellid species are important polyphagous predators within agroecosystems, as *Cycloneda sanguinea* (L.) is a voracious predator of pests such as aphids, mealybugs, and eggs of Lepidoptera (Bruck and Lewis, 1998; Smith et al., 2004). These natural enemies are reported to be affected by the toxins of Bt cotton, for example, Funichello et al. (2012) reported changes in the biological parameters of aphids when fed from Bt cotton.

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The spatial dispersion of a population in a given ecosystem or agroecosystem basically corresponds to three models: Aggregate (or contagious), random (or by-chance), or uniform (or regular) (Young and Young, 1998). To determine the spatial arrangement pattern of a given species, it is necessary to obtain numerical data of individuals in the ecosystem to be studied, and the ecosystem in question must allow sampling (Fernandes et al., 2003). These samples may be used to infer the sample distribution pattern or the characteristics of distribution (Young and Young, 1998). Indices of aggregation and frequency distributions are utilized for description of the distribution patterns of a population.

Studies on the frequency distributions of different insect species in different crops are important to understand the real spatial distribution of these individuals by adopting appropriate sampling criteria to estimate population parameters (Barbosa et al., 1992). According to the study of Kuno (1991), the area under the study must first be divided into several units or grids of same size and then the occupation model of the area must be described by individuals in the population as a frequency distribution of the individuals observed in each grid.

As the spatial distribution of *C. sanguinea* related to a targeted field population (Hagen, 1962), we tested the hypothesis that lack of *S. frugiperda* in maize Bt would influence the spatial distribution of adult *C. sanguinea* as compared with conventional maize populations infested with *S. frugiperda*.

Material and Methods

The experiment was conducted during the autumn-winter season of 2012 as a part of Fazenda Experimental by Universidade Federal da Grande Dourados (UFGD) in Dourados, Mato Grosso do Sul, Brazil. Fields were planted with Bt transgenic corn (event MON89034) expressing the toxins Cry1A.105 and Cry2Ab2 and with non-Bt commercial field corn. Each field was divided into 100 plots of 25 m^2 (5 × 5 m) area, and, five plants were tested in each plot, totaling 500 plants per field. We counted the total number of *C. sanguinea* adults every week to total six samples for each field (Bt or non-Bt).

Statistical analyses to determine the spatial distribution pattern of the insect population considered the means and variances of the number of individuals found in each plot from the two working areas, and the following dispersion indices were used for the same:

Variance/Mean Ratio Index (Southwood 1966)

This ratio (I) is an index that measures the deviation of a random data arrangement. I = 1 indicates random or by chance spatial arrangement, I < 1 indicates regular or uniform spatial arrangement, and I > 1 indicates aggregated or contagious distribution (Rabinovich, 1980). According to (Southwood, 1966), the limitation of this index is in the influence of the size of the sampling unit on the number of individuals observed, which is significantly affected by the provisions of infection.

Morisita index

The Morisita index ($I\delta$) is relatively independent of the mean and number of samples. $I\delta$ = 1 indicates a random distribution, $I\delta$ > 1 indicates a contagious distribution, and $I\delta$ < 1 indicates a regular distribution.

The limitation of this index is that it is significantly influenced by the sample size (N), as, for safe use, the number of sampling units must be the same for all fields being compared.

Exponent k of the Negative Binomial Distribution

The exponent k is a suitable dispersion index when the size and numbers of sample units are the same in each sample; this is often influenced by the size of the sampling units. This parameter is an inverse measure of the degree of aggregation. In this case, negative values indicate a normal or uniform distribution, positive values near zero indicate an aggregated arrangement, and higher values up to eight indicate a random distribution (Southwood,1966; Elliot, 1977). Considering this aspect, (Poole, 1974) utilized a different interpretation, where 0 < k < 8 indicates an aggregated distribution, and 0 < k > 8 indicates a random distribution.

The theoretical frequency distributions used to assess the spatial distribution of the observed species are as follows (Young and Young, 1998):

Poisson distribution

Also known as random distribution, it is characterized by presenting variance equal to the mean.

Negative binomial distribution

It shows greater variance than the mean, thereby indicating an aggregated distribution, and it has two parameters: the mean (m) and the parameter k (k > 0).

Chi-square test

To verify the fit test of the collected data to theoretical distributions of frequency, the Chi-square test for adherence was used for comparing the total number of frequencies observed in the sample area with the expected frequencies (Young and Young, 1998), where these frequencies were defined by the product of the probabilities for each class and the total number of sampling units used. In the present study, Chi-square test was used to fix the minimum expected frequency equal to one.

RESULTS AND DISCUSSION

Dispersion indices

The Chi-square test on the data related to mean-variance index indices and Morisita gave values equal to one in three samples collected from Bt corn, with the three remaining values much greater than one. In conventional farming, only the fourth sample showed a value of Chi-square, indicating that the calculated indices were much greater than one and the remaining equaled one. Parameter values set K aggregation in four samples for both the cultivars, as it was the sixth sampling adjusting uniformity (Table 1).

Table 1. Statistical analysis (sample mean and variance) and dispersion indexes for *Cycloneda sanguinea* (Coleoptera: Coccinelidae) on Bt and non-Bt maize, Dourados, MS, 2012.

Cultivar	Index	Sampling Period (D.A.E)					
		7	14	21	28	35	42
Bt	$\dot{m}$	0.31	0.53	0.49	0.63	0.72	0.43
	S^2	0.418	0.615	0.515	0.842	0.729	0.672
	I	1.349	1.161	1.051	1.336	1.012	1.562
	$I\delta$	2.151	1.306	1.105	1.536	1.017	2.326
	K	0.889^{ag}	3.295^{ag}	9.585^{al}	1.876^{ag}	58.32^{al}	0.765^{ag}
	X^2	133.516*	114.924^{ns}	104.061^{ns}	132.238*	100.222^{ns}	154.674*
Nbt	$\dot{m}$	0.39	0.62	0.8	1.12	0.6	0.27
	S^2	0.48	0.62	0.93	1.48	0.67	0.26
	I	1.238	1.003	1.162	1.322	1.111	0.962
	$I\delta$	1.619	1.005	1.203	1.287	1.186	0.855
	K	1.64^{ag}	211.42^{al}	4.95^{ag}	3.481^{ag}	5.4^{ag}	-7.076^{un}
	X^2	122.538^{ns}	99.290^{ns}	115^{ns}	130.857*	110^{ns}	95.22^{ns}

$\hat{m}$ = sample mean of adults per sampling unit; S^2 = variance; I= variance/mean relationship; $I\delta$ = Morisita index; K = K exponent; X^2*= significant at 5% probability and ^{ns} non-significative in chi-square test; **ag** = aggregated; **al** = random; **un** = uniform; **D.A.E** = days after plant emergence.

The dispersion indexes indicated random distribution of adult *C. sanguinea* occurring in non-Bt corn. However, these indices were inconclusive when observing the behavior of adults in Bt corn, because the Chi-square values calculated both the aggregate distributions as random (Table 1). Thus, it was necessary to calculate the indices of frequency, which is standard in studies of spatial distribution of arthropods (Maruyama et al., 2002; Fernandes et al., 2003; Martins et al., 2010; Rodrigues et al., 2010).

Theoretical frequency distributions

Tests frequency calculated from the data of class number of individuals in the field did not indicate a good fit to the negative binomial distribution of spatial arrangement of individuals in the population of *C. sanguinea* in both the cultivars of maize (Bt and non-Bt) (Table 2). After adjusting to the Poisson distribution, four samplings were well adjusted to the test in Bt maize, whereas all conventional maize samples had a good fit to this frequency distribution.

From the results obtained by fitting the data to the field frequency indexes, adult *C. sanguinea* was found to present random distribution. Furthermore, the absence of larvae of *S. frugiperda* in cultivating Bt maize did not contribute to the spatial distribution of the predator in both the cultivars with similar results. Therefore, we assumed that indirect factors related to the absence of prey in Bt corn would not influence the spatial distribution of *C. sanguinea*. For example, Funichello et al. (2012) observation that the biological characteristics of *C. sanguinea* are negatively influenced when these predators are fed prey from Bt crops do not interfere in the behavioral aspects of the population, at least with regard to their spatial distribution in cotton.

Guerreiro et al. (2005) reported that spatial distribution of adults and nymphs of *Doru luteipes* (Scudder, 1876) (Dermaptera: Forficulidae) occurs in the aggregate and is dependent on the spatial distribution of prey *S. frugiperda*. However, they found that the hypothesis changed these results in the absence of a preferred prey, as in the present study. Other factors that influences the behavior of *C. sanguinea*, for example, the local landscape and liking for preys such as aphids and scale insects (Elliott et al., 1999; Garcia et al., 2004), and it not being considered as a target of Bt maize (Lumbierres et al., 2004) in both the cultivars used in this study may be related to the distribution of horizontal type randomly.

As the spatial distribution of both the genotypes of *C. sanguinea* adult baker is random, the absence of prey *S. frugiperda* did not influence the result. Thus, the assumption that the spatial distribution of predators is related to the distribution of prey was not confirmed to *C. sanguinea* in maize.

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Table 2. Chi-square test to *Cycloneda sanguinea* (Coleoptera: Coccinelidae) (Poisson and Negative Binomial) on Bt and non-Bt maize cultivars in Dourados, MS, 2012.

Cultivar	Sampling Period (D.A.E)	Poisson		Negative Binomial	
		X^2	DF(nc-2)	X^2	DF(nc-3)
Bt	7	6.076*	1	2.589^{ns}	1
	14	0.707^{ns}	2	1.136^{ns}	1
	21	7.461*	2	7.068**	1
	28	1.630^{ns}	2	0.374^{ns}	2
	35	3.840^{ns}	2	3.981*	1
	42	2.325^{ns}	1	6.979*	1
Nbt	7	1.448^{ns}	1	2.187^{ns}	1
	14	1.154^{ns}	2	1.1479^{ns}	1
	21	1.189^{ns}	2	2.731^{ns}	3
	28	3.734^{ns}	2	8.41*	3
	35	1.402^{ns}	2	2.774^{ns}	1
	42	0.010^{ns}	1	0.027^{i}	-

^{ns} – non-significative at 5% probability, * - significative at 5% probability, ** - significative at 1% probability, ^{i} = insufficient of classes, X^2 – value of the calculated chi-square, DF – degree of freedom, nc – number of classes observed at field; **D.A.E** = days after plant emergence.

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African Journal of Biotechnology

Full Length Research Paper

Bioremediation of soil contaminated with spent and fresh cutting fluids by *Pleurotus pulmonarius* (Fries) Quelet

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Contamination of soil with industrial cutting fluids containing heavy metals and petroleum hydrocarbons has detrimental effects on ecosystems. As such contaminants constitute risk to human health; they can enter the food chain through agricultural products or contaminated drinking water. This growing concern about contamination with cutting fluids makes remediation process imperative. White rot fungus, *Pleurotus pulmonarius* was investigated in this pilot study for its potential to bioremediate contaminants such as heavy metals and total petroleum hydrocarbon at 10, 20 and 30% contamination levels. Control experiment (0% contamination level) was also set up. The effects of incubation periods on the contents of cutting fluid contaminated soil inoculated with *P. pulmonarius* after two months was equally studied. Bioremediation efficiently occurred at 10% contamination level. At this level, soil pH was 6.9 and organic carbon, organic matter and phosphate contents increased by 22.4, 21.4 and 3.5%, respectively for spent cutting fluid and by 30.0, 30.1 and 50.0% for fresh cutting fluid respectively after two months of incubation with the fungus. Copper, manganese and nickel decreased significantly by 35.3, 25.0 and 1.5%, respectively for spent cutting fluid contaminated soil and by 10.5, 54.4 and 55.9% respectively for fresh cutting fluid contaminated soil. High percentage degradation of Total Petroleum Hydrocarbon (TPH) at 10% contamination level was about 90.0% for both fresh and spent contaminated soil samples. The cation exchange capacity increased by 50.8 and 38.4% for spent and fresh cutting fluid contaminated soil samples respectively. These results indicate the efficiency of *P. pulmonarius* as a bioremediating agent of heavy metals and TPH in contaminated soil.

Key words: Bioremediation, cutting fluids, total petroleum hydrocarbon, cation-exchange analysis, heavy metals.

INTRODUCTION

Cutting fluids are used in the engineering industry to improve machine tools performance and productivity. They are generally composed of oil and organic compounds mixed with water. Once the mixture has been used for a period of time, it degrades and generates a toxic fluid as waste (Bio-wise, 2001). The fluid includes chemicals that inhibit metal corrosion and microbial activities (biocides) while lubricating and cooling the metal cutting process (Van der Gast et al., 2002). Cutting fluids are difficult to degrade due to the presence of emulsified oil and their high molecular weight components. Different methods have been used to treat cutting fluids. One of

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the methods involves coagulation with chemical solutions and is applicable only to emulsified oil. Another method is membrane separation in which case fouling of membrane element leads to huge reduction in efficiency. The unavailability of chemical and fouling problem makes the disposal cost rise. The rising disposal costs make it a financial burden on industry, thus resulting in disposal of partially treated cutting fluid on soil. There is therefore a clear need to develop a cost effective and reliable system for treating cutting fluids.

The major mechanism for eliminating spilled oil from the environment is the microbial degradation (Atlas, 1995). This remediation option which involves the use of microorganisms to detoxify or remove organic and inorganic compounds from the environment also offers green technology solution to the problem of environmental degradation. The technological process relied upon microbial enzymatic activities to transform or degrade the contaminants from the environments (Phillip and Atlas, 2005). It is a form of bioremediation that harness fungal mycelium to transform complex or simple chemical compounds into non-hazardous forms thereby resulting in materials of higher nutritive value or simply reducing the final bulk of the product (Grady, 1985). Fungi use is expected to be relatively cheap as they can be cultivated on a number of inexpensive agricultural or forest wastes such as corncobs and sawdust (Lavrovsky, 2004). The natural degradative ability of white rot fungi can be used in the decontamination of polluted soil by ramifying the substratum and digesting it through the secretion of extracellular enzymes which are non specific. The extracellular lignin-degrading enzymes such as manganese peroxidase, laccase and lignin peroxidase can be secreted by white rot fungi to decontaminated polluted soil (Croan, 2000). Okparanma et al. (2011) stated that spent white-rot fungi (*Pleurotus ostreatus*) substrate can be used to biotreat Nigerian oil-based drill cuttings containing Polyaromatic Hydrocarbons (PAH's) under laboratory conditions. Adenipekun (2008) reported an improvement in the nutrient contents of the soil, bioaccumulation of heavy metals, degradation of total petroleum hydrocarbon (TPH), lignin, and increased activity of polyphenol oxidase and peroxidase due to biodegradation of spent cutting fluids by *Pleurotus tuber-regium*.

The most important processes in soil are regarded as ion-exchange, acidification and salinization, oxidation-reduction reactions and metal-organic matter interactions. Ion exchange reaction is important in soil because it controls both the retention of added fertilizer elements and soils capacity to resist changes imposed by the addition of extraneous materials. The ease with which cations are adsorbed or released from soil particle surfaces depends to a large extent on the nature of the exchange cation. Therefore, cation exchange capacity measures the ability of the soil to hold cations at the negative sites by electrostatic force. It has close association with pH and and soil particle surfaces. The concept of soil pH and specific adsorption of cation are important agriculturally. The aim of this study was to remediate cutting fluid contaminated soil with *Pleurotus pulmonarius* in which process, the extent of depletion of soil nutrient content, the extent of accumulation of toxic metals and total petroleum hydrocarbon by the fungus and cation exchange capacity of the soil were investigated.

MATERIALS AND METHODS

Sample and fungus collection

Top soil sample used for this experiment was collected within 1 to 10 cm depth from the nursery site of the Department of Botany, University of Ibadan. The soil was sieved to 2 mm to remove debris. Pure cultures of *P. pulmonarius* was collected from Plant physiology unit of the same Department. Pure spawns of this fungus was prepared according to the method used by Jonathan and Fasidi (2001). Freshly harvested rice straw was collected from International Institute of Tropical Agriculture (IITA), Ibadan and air-dried in the laboratory for seven days to remove moisture content. The dried rice straw was cut into 0.1 to 3 mm size using guillotine. Wheat bran was bought at the feed mill of the popular Bodija Market in Ibadan. Fresh and spent cutting fluid was obtained from the workshop unit in the Departments of Physics and Mechanical Engineering, University of Ibadan. The fluid is suitable for metal working as admixture or emulsion with water.

Pure spawn preparation

The spawn of the fungus was prepared according to the method of Jonathan and Fasidi (2001). Rice straw was soaked in water for 1 h and then sieved until no more water oozed out. Wheat bran was added as an additive to rice straw contained in a 350 ml sterile bottles covered with aluminium foil and autoclaved at 121°C for 15 min. After cooling, the bottles were inoculated with pure cultures of *P. pulmonarius.* All bottles were incubated at 28°C for 2 weeks until the substrate was completely ramified.

Experimental setup for culture conditions

The culture conditions were prepared according to the method of Adenipekun and Fasidi (2005). 400 g of soil were weighed into the 350 cm^2 sterile bottles and then mixed thoroughly with 25 ml of water soluble cutting fluid of varying concentrations (0, 10, 20 and 30%). A mud balance was used in measuring the weight percent volume as density (g/ml) of the fluid. 80 g of moistened rice straw added was laid on the contaminated soil in each bottle separated with a wire gauze and covered with aluminium foil. The bottles were then autoclaved at a temperature of 121°C temperature for 20 min. After cooling, each bottle was inoculated with 10 g of vigorously grown spawn of *P. pulmonarius*. The bottles were incubated at room temperature for two months in an incubator. In the first set of control treatment, cutting fluid was not added to the soil while in the second set different percentages of cutting fluid were added to the soil and not inoculated with the fungus. At the end of the period of incubation, the mycelium ramified substrate was carefully separated from the soil layer ensuring that soil particles did not mix with it.

The different treatments were replicated three times and incubated at 28±2°C in the dark for 60 days. The contaminated soil samples were analyzed for the physicochemical parameters after drying.

Table 1. Nutrient contents of spent cutting-fluid contaminated soil incubated with *P*. *pulmonarius* for 2 months.

Level of contamination (%)	Incubation period (month)	Organic carbon (%)	Organic matter (%)	Total nitrogen (mg/kg)	Phosphorus (mg/kg)	Potassium (mg/kg)	pH
Control (0)	0	1.96[b]	3.38[b]	98.0[a]	11.8[b]	146[b]	7.0[a]
	2	2.32[a]	3.66[a]	98.0[a]	12.9[a]	160[a]	6.8[a]
10	0	2.55[b]	4.40[b]	12.4[a]	14.3[a]	239[a]	6.9[a]
	2	3.12[a]	5.34[a]	10.9[b]	14.8[a]	341[b]	6.9[a]
20	0	2.26[a]	3.90[a]	11.8[a]	16.2[a]	343[a]	6.9[a]
	2	2.65[b]	4.27[a]	10.8[a]	18.0[a]	395[a]	6.8[a]
30	0	2.92[a]	5.52[a]	14.8[a]	17.5[a]	176[a]	7.0[a]
	2	3.20[a]	5.03[a]	14.4[b]	17.6[a]	266[a]	6.7[a]

Each value is a mean of three replicates. Values in the same column with different letters are significantly different (P≤0.05).

Table 2. Nutrient contents of fresh cutting fluid contaminated soil incubated with *P. pulmonarius* for 2 months.

Level of contamination (%)	Incubation period (month)	Organic carbon (%)	Organic matter (%)	Total nitrogen (mg/kg)	Phosphorus (mg/kg)	Potassium (mg/kg)	pH
Control (0)	0	2.60[a]	4.49[a]	13.7[a]	13.7[a]	179[a]	6.5[a]
	2	3.18[a]	5.48[a]	13.5[a]	14.8[a]	292[b]	6.5[a]
10	0	2.43[b]	4.18[b]	10.9[a]	16.7[a]	171[b]	6.6[a]
	2	3.16[a]	5.44[a]	12.3[b]	17.2[b]	297[a]	6.9[a]
20	0	2.15[b]	4.13[a]	11.8[a]	14.9[a]	343[a]	6.7[b]
	2	2.40[a]	3.70[a]	10.8[a]	16.6[a]	304[a]	6.9[a]
30	0	2.55[a]	4.39[b]	14.8[a]	17.4[a]	312[a]	6.6[a]
	2	2.95[b]	5.09[a]	14.4[b]	17.6[a]	331[a]	6.9[b]

Each value is a mean of three replicates. Values in the same column with different letters are significantly different (P≤0.05).

Physicochemical analysis of soil

The pH was determined for all the air-dried soil samples in soil water (1:1) suspension using a Jenway 3510 pH meter according to a method adapted by MAFF (1986). Total organic carbon in soil was determined by oxidation with excess amount of potassium dichromate and the unreacted potassium dichromate titrated with standard ferrous sulphate. Total nitrogen was determined by the kjeldhal method. Ascorbic acid and flame photometry methods were adopted for the determination of phosphorus and potassium respectively according to the official methods of analysis (AOAC, 2003). The exchangeable cations of the soil were extracted with ammonium acetate solution and the 'cation exchange capacity' was measured by the methods outlined by Sparks et al. (1996). Total petroleum hydrocarbon (TPH) was determined using the method of the Inter-governmental Oceanographic Commission (IOC) adapted by Onianwa and Essien (1999). The concentrations of toxic metals (Mn, Pb, Ni, Cu) in soil samples were determined by atomic absorption spectrophotometry after digestion with aqua regia. Reagent blanks were used to estimate analytical bias. Test for significant differences between metal concentrations, nutrient contents and exchangeable fractions at different incubation periods were carried out using ANOVA.

RESULTS

Tables 1 and 2 show the nutrient contents of spent and fresh cutting-fluid contaminated soil samples incubated respectively with *P*. *pulmonarius*. After two months of incubation, the control experiment revealed an increase in organic carbon contents, organic matter, phosphorus content and potassium. The same trend of increase in contents of these parameters was observed after two months for 10, 20 and 30% of contamination with respective spent and fresh cutting fluids. There was a slight reduction in soil pH but the difference was not significant (P≤0.05) upon treatment with *P*. *pulmonarius* for all the levels of contamination. The difference was significant at 0, 10 and 20% for organic carbon, organic matter and potassium. The levels of organic carbon, organic matter,

Table 3. Heavy metal contents (mg/kg) of spent cutting fluid contaminated soil incubated with *P. pulmonarius* for 2 months.

Level of contamination (%)	Incubation period (month)	Mn	Pb	Ni	Cu
Control (0)	0	0.066[a]	0.047[a]	0.055[a]	0.021[a]
	2	0.061[b]	0.044[a]	0.055[a]	0.017[b]
10	0	0.072[a]	0.022[a]	0.065[a]	0.034[a]
	2	0.054[b]	0.055[a]	0.064[a]	0.022[a]
20	0	0.060[a]	0.042[a]	0.053[a]	0.037[a]
	2	0.064[a]	0.042[a]	0.049[a]	0.027[b]
30	0	0.066[a]	0.036[a]	0.025[a]	0.039[a]
	2	0.031[b]	0.036[a]	0.064[b]	0.032[b]

Each value is a mean of three replicates. Values in the same column with different letters are significantly different (P≤0.05).

Table 4. Heavy metal contents (mg/kg) in the fresh cutting fluid contaminated soil incubated with *P. pulmonarius* for 2 months.

Level of contamination (%)	Incubation period (month)	Mn	Pb	Ni	Cu
Control (0)	0	0.044[a]	0.021[a]	0.036[a]	0.029[a]
	2	0.034[b]	0.023[a]	0.034[a]	0.023[b]
10	0	0.057[a]	0.009[a]	0.059[a]	0.019[a]
	2	0.026[b]	0.018[a]	0.026[b]	0.017[b]
20	0	0.065[a]	0.024[a]	0.064[a]	0.025[a]
	2	0.064[a]	0.024[a]	0.063[a]	0.021[b]
30	0	0.065[a]	0.035[a]	0.051[a]	0.033[a]
	2	0.062[a]	0.024[a]	0.072[a]	0.028[b]

Each value is a mean of three replicates. Values in the same column with different letters are significantly different (P≤0.05).

phosphorus and potassium after three months were found to be significantly different from the initial levels for only 10% of contamination. This pattern was observed for both cutting and fresh fluids (Tables 1 and 2). Heavy metals contents in spent and fresh cutting fluid polluted soil samples incubated respectively with *P. pulmonarius* were shown in Tables 3 and 4. Reduction in Mn, Ni, Cu levels were observed after two months of incubation at 10, 20 and 30% contamination of the soil with spent and fresh cutting fluid respectively. The reduction in metal levels was also noticed with the control sample for these metals.

The reduction for Mn, Ni and Cu at all levels of contamination was found to be significant at P≤0.05. There was a slight reduction in lead concentrations from 0.047 to 0.044 mg/kg for the control sample while the concentrations remained constant for other levels (10, 20 and 30%) of contamination. There was removal of total petroleum hydrocarbons (TPH) from spent and fresh cutting fluid contaminated soil samples as shown in Figures 1 and 2. TPH loss decreases as the concentration of cutting fluid increases. The highest level of degradation was obtained at 10% level of contamination while the least degradation was obtained at 30% level of contamination. Table 5 shows the cation exchange capacity (CEC), which is a useful indicator of soil fertility. The more the value of cation exchange capacity, the better the fertility. The increase in CEC values at 10% level of contamination with both spent and fresh cutting fluid was to be significant from each other at P≤ 0.05.

DISCUSSION

The use of fungi in the remediation of polluted soil was preferred to bacteria because the former have the lignin degrading systems which act extracellularly (Barr and Aust, 1994). Strains like *Pleurotus* spp seem to be more suitable for application in soil remediation; although, they can also produce their characteristic ligninolytic enzymes

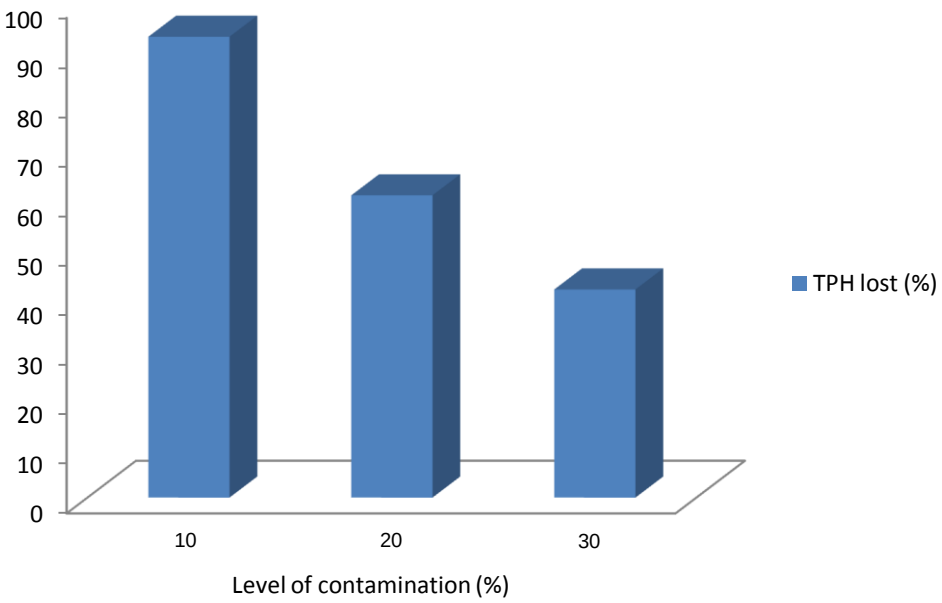

Figure 1. Loss of TPH (%) of the spent cutting fluid contaminated soils incubated with *P. pulmonarius* for 2 months.

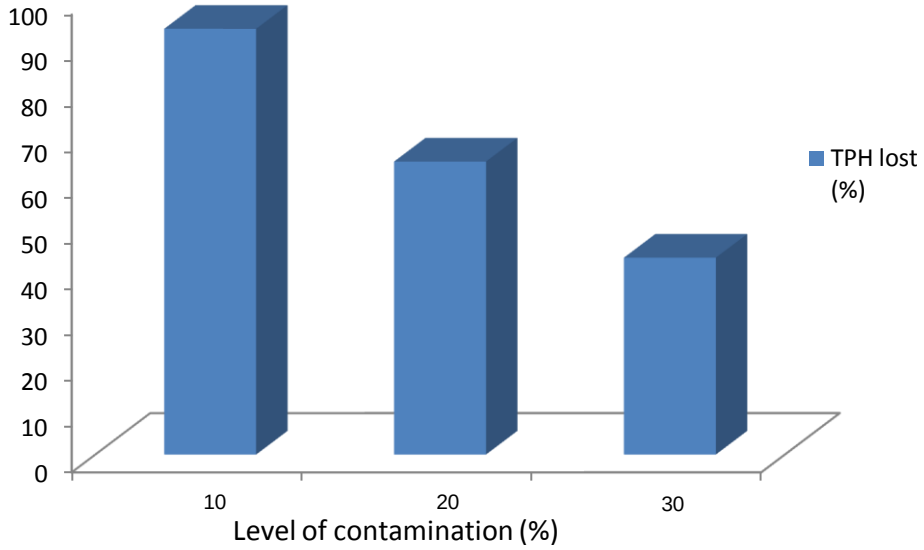

Figure 2. Loss of TPH (%) of the fresh cutting fluid contaminated soils incubated with *P. pulmonarius* for 2 months.

Table 5. Results of cation exchange capacity (meq/100 g) at different levels of contamination with spent and fresh cutting fluids.

Spent cutting fluid			Fresh cutting fluid		
Cutting fluid concentration (%)	**0 month**	**2 months**	**Cutting fluid concentration (%)**	**0 month**	**2 months**
Control (0)	3.96[a]	6.11[a]	Control (0)	3.96[a]	6.21[a]
10	7.30[a]	11.0[b]	10	7.30[a]	10.1[b]
20	8.12[a]	7.94[a]	20	8.43[a]	8.32[a]
30	8.62[a]	8.35[a]	30	8.83[a]	8.47[a]

Each value is a mean of three replicates. Values in the same column with different letters as superscript are significantly different ($P \leq 0.05$).

in the soil environment (Kubatova et al., 2001; Lang et al., 1998). In this study, the organic matter and organic carbon were found to be higher in fresh cutting fluid than in spent cutting fluid. This is expected as fresh cutting fluid contains more carbon and phenolic compounds than the spent one (Van der Gast et al., 2002). The high organic matter in fresh cutting fluid arising from greater percentage of phenolic compounds will stimulate the fungus to secrete more enzymes than in spent fluid. The reduction in total nitrogen levels at different levels of contamination except at 10% level for fresh cutting fluid contamination soil suggests that the fungus must have utilized nitrogen for growth during the incubation period. This is in agreement with the findings of Benka-Coker and Ekundayo (1995) who reported low levels of nitrogen and phosphorus from a crude oil spill site in the Niger Delta of Nigeria. In this study, the pH of the soil which ranges from 6.7 to 7.0 also influenced the occurrence of biotransformation process of cutting fluid contaminated soil. This agrees with the findings of Bishnoi et al. (2008) who reported that optimum pH 7.0 support biodegradetion of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium* in sterile and unsterile soil.

The reduction in the heavy metals contents of both contaminated and control soil samples, after 2 months of incubation with *P. pulmonarius* indicates the accumulation of heavy metals such as Cu, Mn and Ni from the soil by the fungus. The fact that the salts of these metals are soluble and are assimilated by microbes, this finding could be related. Fungi have ability to accumulate metals from the environment and have found relevance in the treatment of heavy metals containing effluent (Siegel et al., 1990; Kalac et al., 1996). The TPH loss decreased as the level of contamination increased suggesting the presence of low petroleum (TPH) in the soil even after incubation. TPH is lost at 10% level of contamination through consumption, utilization or breakdown of the cutting fluid contaminated soil into simple compound that are not harmful to the environment. There is possibility of fungus degrading TPH at a level of contamination as low as 1% engine oil concentration if the incubation period is elongated (Adenipekun and Isikhuemhen, 2008). The highest cation exchange capacity value was obtained at 10% level of contamination suggest that bioremediation best occurs at this level of contamination. A soil with cation exchange capacity of 10 meq/100 g and above as obtained at this level of contamination has the potential to be fertile when used for agricultural purposes (Rhoades et al., 1999). This CEC obtained in this study is also in consonant with the finding of Zebulun et al. (2011) in which CEC of 12.2 cmol/kg was obtained after incubating soil contaminated with anthracene with white rot fungus.

In conclusion, white rot fungus *P. pulmonarius* was effective in the remediation of both fresh and spent cutting fluid contaminated soil samples. The remediation of hydrocarbon based compounds by this fungus offers a better and more environmentally friendly technique if properly and thoroughly explored over other chemical treatment methods.

Recommendations

This study therefore recommends that soil contaminated with cutting fluids can be treated with *P.pulmonarius* to reduce heavy metal contents and total petroleum hydrocarbon (TPH) to acceptable levels before final disposal to reduce the level of inherent environmental soil pollution. Such bioremediated contaminated soils can then be useful for agricultural purposes.

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Full Length Research Paper

Moisture dependent physico-mechanical properties of Iranian okra (*Ablemoschus esculentus* L.) seed

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Physical and mechanical properties of mature okra (*Hibiscus esculentus* L.) seeds from Ahvaz in Iran were evaluated. The physical and mechanical properties were evaluated at four moisture content levels of 7.1, 10, 15 and 20% dry basis (d.b). In this moisture range, seed length, width, thickness, geometric diameter, mass of 1000 seeds increased from 5.096 to 5.677 mm, 4.476 to 4.878 mm, 4.239 to 4.608 mm, 4.585 to 5.035 mm and 56.615 to 65.779 g, respectively. The angle of repose, volume, surface area and sphericity increased from 21.2 to 24.3°, 5.367 to 6.396 mm^3, 66.18 to 79.64 mm^2 and 90.07 to 0.92, respectively. The true density, bulk density and porosity decreased from 1096.34 to 1002.16 kg/m^3, 627.4 to 576.2 kg/m^3 and 41.1 to 38.01%, respectively. The rupture force and coefficient of static friction on aluminum, rubber, plywood, iron and galvanized iron sheets increased with increasing moisture content.

Key words: Okra seed, physical properties, moisture content.

INTRODUCTION

Okra (*Hibiscus esculentus* L.), a tropical to subtropical plant that is widely distributed from Africa to Asia, Southern European and America (Oyelade et al., 2003) belongs to the family *Malvaceae*. Okra is primarily a southern vegetable garden plant, grown for its immature pods, which are consumed when cooked either alone or in combination with other foods. There are some special varieties preferred by processing industries and others that are consumed as dried or fresh in Iran. The quality of the seed protein is high. The seeds of mature pods are sometimes used for chicken feed and have been used on a small scale for the production of oil (Martin and Ruberte, 1979; Oyelade et al., 2003).

Dimensions are very important in the design of sizing, cleaning and grading machines. Bulk density and porosity are major parameters in the design of drying and storage systems (Dursun and Dursun, 2005). Knowledge of the coefficient of friction is necessary in designing equipment for solid flow and storage structures. Recent scientific developments have made better the handling and processing of bio-materials through electrical, optical, thermal and other techniques, but little is known about the basic physical characteristics of biomaterials. Such basic knowledge is important not only to the engineers but also to food scientists, plant breeders, processors and other scientists who may find new uses (Mohsenin, 1978).

Several investigators determined the physical properties of seeds at various moisture contents such as Amin et al. (2004) for lentil seed, User et al. (2010) for red pepper seed, Konak et al. (2002) for chickpea seeds and Ogunjimi et al. (2002) for locust bean seed. However, no more published work seems to have been carried out on the physical and mechanical properties of okra seed and their relationship with moisture content. The aim of this

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study was to determine moisture-dependent physical and mechanical properties of okra seed cultivated in Iran to develop appropriate technologies for its processing. The development of the technologies will require knowledge of the properties of this seed.

MATERIALS AND METHODS

The okra seeds were collected from Ahvaz in Iran on May 2012, and kept in cooled bags during transportation to the laboratory. They were cleaned in an air screen cleaner to remove all foreign materials such as dust, dirt and chaff, as well as immature and damaged seeds. The initial moisture content (M_c) of the seeds, as brought from the market, was determined by drying samples in a hot air oven (Memmert-ULE500, Germany) set at 105°C (±1°C) for 72 h and was found to be 7.1% dry basis (d.b.) (AOAC, 2000).

Four levels of moisture contents of okra seeds were selected as 7.1 (initial M_C), 10, 15 and 20% d.b. The samples at the selected moisture contents were prepared by adding a calculated amount of water (Equation 1) and sealing them in separate polythene bags and storing in a refrigerator at 5°C for 7 days. Before each experiment, the required sample was taken out from the refrigerator and kept sealed in an ambient environment for 24 h to equilibrate the water and temperature throughout the sample. The sample is kept in the ambient environment in sealed conditions so there is no chance of change of moisture content (Yalcin, 2007; Kıilickan et al., 2010). Physical and mechanical properties were determined at the moisture contents of 7.1, 10, 15 and 20% d.b.

$$Q = \frac{W_i(M_f - M_i)}{(100 - M_f)} \quad (1)$$

Where, Q is the mass of water to added; M_f is final moisture content of sample; M_i is initial moisture content of sample and W_i is initial mass of sample. The moisture content was checked at the end for all samples.

A digital caliper (AND GF-600. JAPON) with an accuracy of 0.001 mm was used to measure dimensions of the samples. Seed mass was measured by a digital balance (Mitutoyo, JAPON) with an accuracy of 0.001 g. The geometric mean diameter (D_g), Sphericity (Φ), the volume (V) and surface area (S) of the seed were calculated by using the following equations (Mohsenin, 1978):

$$D_g = (abc)^{1/3} \quad (2)$$

$$\phi = \frac{(abc)^{1/3}}{a} \times 100 \quad (3)$$

$$V = \frac{\pi B^2 a^2}{6(2a - B)} \quad (4)$$

$$S = \frac{\pi B a^2}{2a - B} \quad (5)$$

$$B = (bc)^{0.5} \quad (6)$$

Where, a is the major diameter (length); b is the intermediate diameter (width) and c is the minor diameter (thickness) (Figure 1). Surface area of seed is total area (mm^2) around seed surface.

The bulk density (P_b) was determined using the mass/volume relationship, by filling an empty plastic container of predetermined volume (75 cm^3) and tare weight with the grains by pouring from a constant height, striking off the top level and weighing (Ghasemi Varnamkhasti et al., 2008). Using Equation 2:

$$\rho_b = \frac{m}{V_s} \quad (7)$$

Where, m is the total mass of seed in cylindrical (g) and V_s is the volume of cylindrical (cm^3). True density (P_t) of seeds was determined by using the liquid displacement method. Toluene (C_7H_8) was used in place of water because it has lesser extent absorb by seed. Also, its surface tension is low, so that it fills even shallow dips in a seed and its dissolution power is low (Konak et al., 2002; Aydin and Ozcan, 2007). This way, 30 g seed were filled in thirty milliliters toluene and measurement displacement liquid in pipe. True density was obtained as the ratio of seeds weight to the volume of displaced liquid. The porosity is the fraction of the space in the bulk seeds which is not occupied by the seeds. The porosity of okra seed was calculated from the values of true density and bulk density by the following equation (Kabas et al., 2006):

$$\varepsilon = \left(1 - \frac{\rho_b}{\rho_t}\right) \times 100 \quad (8)$$

The angle of repose (ψ) was determined by using a hollow cylindrical mould of 100 mm diameter and 150 mm height. The cylinder was placed on a wooden table, filled with okra seed and raised slowly until it forms a cone of seeds. The diameter (D) and height (H) of the cone were recorded. The angle of repose (ψ) was calculated by the fallowing equation (Mullah, 1992):

$$\psi = \tan^{-1}(2H / D) \quad (9)$$

Incline plane method was used to measure static coefficient of friction on different structural surfaces including (Figure 2): Aluminum, rubber, plywood, galvanized steel and iron sheet. A hollow metal cylinder (100 mm diameter and 100 mm high) opened at both ends was filled with samples at the specific moisture content. The cylinder was then placed on an adjustable tilting plate without allowing the metal cylinder to touch the inclined surface. The tilting surface was then raised slowly and gradually by a screw mechanism until the cylinder started to slide down. At this point, the angle of tilt was measured and the friction coefficient was calculated as the tangent of that specific tilt angle (Dutta et al., 1988; Suthar and Das, 1996; Gezer et al., 2002):

$$\mu = \tan(\alpha) \quad (10)$$

Where, μ is the coefficient of friction and α is the angle of tilt in degree.

Rupture force (F) of okra seed was determined by the testing machine (H50 K-S, Hounsfield, England), equipped with a 5 kg compression load cell and integrator. The measurement accuracy was ±0.001 N in force and 0.001 mm in deformation. The individual seed was loaded between two parallel plates of the machine and compressed along with thickness until rupture occurred as is denoted by a rupture point in the force–deformation curve. The

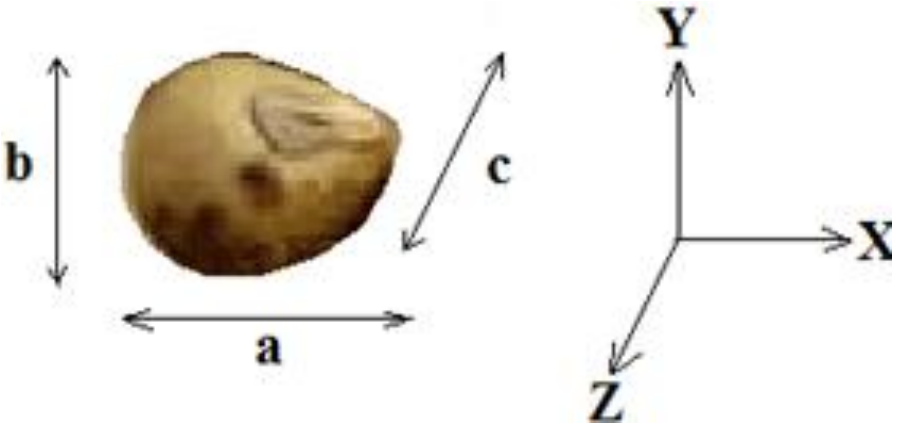

Figure 1. Dimensions of okra seed. a, Length; b, width; c, thicknes.

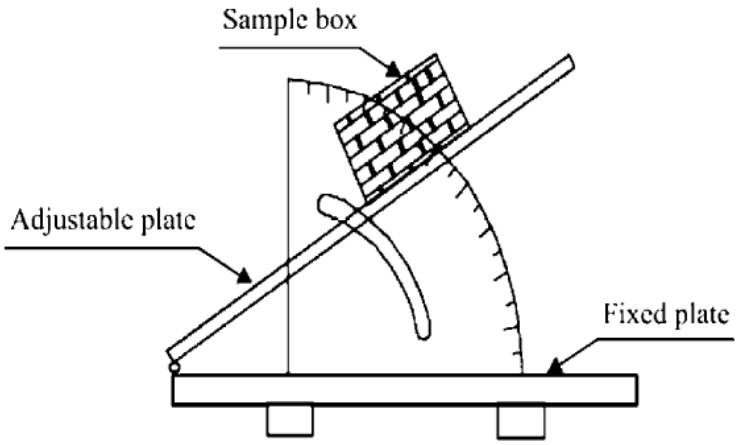

Figure 2. Apparatus for measuring static coefficient of friction.

rupture point is a point on the force–deformation curve at which the loaded specimen shows a visible or invisible failure in the form of breaks or cracks. This point is detected by a continuous decrease of the load in the force-deformation diagram. While the rupture point was detected, the loading was stopped. These tests were carried out at the loading rate of 3 mm/min for all moisture levels (Aydin and Ozcan, 2007).

Statistical analysis

For each of the moisture level, 100 okra seeds were randomly selected for measurement of properties. All the data were analyzed using the SPSS statistical software. Data means were compared using one way analysis of variance (ANOVA). Means that differed significantly were separated using Duncan's multiple range test. Significant differences were accepted at P<0.05.

RESULTS AND DISCUSSION

Seed dimensions and size distribution

The average of dimensional and technological properties of 100 okra seeds at different moisture content is presented in Table 1. Linear relationship was observed between moisture content and axial dimensions as follows:

$$a = 0.197M_C + 4.901 (R^2 = 0.996) \quad (11)$$

$$b = 0.135M_C + 4.35 (R^2 = 0.992) \quad (12)$$

$$c = 0.125M_C + 4.11 (R^2 = 0.996) \quad (13)$$

$$D_g = 0.15M_C + 4.444 (R^2 = 0.995) \quad (14)$$

According to the results from Figure 3, major diameter, intermediate diameter and minor diameter of okra seeds increased linearly with increase in moisture content. For the increase in moisture contents from 7.1 to 20% d.b., the increase of the major diameter, intermediate diameter, minor diameter and geometric mean diameter were 11.41% (5.096 to 5.677 mm), 8.99% (4.476 to 4.878 mm), 8.71%(4.239 to 4.608 mm) and 9.7% (4.589 to 5.035 mm), respectively. The increase of the major diameter

Table 1. Effect of moisture content on physical and mechanical properties of okra seed.

Property	Moisture content (%, d.b.)			
	7.1	10	15	20
Length (mm)	5.096±0.41	5.289±0.43	5.516±0.44	5.677±0.43
Width (mm)	4.476±0.32	4.629±0.25	4.775±0.28	4.878±0.36
Thickness (mm)	4.239±0.34	4.359±0.4	4.505±0.38	4.608±0.33
Geometric mean (mm)	4.585±0.29	4.743±0.31	4.914±0.34	5.035±0.35
Volume (mm^3)	5.367±0.33	5.703±0.38	6.108±0.35	6.396±0.41
1000 seed mass (g)	56.615±1.72	60.233±1.84	63±1.87	65.779±1.91
Porosity (%)	41.1±1.14	40.01±1.12	39.1±1.08	38.01±1.03
True density (kg/m^3)	1096.34±11.28	1067.14±10.64	1035.27±10.38	1002.16±9.25
Bulk density (kg/m^3)	627.4±32.12	610.1±31.35	590.7±30.18	576.2±28.87
Sphericity (%)	90.07±7.19	90.9±7.65	91.5±8.14	92.01±9.06
Surface area (mm^2)	66.18±4.62	70.69±4.98	75.86±5.39	79.64±6.47
Angle of repose (degree)	21.2±1.85	22.1±1.96	23.2±2..05	24.3±2.17
Static coefficient of friction				
Steel sheet	0.389±0.11	0.431±0.12	0.463±0.13	0.493±0.16
Galvanized steel sheet	0.372±0.15	0.395±0.18	0.408±0.16	0.419±0.19
Aluminum	0.347±0.07	0.361±0.11	0.381±0.13	0.399±0.17
Plywood	0.493±0.18	0.534±0.16	0.564±0.17	0.582±0.21
Rubber	0.524±0.15	0.562±0.16	0.591±0.19	0.625±0.22
Rupture force (N)	13.75±1.11	12.77±1.03	11.54±0.97	9.44±0.92

was more than the minor and intermediate diameter. Bagherpour et al. (2010) and Kiani Deh Kiani et al. (2008) found similar results for lentil seed and red bean grain, respectively.

Mass, volume and densities

As shown in Figure 4 mass of 1000 seeds was found to increase from 56.615 to 65.779 g as moisture content increased from 7.1 to 20% d.b. This parameter is useful in determining the equivalent diameter that can be used in the theoretical estimation of seed volume and in cleaning using aerodynamic forces. The increase of 1000 seeds mass is in conformity with the findings of Amin et al. (2004), Kiani Deh Kiani et al. (2008), Gharibzhedi et al. (2010) and Tabatabaeefar (2003) for lentil, red bean grains, sesame seed and wheat, respectively. For the increase in moisture contents, the increase of the volume was 19.17% as shown in Figure 4 as follows:

$$m_{1000} = 3.025M_C + 53.84\ (R^2 = 0.995) \quad (15)$$

$$V = 0.349M_C + 5.02\ (R^2 = 0.996) \quad (16)$$

The effect of the increase in moisture contents was increased in the volume (19.17%) to increase the mass (18.39%). Therefore, the decrease in the true density was higher than the decrease in bulk density. The bulk and true densities of seeds decreased with increase in moisture content. The bulk and true density was found to decrease from 627.4 to 576.2 kg/m^3 (8.88%), 1096.34 to 1002.2 kg/m^3 (9.39%), as moisture content increased from 7.1 to 20% d.b. as shown in Figure 5. The following linear relationships were developed for bulk density and true density.

$$\rho_b = -17.3M_C + 644.3\ (R^2 = 0.997) \quad (17)$$

$$\rho_t = -31.44M_C + 1128\ (R^2 = 0.998) \quad (18)$$

Similar decreasing moisture content has been observed for other products (Gharibzahedi et al., 2010; Cagaty et al., 2006).

Porosity

Porosity was found to decrease from 41.1 to 38% with the moisture content increase from 7.1 to 20% d.b. as shown in Figure 6. A linear relationship was obtained as follows:

$$\varepsilon = -1.02M_C + 42.1\ (R^2 = 0.998) \quad (19)$$

This could be attributed to the expansion and swelling of seeds that might have resulted in more voids between the seeds and increased bulk volume. This is also exhibited in the reduction of bulk density with increase in

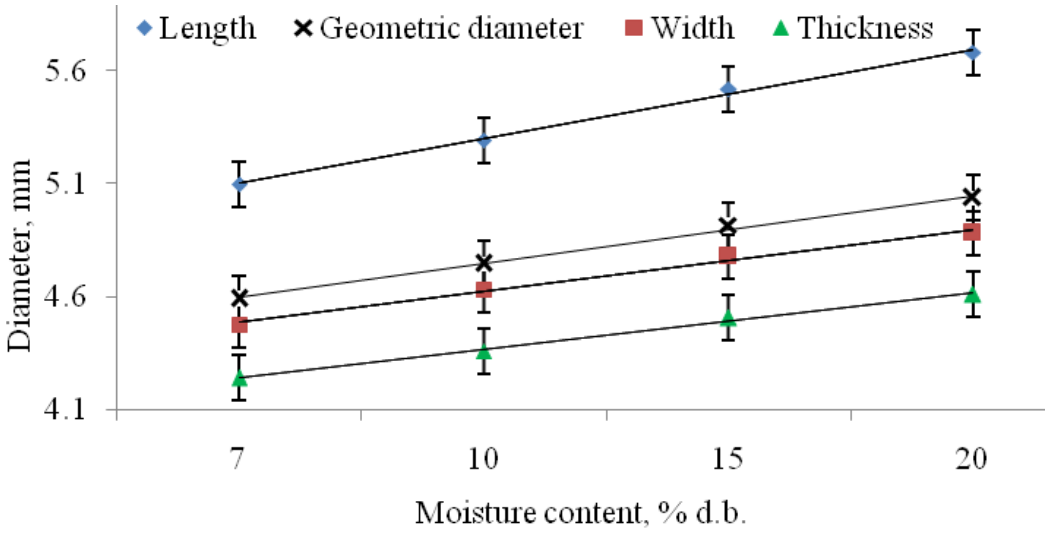

Figure 3. Effect of moisture content on dimensions of okra seed.

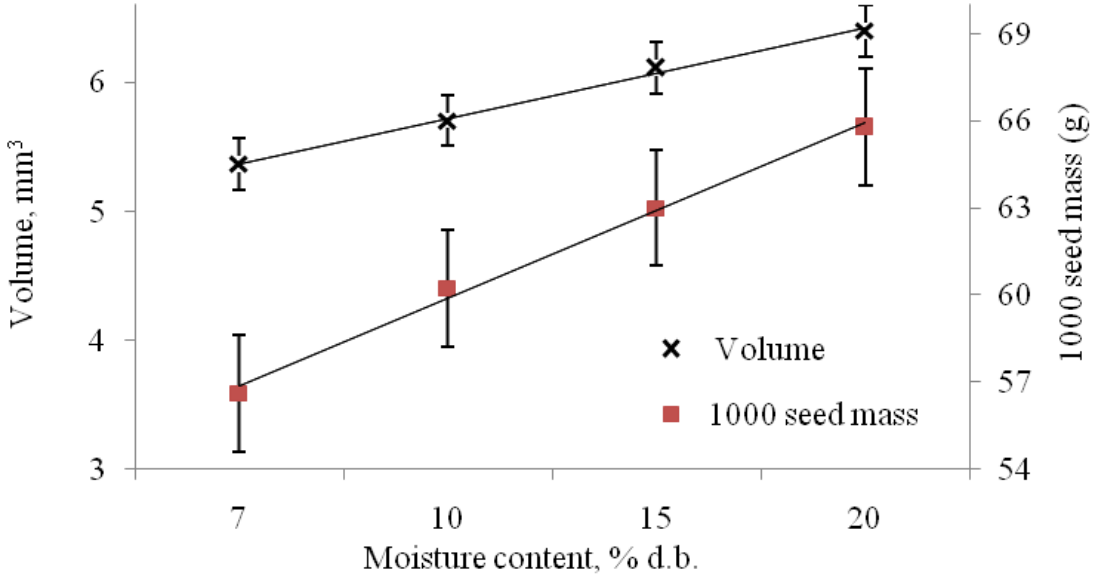

Figure 4. Mass of 1000 seeds and volume of okra seed as a function of moisture content.

moisture content. Similarly, for lentil, Bagherpour et al. (2010) stated that as the moisture content increased so did the porosity value.

Sphericity

The sphericity of okra seed was found to increase from 90.07 to 92.01% with the increase in moisture content (Figure 6). The following a linear relationship was developed for Sphericity.

$$\phi = .0.006M_C + 0.895 (R^2 = 0.986) \quad (20)$$

Similar trends have been reported by Gharibzhedi et al. (2009) for sesame seed Sacilik et al. (2003) for hemp seed.

Surface area

According to the results, surface area of okra seeds increased linearly with increase in moisture content as shown in Figure 7. For the increase in moisture contents from 7.1 to 20% d.b., the increase of the surface area was 20.34% (66.18 to 79.64 mm^2). Similar increases have been reported by Gharibzahedi et al. (2010) and Sacilik et al. (2003) for black cumin and hemp seed,

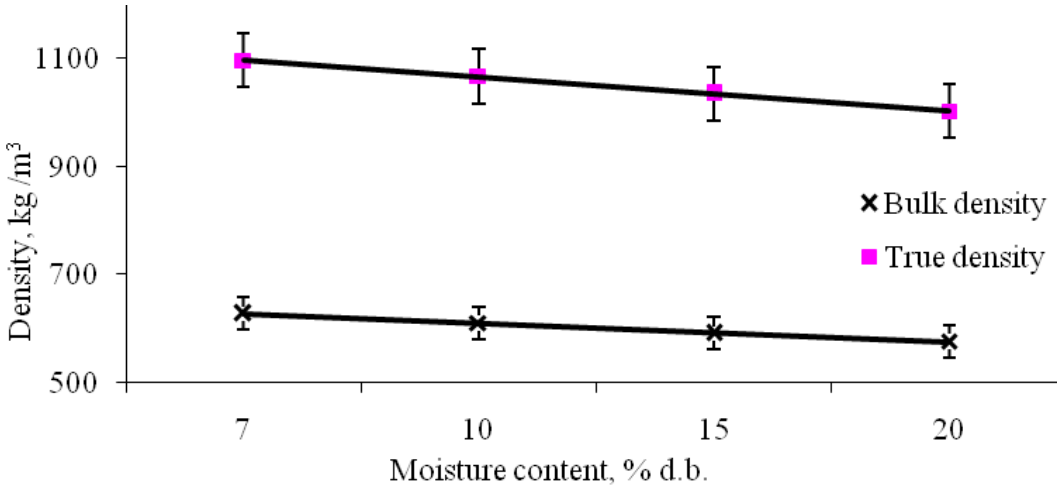

Figure 5. Effect of moisture content on true and bulk density of okra seed.

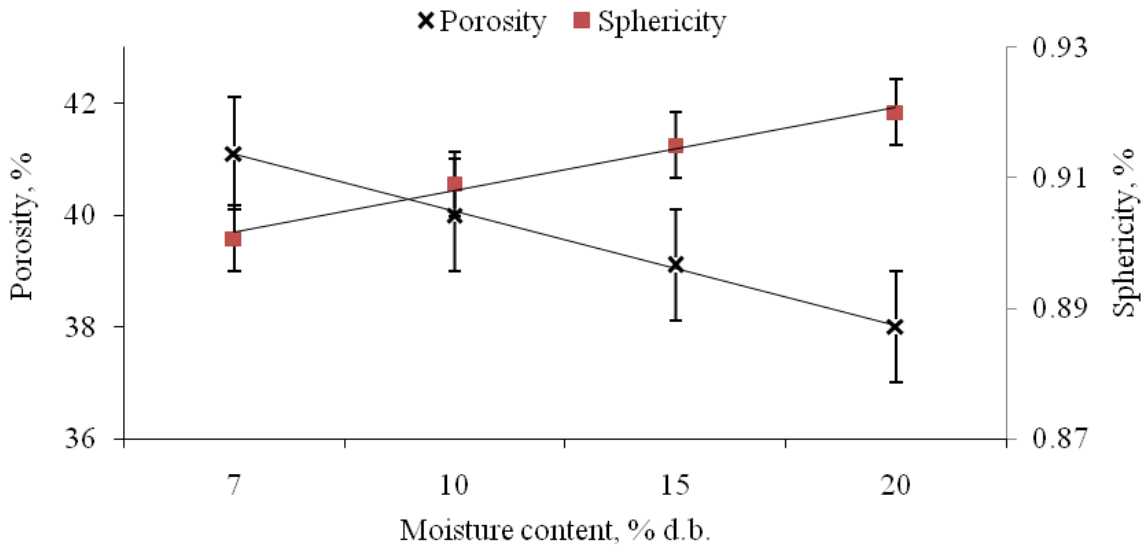

Figure 6. Porosity and sphericity of okra seed as a function of moisture content.

respectively. The relationship between surface area and moisture content of okra seeds was found to be as follows:

$$S = 4.55M_C + 61.70 \ (R^2 = 0.996) \quad (21)$$

Angle of repose

The angle of repose of okra seeds increased with increase in moisture content as shown in Figure 7. It increased from 21.2 to 24.3° in the moisture range of 7.1 to 20 % d.b. The increase in the angle of repose with increase in moisture content of the seed could be the cause of the higher surface area which may increase the internal friction of the seeds. The obtained results are similar to those reported by Amin et al. (2004) and Sacilik et al. (2003) for lentil seed and hemp seeds, respectively. The angle of repose is importance in designing hopper openings, sidewall slopes of storage bins, chutes for bulk transporting of seeds and it is particularly useful for calculating the quantity of granular materials which can be placed implies or flat storages (Gharibzahedi et al., 2009). Therefore, seed moisture content should be taken into account while designing transport and storage equipment. The relationship existing between moisture content and angle of repose appears a linear relationship was obtained as follows:

$$\psi = 1.04M_C + 20.1 \ (R^2 = 0.997) \quad (22)$$

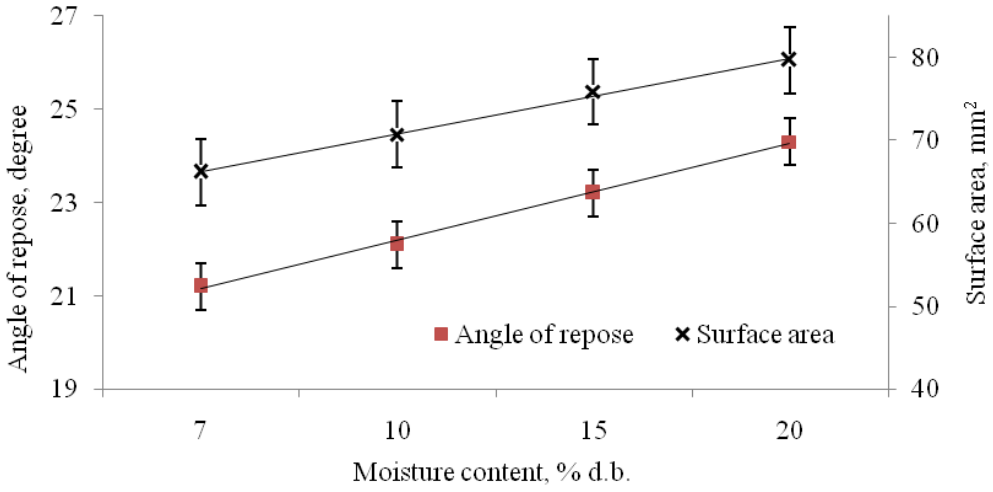

Figure 7. Effect of moisture content on surface area and angle of repose of okra seed.

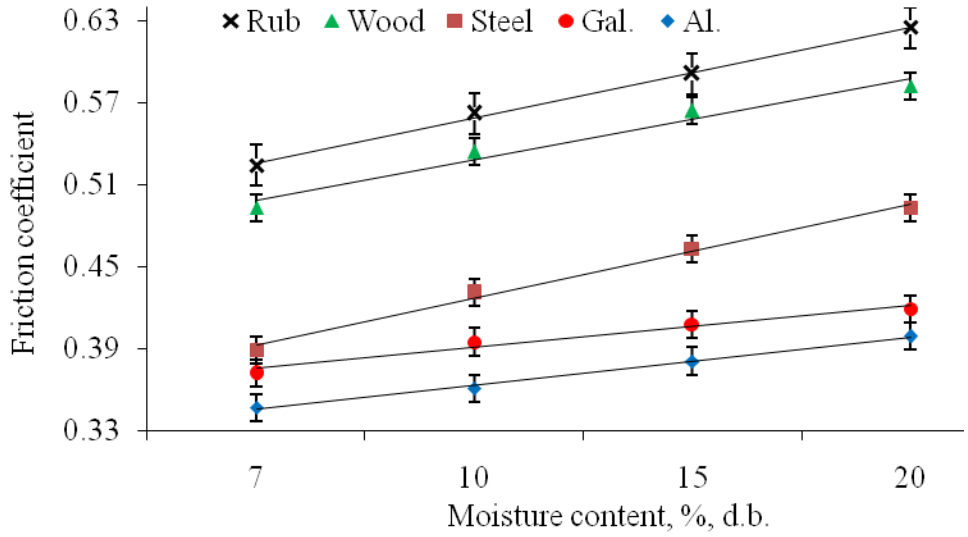

Figure 8. Effect of moisture content on static coefficient of friction of okra seed.

Static coefficient of friction

The static coefficient of friction of okra seed on five surfaces (plywood sheet, rubber sheet, galvanized steel sheet, aluminum sheet and steel sheet) against moisture content in the range 7.1 to 20% d.b. are presented in Figure 8. According to the results, the static coefficient of friction increased with increase in moisture content for all the surfaces. This is due to the increased adhesion between the seed and the material surfaces at higher moisture values. At all moisture contents, the least static coefficient of friction were on aluminum sheet. This may be owing to smoother and more polished surface of the aluminum sheet than the other materials used. A higher variation in the relationship of coefficient of static friction with moisture content was detected on steel sheet (26.73%) than on rubber (19.21%), plywood (18.01%), aluminum (14.98%) and galvanized steel (12.63%). The relationship between static friction coefficient and moisture content of okra seed was found to be as follows:

$$\mu_{steel} = 0.034M_C + 0.358 (R^2 = 0.993) \quad 23$$

$$\mu_{gal} = 0.015M_C + 0.36 (R^2 = 0.968) \quad 24$$

$$\mu_{wood} = 0.029M_C + 0.469 (R^2 = 0.971) \quad 25$$

$$\mu_{rub} = 0.033M_C + 0.492 (R^2 = 0.997) \quad 26$$

$$\mu_{Al} = 00.17M_C + 0.328 (R^2 = 0.995) \quad 27$$

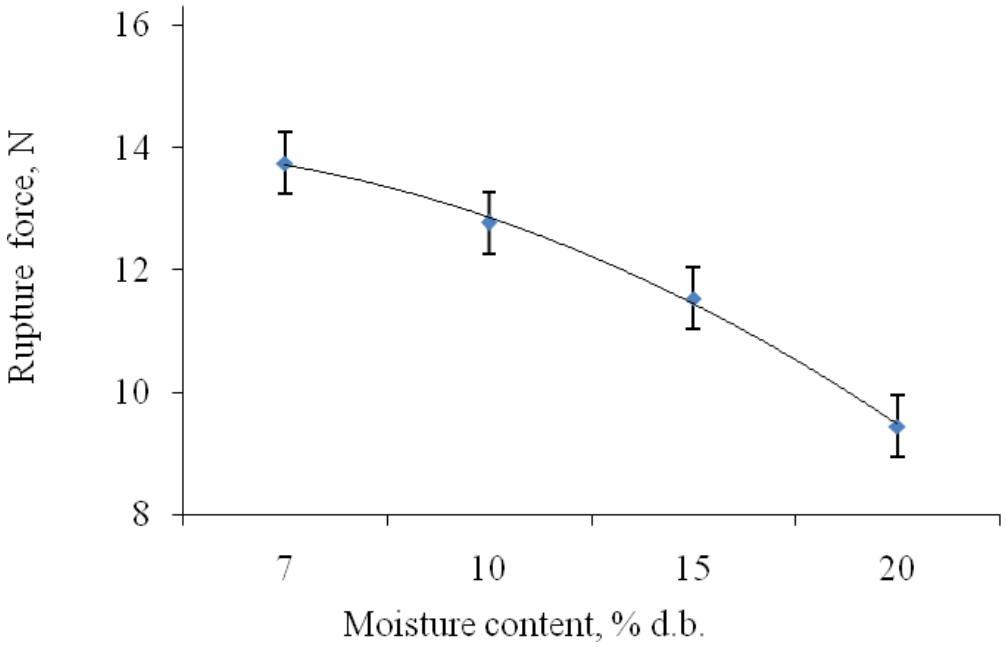

Figure 9. Rupture force of okra seed as a function of moisture content.

Similar trend has been observed by Coskun et al. (2006), Tabatabaeefar (2003), Tekin et al. (2006) and Kingsly et al. (2006) for sweet corn, wheat, bombay bean and anardana seeds respectively.

Rupture forces

The rupture force was found to decrease from 13.75 to 9.44 N (31.31%, decreases), as moisture content increased from 7.1 to 20% d.b. as shown in Figure 9. The small rupture forces at higher moisture content might have resulted from the fact that the seed became more sensitive to rupture at high moisture. These findings are similar to those reported for lentil seed (Bagherpour et al., 2010) and pine nut (Vursavus and Ozguven, 2005). The following a second-order polynomial relationships were developed for rupture forces:

$$F = -0.279M_C^{\,2} - 0.018M_c + 14.01 \quad (R^2 = 0.998) \qquad 28$$

Conclusions

Some physical and mechanical properties of okra seed were measured. The following conclusions may be made based on statistical analysis of the data: The average dimensions for each of the three principal diameters (major, intermediate and minor), geometric mean diameter, mass of 1000 seeds, volume, sphericity, porosity, surface area, and angle of repose increased significantly in the moisture range from 7.1 to 20% d.b. True density decreased from 1096.34 to 1002.16 kg/m^3 and the bulk density decreased from 627.4 to 576.28 kg/m^3. Also, the coefficient of static friction on all surfaces (plywood sheet, rubber sheet, galvanized steel sheet, aluminum sheet and steel sheet) of okra seeds increased in the moisture range from 7.1 to 20% d.b. Finally, the average rupture force of okra seeds decrease from 13.75 to 9.44 N.

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Full Length Research Paper

Effect of soil moisture management on the quality of wax apple

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Wax apple (Syzygium samarngense Merr.et Perry) was one of the economically planted fruits in Taiwan. This research was conducted to evaluate the effects of different soil moisture management on increasing wax apple quality. It was preceded at two different soil properties (shallow soil and alluvial soil) in Pingtung, Taiwan. Three different treatments were used at the two areas in the fruit setting stage: (1) the soil was furrowed 20 cm and immersed in water (5 cm) (FI); (2) soil moisture was monitored by tensiometer, from 15 to 25 cbar (TM); (3) Drought (DR) and the treatment of farmer usage as check (FU). The results show that the effect of TM treatment on soil available elements, the concentration of leaf elements and fruit color were all highest, and the fruit cracking percentage was lower than in other treatments in shallow soil. On the other hand, the effect of FI treatment on soil available elements, the concentration of leaf elements and fruit color were all highest, and the fruit cracking percentage was lower than for other treatments in alluvial soil. To improve the quality of wax apple, it may be treated by different management of soil moisture for the two kinds of soils at the fruit setting stage.

Key words: Wax apple, soil moisture, nutrition, fruit quality, fruit cracking.

INTRODUCTION

Wax apple is a kind of tropical fruit. In Taiwan, they are cultivated at the farmlands of Kaohsiung and Pingtung County. High quality wax apples like" Black Pearl" and "Black Diamond" have been well known for more than a decade. However, the influences of factors including climate (temperature, sunlight, and rainfall etc.), cultivation and management (pruning, flower and fruit thinning etc.), and supply of different nutrition significantly act on the growth and development, sugar content, color, and even abscission and cracking of the wax apple (Kuo et al.,2004). Besides color and sugar degree, fruit cracking is another reference index for the quality of wax apple. Sugar content and color have been notably improved in the past years. However, fruit cracking is always a quality defects. High ratio of fruit cracking has a negative impact on the marketing of wax apple (Lai, 2005). We cannot wait any more to solve this problem. There have been a bunch of achievements in fighting against fruit cracking for other fruits. For example, GA_3 (50 ppm) + NAA (50 ppm) is great for loquat in preventing fruit cracking (Yen, 1989).

Calcium fertilizer improves the fruit cracking and malformed fruits of tomato as a result of calcium deficiency (Asen, 1976). For Fuyu sweet persimmon, an appropriate rates of N- and K-Fertilizer (N: K2O=300:300 g/year/tree) is better than the high N- and K-Fertilizer rates (N: K2O=450:300 g/year/tree), which is commonly adopted by the farmers, in reducing fruit cracking at tampuk for 25% (Lai, 2001). The control of moisture content also has its impacts on fruit cracking. Markakis (1974) showed that it was possible to control the fruit cracking of pineapple by controlling the moisture content of the soil. During the period of fruit growing, instant rainfalls and bad management

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of moisture content in the soil may result in severe fruit cracking for Murcott Orange (Garcia-Luis, 1994). Soil should be kept from being excessively dry and with appropriate water content (Chang et al., 2005).

For wax apple, most of the studies on the fruit cracking are based on the utilization of nutritional agents and the cultivation management of the parts above ground. For example, Lai (2005) showed that the fruit string at the outside of the crown demonstrated the highest fruit cracking ratio on a horizontal basis. Spraying 0.3% $CaCl_2$ or $CaSO_4$ on the surface of leaves had a remarkable effect against fruit cracking. Yen et al. (2004) believed that spraying Chung-Hsin 100(CH-100) plant extract's 1000X solution in the winter helped to reduce the fruit cracking ratio by 25%. Wu (2002) found that plastic packaging demonstrated a higher fruit cracking ratio (12 to 63%) than paper packaging (10 to 45%). Rainfalls in the mature stage of wax apple result in significant fruit cracking. This is because of the fast alternation between dryness and moisture at the roots (Lai, 2005). In this study, the impacts of soil's moisture were discussed.

MATERIALS AND METHODS

The soil properties of experimental orchards

The experimental orchards were located at Kaosu and Nantzu, Pingtung, Taiwan. The soil in Kaosu is among Shashuipu series. It is a shallow soil with very good drainage properties accumulated by clay slates. This area can be characterized by rock layer or stone block layer beneath the surface soil. The soil in Nantzu belongs to Lantau series. Its alluvial soil with limited drainage capability, is based on concretion of iron and manganese accumulated by the ancient alluvial deposits of clay slates, which are alkalescent.

Experiments of water content control for soil

During mid-harvest period (December 2004), the following methods were applied accordingly: 1) FI: Digging of a channel which is 20 cm in depth on the soil around the tree. The water level in this channel was kept as 5 cm in depth; 2) TM: The moisture content of the root system at 30 cm under the ground was monitored with a buried tensionmeter as a reference for irrigation. If the reading of tensionmeter is greater than 25 cbar, the irrigation starts. If the reading is smaller than 15 cbar, the irrigation stops; 3) DR: no more irrigation was applied in and after the mid-harvest period; 4) FU (control group): The land was irrigated every 4 to 5 days to keep the soil moist.

Before the experiment and during the mid-harvest period, soil around treated plants and the 2nd mature leaf pair of the summer shoot of a non-fruit branch were analyzed. Sugar content, average fruit length, average fruit width, average fruit weight, and cracking ratio in the mature stage were investigated.

Pretreatment and analysis of soil and plants

Pretreatment and analysis of soil

After getting back soil samples, they were air dried, ground and were filtered through a 2 mm sieve. The soil was analyzed: 1) pH: water: soil = 1:1, measured by a pH meter (McLean, 1982); 2) organic content: measured by wet oxidation method described by Nelson and Sommer (1982); 3) Ca, Mg and K: K, Ca and Mg from the soil were extracted by 0.1 N HCl. The content of Ca and Mg was measured by an Atomic Absorption (AA) Spectrometer (Shimatzu, 6601F) and the content of K by a flame photometer (Corning, 401) (Baker and Suhr, 1982); 4) P was measured by molybdenum blue method (Bray No.1) (Murphy and Riley, 1962); 5) Microelements of Fe and Mn were extracted from the soil by 0.1 N HCl. Its content can be measured by an AA spectrometer (Cope and Evans, 1985).

Pretreatment and analysis of leaves (Chang, 1981)

The dusts and chemical residuals on the leaves were cleaned by tap water and put into an oven (70-75°C) which were ground after 2 to 3 days and put into a bottle. The leaves were resolved by concentrated sulphuric acid and analyzed by the following methods: 1) N: was measured by Kjeldahl method; 2) P was measured by molybdenum yellow method (Bray No.1); 3) K, Ca, Mg: Ca and Mg contents were measured by an AA spectrometer (Shimatzu, 6601F) and the K content was measured by a flame photometer (Corning, 401); 4) Fe, Mn: The concentrations of Fe and Mn were measured by an AA spectrometer.

Investigation of fruit properties and cracking ratio

In the mature stage, 20 fruits in similar size and treated by identical method were sent for the measurement of sugar content, fruit length, fruit width, and fruit weight. Color measurement was based on the L value (lightness), a value (red value), and b value (yellow value) of the widest part of the fruit by a color difference meter for every 10 fruits treated by identical method. 20 samples taken from the same tree and 60 samples in total for every treatment were investigated for cracking ratio.

RESULTS AND DISCUSSION

The influence of water content management on soil properties, leaf element concentration, and fruit quality in wax apple farm in Kaosu orchard

Table 1 demonstrates the fundamental physical and chemical properties of the soil in Kaosu before the experiment (November 2005). The soil was sandy loam. pH was 3.75. Organic content was 2.58%, P content was 108 mg/kg, K content was 121 mg/kg, Ca content was 1009 mg/kg, Mg was 140 mg/kg, Fe was 319 mg/kg and Mn was 74 mg/kg. In the harvest period (January 2006), soil pH increased a bit in the control group but decreased in all other three treatment groups. Group DR had the maximum descent of soil pH (0.34 unit), which is significantly different from the situation before the experiment. Lien (1994) thought that if the weather during the cultivation was drought, the soil would be dry and the ammonium nitrogen would become nitrate, which decreased the soil pH. Therefore, soil pH may be impacted by the environmental factors like water content and nitrogen content. As a result, the pH of DR group dropped significantly. For group FI and TM, the soil pH decreased a bit (0.16-0.18 unit) but is not considered to be significant compared to the control group.

Table 1. The influence of different soil moisture managements on soil properties in Kaosu,Pingtung.

Parameter	pH	OM (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})
Before treatment (November, 2005)								
	3.75^{a}	2.58^{a}	108^{b}	121^{a}	1009^{a}	140^{a}	319^{ab}	74^{a}
Harvesting stage (January, 2006)								
FI	3.57^{ab}	2.81^{a}	143^{a}	138^{a}	907^{a}	147^{a}	343^{a}	84^{a}
TM	3.59^{ab}	2.68^{a}	157^{a}	145^{a}	1142^{a}	164^{a}	336^{a}	77^{a}
DR	3.41^{b}	2.97^{a}	129^{ab}	93^{b}	757^{b}	125^{a}	301^{b}	49^{b}
FU	3.78^{a}	2.74^{a}	127^{ab}	116^{ab}	1113	151^{a}	318^{ab}	66^{ab}

FI, Furrowing and Immersing; TM, Tensiometer monitoring; DR, Drought; FU, Farmer usage.

Table 2. The influence of different soil moisture managements on leaf elements concentration in Kaosu,Pingtung.

Parameter	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn
Before treatment (November, 2005)									
	1.24^{a}	0.13^{a}	1.10^{a}	18953^{b}	1183^{a}	190^{ab}	81.3^{b}	23^{a}	37.8^{b}
Harvesting stage (January, 2006)									
FI	1.30^{a}	0.13^{a}	1.15^{a}	22380^{a}	1233^{a}	233^{a}	140^{a}	16.3^{a}	53.5^{a}
TM	1.31^{a}	0.14^{a}	1.15^{a}	21173^{a}	1355^{a}	244^{a}	143^{a}	16.8^{a}	58.8^{a}
DR	1.22^{a}	0.14^{a}	1.10^{a}	19925^{a}	1120^{a}	228^{a}	125^{a}	14.3^{a}	49.3^{a}
FU	1.28^{a}	0.11^{a}	1.15^{a}	20985^{a}	1085^{a}	245^{a}	138^{a}	16.5^{a}	51.5^{a}

FI, Furrowing and Immersing; TM, Tensiometer monitoring; DR, Drought; FU, Farmer usage.

For organic content, no organic fertilizer is applied in the period from pre-experiment to harvest. Therefore, the organic content increases in all of the groups but such increases are not significant. Among all the groups, group DR demonstrated a highest increase in organic content and group TM showed the lowest. Possible reason is that the decomposition of organics slows down if the water is insufficient in the soil in group DR. In group FI, TM, and the control group, soil water content was sufficient to support a normal decomposition of organics. P contents in the soil for all the groups increased, especially for group TM. For K content, group TM and FI demonstrated an increase but group DR and the control group demonstrated a descent. Among them, group TM had the highest K content and the group DR had the lowest. For Ca content, group TM and the control group demonstrated an increase but group FI and DR showed a descent. Among them, group RM had the highest Ca content while group DR had the lowest.

For Mg content, group DR turns out to be lower than pre-experiment period. Group FI, TM, and the control group were higher. For Fe content, group DR and the control group showed a lowered value than the pre-experiment period but group TM and FI demonstrated an increase. Among them, group TM hads the highest Fe content and group DR had the lowest. For Mn content, group TM and FI demonstrated an increased value in the harvest period, but the content in group DR and the control group dropped. Group FI showed the highest Mn content among all while group DR showed the lowest. As a whole, group TM hads an increased level of nutrition elements in the harvest period than in the pre-experiment period. It indicates that method TM helps to increase the effect of nutrition elements in the soil in Kaosu. Table 2 tells us the nitrogen concentration of the leaves from Kaosu in harvest period. Except for the descent in group DR, all other three groups showed an increase, when being compared to the pre-experiment period. Group TM shows a maximum increase in nitrogen concentration. For P concentration, the control group showed a descent in the harvest period and group FI keeps a balance. The rest showed an increase.

For K concentration, group DR had a descent but the rest had an increase (0.05%). For Ca concentration, values of all groups increased, especially in group FI. For Mg concentration, all of the groups increased except for group DR. Group TM showed a most significant increase. For Fe, Mn, and Zn concentrations, values increased in all of the groups, especially in group FI. On the contrary, Cu concentration showed a decent in all of the groups, especially in group DR. To summarize, group TM had a general increase in nutrition elements when compared to pre-experiment period and the other groups in the harvest period. Group DR demonstrated a lowered level of nutrition elements in both situations. This is probably because of slowed resolution of nutrition in the soil. Table 3 shows

Table 3. The influence of different soil moisture managements on waxapple quality in Kaosu, Pingtung.(January,2006).

Treatment	Sugar degree (°Brix)	Fruit length (cm)	Fruit width (cm)	Fruit weight (g/granule)	Cracking percentage (%)	Color		
						L	a	b
FI	8.8[a]	62.3[a]	72.1[a]	134.1[a]	38.3[b]	29.9[a]	12.6[ab]	5.2[a]
TM	9.0[a]	63.2[a]	67.5[a]	115.4[a]	35.0[b]	29.9[a]	15.5[a]	6.3[a]
DR	8.1[b]	65.4[a]	68.8[a]	117.6[a]	46.7[a]	30.4[a]	10.9[b]	5.2[a]
FU	9.2[a]	62.0[a]	68.2[a]	123.9[a]	43.3[a]	31.9[a]	13.0[ab]	5.4[a]

FI, Furrowing and Immersing; TM, Tensiometer monitoring; DR, Drought; FU, Farmer usage.

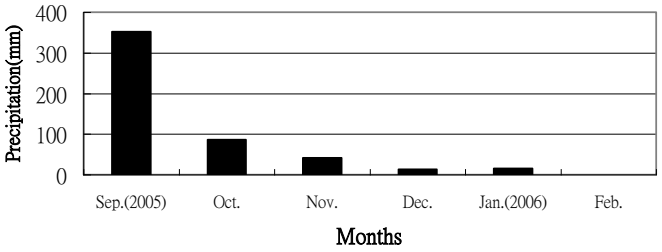

Figure 1. The average precipitation of month from September, 2005 to February, 2006 (Data from Kaohsiung district agricultural research and extension station).

that group TM and the control group had the highest sugar content in the fruit in the harvest period. Group DR turned out to be the lowest.

For fruit length, the longest value shows up in group DR and the shortest value was in group TM. Group FI demonstrated the widest value in fruit width. But the narrowest value was from group TM. For average fruit weight, group FI had the heaviest value, but group TM was the lightest. For cracking ratio, the highest value was from group DR and the lowest value was from group TM. Based on the above data, dry treatment may result in insufficient water content and therefore have a negative impact on the photosynthesis in the leaves, which produces carbohydrates. As a result, the sugar content in this group was lower than others. In group TM, the water content in the soil was always favorable. That is why the sugar content was high in this group. Although the fruits in group DR were better in length and width than that in any other group, the actual fruit density was lower than other groups due to lighter fruit weights in the group. For cracking ratio, group DR had a high value, as shown in Figure 1. A significant drop of rainfall brought drought to the soil since November 2005.

Bad fruit cracking is probably a result of drastic alteration of soil moisture by the sudden shower at the end of December 2005 and at the mid-January of 2006 before the harvest. Among all the groups, group TM showed the lowest cracking ratio. It indicates that TM method is better than others in controlling cracking ratio when cultivating wax apple in Kaosu. It will be seen from this that water control in the soil increases the effects of soil nutrition in Kaosu and helps the absorption of these nutrition. Thus, the fruit quality is improved. Although method TM is not as good as the others in fruit length, width, and weight, it does improve fruit's sugar content and lower the cracking ratio. For the L value of fruit color, the control group has the highest value while group FI and TM had the lowest. For a value (red), group TM was the highest and group DR was the lowest. Similar situation can be found for b value (yellow). It indicates that although method TM is not so good for fruit lightness, it is best for the improvement of red and yellow components in the fruit color. Method DR are worst for both red and yellow colors.

The influence of water content management on soil properties, leaf element concentration, and fruit quality in wax apple farm in Nantzu orchard

As shown in Table 4, the soil pH in all groups in Nantzu dropped in harvest period than in the pre-experiment period, especially in group DR (0.17 units). For organic content, slight descents were found in these groups. The lowest value was shown in group FI. The differences of organic content were not significant among these groups. This is because the soil in Nantzu is good in water retention. As a result, the decomposition rates of organics are quite close. The P content in the soil was increased in all groups, compared to the pre-experiment period. Group FI had the highest P content and group DR had the lowest. For K content, all groups except for group DR had increases, especially for group FI. For Ca content, all groups except for group FI were lowered, especially for the control group. For Mg content, values increased in group Fi and TM, but lowered in group DR and the control group. For Fe content, the values increased in all of the groups, especially in the group FI. For Mn content, the values dropped slightly in all of the groups. As a whole, nutrition elements in the soil were good in group FI but bad in group FR.

For nutrition elements on the leaves, concentrations of P, K, Ca, Mn, and Zn were highest in group FI. They were even higher than the concentrations in pre-experiment period. For all the measured nutrition elements except Cu, the values were lowest in group DR. They were even lower than the concentrations in pre-experiment period. Therefore, in Nantzu area, method FI is best for the soil

Table 4. The influence of different soil moisture managements on soil properties in Nantzu,Pingtung.

Parameter	pH	OM (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})
Before treatment (November, 2005)								
	4.01^{a}	2.44^{a}	92^{b}	143^{a}	1116^{a}	116^{b}	201^{b}	38^{a}
Harvesting stage (January, 2006)								
FI	3.93^{a}	2.39^{a}	105^{ab}	143^{a}	1077^{a}	149^{a}	235^{ab}	32^{a}
TM	3.87^{a}	2.34^{a}	130^{a}	147^{a}	1217^{a}	159^{a}	310^{a}	38^{a}
DR	3.76^{a}	2.43^{a}	96^{b}	138^{a}	1057^{a}	10^{b}	213^{b}	23^{b}
FU	3.81^{a}	2.36^{a}	114^{ab}	140^{a}	1030^{a}	114^{b}	226^{b}	33^{a}

FI, Furrowing and Immersing; TM, Tensiometer monitoring; DR, Drought; FU, Farmer usage.

Table 5. The influence of different soil moisture managements on leaf elements concentration in Nantzu,Pingtung.

Parameter	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn
Before treatment (November, 2005)									
	0.72^{b}	0.12^{a}	1.05b	8768^{b}	1418^{b}	173^{a}	243^{ab}	15^{a}	73.5^{a}
Harvesting stage (January, 2006)									
FI	1.08^{a}	0.14^{a}	1.10^{b}	9368^{ab}	1438^{b}	143^{ab}	245^{a}	13^{a}	80.3^{a}
TM	1.04^{a}	0.14^{a}	1.05^{b}	10764^{a}	1443^{b}	156^{a}	255^{a}	14^{a}	83.5^{a}
DR	0.93^{ab}	0.13^{a}	1.05^{b}	8398^{b}	1386^{b}	102^{b}	219^{b}	14^{a}	70.6^{a}
FU	1.16^{a}	0.15^{a}	1.25^{a}	10550^{a}	1763^{a}	135^{ab}	233^{ab}	18^{a}	76.3^{a}

FI, Furrow and Imerse; TM, Tensiometer monitoring; DR, Drought; FU, Farmer usage.

Table 6. The influence of different soil moisture managements on waxapple quality in Nantzu, Pingtung.(January,2006).

Treatment	Sugar degree (^{o}Brix)	Fruit length (cm)	Fruit width (cm)	Fruit weight (g/granule)	Cracking percentage (%)	Color		
						L	a	b
FI	10.5^{a}	66.8^{a}	74.5^{a}	136.2^{a}	10.3^{c}	30.2^{a}	14.3^{a}	4.8^{a}
TM	8.4^{b}	65.4^{a}	71.0^{a}	130.7^{a}	25.2^{b}	33.3^{a}	10.5^{b}	5.2^{a}
DR	8.0^{b}	64.5^{a}	68.3^{a}	127.3^{a}	33.3^{a}	31.4^{a}	11.3^{b}	4.2^{a}
FU	9.4^{a}	66.6^{a}	73.1^{a}	131.9^{a}	30.0^{a}	32.3^{a}	12.5^{ab}	5.1^{a}

FI, Furrowing and Immersing; TM, Tensiometer monitoring; DR, Drought; FU, Farmer usage.

nutrition. That is why the nutrition concentrations were high in the leaves (Table 5). For fruit quality (Table 6), group FI demonstrates the highest in sugar content while group DR was the lowest. For fruit size and weight, group FI was the best and group DR was the worst. For cracking ratio, group FI showed the lowest (10.3%) and group DR showed the highest (33.3%). As shown in Figure 1, the rainfall dropped in November 2005. Bad fruit cracking is probably a result of drastic alteration of soil moisture by the sudden shower at the end of December 2005 and at the mid-January of 2006 before the harvest. The situation of fruit cracking was very bad in group DR. The L value of fruit color was highest in group TM and lowest in group FI. The a value (red) was highest in group FI and lowest in group TM. The b value (yellow) was highest in group TM and lowest in group DR. To conclude, method TM was best for lightness and yellow component of the fruit color in this area. Method FI and DR weare not so ideal. However, method FI had a best performance in red color component.

Conclusion

The quality of wax apple covers factors like color, sugar content, and fruit cracking etc. Climate and cultivation management are two factors that may impact fruit quality. Since the climate factor is hard to control, more efforts can be paid to cultivation management to improve the fruit

quality. Among all cultivation managements, the improvement of tree circumstances is important. Enhanced fertilization and better water management are good to improve the quality of wax apple. In this study, different water content managements were conducted on the wax apple farms with different soil characteristics. It was found that significant differences can be found in soil properties, leaf element concentrations, as well as the fruit quality. Only the influence of water management is discussed in this study. As a matter of fact, there are much more factors that may have influences on the fruit quality of wax apple. Further study will be carried out.

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In vitro somatic embryogenesis of high yielding varieties of rice (*Oryza sativa* L.)

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Rice (*Oryza sativa* L.) belongs to the family Gramineae and is the staple food for half of the world's population and occupies almost one-fifth of the total land area covered under cereals. Now-a-days, the production of rice is hampered due to climatic changes. Therefore, it is essential to develop variety which is tolerant to abiotic and biotic stresses. The present investigation was conducted to establish an efficient and simple protocol for regeneration of four agronomically important *indica* rice varieties (Khandagiri, Udayagiri, Swarna and Pratikhya). Somatic embryogenesis were achieved from immature zygotic embryos on Murashige and Skoog (MS) medium supplemented with 3 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 1.0 mg/l kinetin and 3% (w/v) sucrose within 4 weeks of culture. The secondary somatic embryogenesis was also achieved in subsequent subculture on MS medium supplemented with 2 mg/l 2,4-D and 2.0 mg/l kinetin and 200 mg/l L-proline. The percentage of embryogenic calli proliferation were 82.4, 83.7, 88.4 and 84.4 in variety Khandagiri, Udayagiri, Swarna and Pratikhya respectively on MS basal medium supplemented with 3.0 mg/l 2,4-D, 2.0 mg/l Kinetin and 200 mg/l L-proline. Inclusion of higher concentration of L-proline (400 mg/l) in the induction medium, the growth of calli was reduced. The maximum percentage of somatic embryo germination took place in medium supplemented with 2.0 mg/l kinetin, 0.25 mg/l NAA and 50 mg/l adenine sulfate within 4 weeks of culture. The regenerated plantlets were transferred to pots for acclimatization. About 80% of plants were survived in the greenhouse condition.

Key words: Somatic embryogenesis, immature zygotic embryos, *Indica* rice, plant regeneration.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops of family Gramineae cultivated for more than 10,000 years (Sasaki, 2005). Currently, this crop supports more than 50% of the world population (Christou, 1997). Rice consumers are increasing at the rate of 1.8% every year (FAO/STAT, 2001). But the rate of growth in rice production has slowed down. It is estimated that rice production has to be increased 50% by 2025 (Khush and Virk, 2000). The available cultivable land is being reduced day by day due to unplanned industrialization and population growth. Both abiotic and biotic stresses making a great impact for reduction of production. Thus, there is a constant need to improve crops to overcome all these hazards. Resistant varieties have to be developed to meet the demand. Induction of tolerance requires an efficient breeding system. *In vitro* system is an alternative method for genetic improvement of rice. Efficient plant regeneration system leads to success in the crop improve-

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ment through genetic transformation. It is often difficult to establish embryogenic cell cultures and to regenerate plants from these cultured cells specially those belonging to *Indica* subspecies (Jain, 1997). Even within the *Indica* group, there are significant variations in the *in vitro* culture responses among the different genotypes (Khanna and Raina, 1998). The recalcitrant nature of this subspecies has, in fact, been a major limiting factor in the transfer of available useful genes (Toenniessen, 1991). Therefore, the identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration *in vitro* are key steps in rice genetic improvement program through application of biotechnology (Hoque and Mansfield, 2004).

Some of the notable information on transgenic indica rice are reported (Lin and Zhang, 2005; Karim et al., 2007; Yang et al., 2010, 2012). This present investigation deals with the development of efficient protocol in upland (Khandagiri and Udayagiri), lowland (Swarna and Pratikhya) indica rice varieties which is suitable for genetic transformation experiment.

MATERIALS AND METHODS

Plant materials

Semi-mature seeds of *O. sativa* vars. Khandagiri and Udayagiri (upland) and Swarna and Pratikhya (lowland) were collected from rice germplasm centre of Orissa University of Agriculture and Technology, Bhubaneswar, India. The seeds were washed with 2% bavistin (w/v) for 30 min, dehusked and surface sterilized with 70% ethanol for 2 min followed by washing twice with sterilized distilled water with a drop of Tween 20 with continuous shaking for 15 min. Further, the seeds were treated with 0.2% (w/v) $HgCl_2$ solution for 5 min followed by rinsing 4 to 5 times with sterile distilled water and blot dry on sterilized filter paper. Immature embryos were aseptically cultured on MS (Murashige and Skoog, 1962) medium supplemented with various concentrations of benzyl aminopurine (BA) or kinetin (Kn) (0, 0.25, 0.5, 1.0, 1.5, 2.0 mg/l), naphthalene acetic acid (NAA) or 2, 4-D (0, 0.5, 1.0, 1.5, 2.0, 3.0 mg/l) alone or in combinations for embryogenic callus culture. The pH of the media was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl prior to addition of 0.8% (w/v) agar (Himedia, India). Routinely, 20 ml of molten medium was dispensed into 25 × 150 mm glass test tubes (Borosil, India), capped with non-absorbent cotton plugs. The cultures were sterilized at 121°C and 15 psi for 15 min. The cultures were incubated under 16 h photoperiod and fully dark at 25 ± 2°C for 4 to 6 weeks.

Induction of embryogenic calli

After four weeks of incubation, data were taken on total callus induction frequency (embryogenic and non-embryogenic). Only embryogenic calli were subcultured to fresh medium supplemented with different concentrations of BA or kinetin and 2,4-D or NAA (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) singly or in combinations for induction of somatic embryo. In another experiment, different concentrations of L-proline (0, 50, 100, 150, 200 and 300 mg/l) was added in the embryogenic culture medium to enhance the embryogenic potential. The cultures were incubated under dark at 25 ± 2°C. Subculturing was made every 4 week intervals. The media were solidified with 0.8% (w/w) agar-agar. Morphological changes were recorded through visual observations at every 4- week intervals. The embryogenic response and number of somatic embryos per culture were recorded. Each treatment had 20 replicates and the experiment was repeated three times. Data were also recorded in respect of embryogenic frequency, number of somatic embryos and frequency of normal embryos per culture. The data were analyzed statistically by the Duncan's multiple range test (Duncan, 1955). Means followed by the same letter within columns were not significantly different at $P < 0.05$.

Germination of somatic embryos

The group of somatic embryos was transferred to MS basal medium supplemented with different concentration of growth regulators for maturation and germination. Regeneration medium was based on the formulation of MS basal salt supplemented with NAA 0.2 to 1.0 mg/l, Kn 1.0 to 4 mg/l, 30 g/l (w/v) sucrose and gelled with 8.0 g/l (w/v) agar. The pH of the medium was adjusted to 5.8 before sterilization. For regeneration, eight-week-old somatic embryos were placed into test tubes, each containing 15 ml regeneration medium. All such cultures were kept under 16 h photoperiod at 25 ± 2°C with 3000 lux light intensity. Data on percent of embryo germination/ culture, number of germinated plant/culture were statistically by the Duncan's multiple range test (Duncan, 1955).

Acclimatization

About 250 somatic embryos derived plantlets were transferred to the earthen pots with soil mixture at the ratio of 1: 1: 1 (sand: soil: FYM) and kept in the greenhouse with 85% relative humidity. The watering was given at every two day intervals till new growth occurring in the plantlets.

RESULTS AND DISCUSSION

Production of embryogenic calli with high regeneration capacity is a prerequisite for highly efficient transformation of rice. Many efforts have been made to identify suitable ex-plants in rice to induce embryogenic calli under appropriate culture condition. Different growth regulators like auxins and cytokinins were used to develop the embryogenic calli from semimature embryos of upland and lowland rice varieties. The medium devoid of growth regulators did not show any positive impact on callus development. The embryogenic calli were initiated on basal MS medium supplemented with 2.0 to 3.0 mg/l 2,4-D and 1.0 to 1.5 mg/l kinetin within 4 weeks of culture. The growth of the calli was depended on the genotypes tested. The cultures were incubated in the 16 h photoperiod did not show any growth of the calli rather it became dried. The cultures incubated in continuous dark for 4 to 8 weeks, the yellowish white, friable callus were developed. The maximum percentage of calli development was achieved in MS medium supplemented with 3.0 mg/l 2,4-D and 1.0 mg/l kinetin (Figure 1A). The percentage embryogenic calli development was 73.4, 74.7, 75.4 and 80.6 in variety Khandagiri, Udayagiri, Swarna and Pratikhya respectively (Table 1). Among the two cytokinins used, kinetin has significant impact on embryogenic calli development as compare with BA + 2,4-D. The medium having BA + NAA and Kn + NAA did

Table 1. Effect of auxins and cytokinins on embryogenic calli development from semimature zygotic embryos of *O. sativa* varieties Khandagiri, Udayagiri, Swarna and Pratikhya after 4 weeks of subculture.

MS + growth regulator (mg/l)				Development of embryogenic calli (%) [mean (average) ±SE]*			
Kn	**BA**	**2,4-D**	**NAA**	**Khandagiri**	**Udayagiri**	**Swarna**	**Pratikhya**
0	0	0	0	0	0	0	0
0	0.25	0.5	0	12.3 ± 0.6^{a}	18.7 ± 0.5^{b}	15.1 ± 0.6^{a}	15.4 ± 0.6^{a}
0	0.25	1.0	0	18.2 ± 0.8^{c}	24.3 ± 0.6^{c}	22.2 ± 0.8^{c}	27.2 ± 0.8^{c}
0	0.25	2.0	0	33.6 ± 0.7^{e}	46.7 ± 0.8^{f}	45.6 ± 0.7^{h}	42.6 ± 0.7^{f}
0	0.5	2.0	0	46.8 ± 0.6^{g}	51.9± 0.5^{h}	46.8 ± 0.6^{h}	48.8 ± 0.6^{g}
0	0.5	3.0	0	51.2 ± 0.8^{h}	52.1 ± 0.9^{h}	51.2 ± 0.8^{i}	58.2 ± 0.5^{i}
0.25	0	1.0	0	38.4 ± 0.8^{f}	37.3 ± 0.7^{e}	38.4 ± 0.8^{f}	41.7 ± 0.6^{f}
0.50	0	2.0	0	46.2 ± 0.7^{g}	49.7 ± 0.6^{g}	46.2 ± 0.7^{h}	43.2 ± 0.9^{f}
0.50	0	3.0	0	66.6 ± 1.0^{j}	67.8 ± 1.0^{j}	66.6 ± 1.0^{l}	61.6 ± 1.0^{j}
1.0	0	3.0	0	73.4 ± 0.6^{k}	74.7 ± 0.8^{k}	75.4 ± 0.6^{m}	80.6 ± 0.6^{k}
0	0.25	0	1.0	15.6 ± 07^{b}	15.6 ± 0.7^{a}	19.6 ± 0.7^{b}	19.0 ± 0.7^{b}
0	0.25	0	1.0	26.4 ± 0.8^{d}	31.6 ± 0.6^{d}	30.4 ± 0.8^{d}	32.4 ± 0.7^{d}
0	0.5	0	3.0	34.4 ± 0.9^{e}	39.5 ± 0.6^{e}	34.4 ± 0.9^{e}	36.4 ± 0.8^{e}
0	1.0	0	2.0	40.1 ± 1.0^{f}	44.4 ± 1.1^{f}	40.1 ± 1.0^{g}	46.1 ± 1.0^{g}
0	1.5	0	2.0	56.6 ± 0.4^{i}	57.6 ± 0.7^{i}	59.6 ± 0.4^{k}	58.6 ± 0.7^{i}
0.25	0	0	2.0	46.9 ± 1.1^{g}	45.7 ± 1.1^{f}	46.6 ± 1.1^{h}	41.4 ± 1.1^{f}
0.25	0	3.0	0	58.4 ± 1.5^{i}	57.5± 0.9^{i}	54.4 ± 1.2^{j}	55.4 ±1.8^{h}

*20 replication/treatment; repeated thrice.
Means followed by the same letter within columns were not significantly different at $P < 0.05$.

not show any positive growth of the callus. However, the response of callus growth was different in different genotypes of rice tested. Similar response was also reported by many researchers by using different explant source that is, immature and mature embryos, leaf blade, coleoptile, meristematic cells and roots (Yan and Zhao, 1982; Koetje et al., 1989; Lee et al., 2002, 2004; Mandal et al., 2003).

Lee et al. (2002) reported that immature embryos were high responsive with embryogenic potential as compared with other explants. In most cases, 2,4-D as a strong synthetic auxin was sufficient to initiate and sustain embryogenic callus growth in rice and other monocots (Lee et al., 2002; Ozawa et al., 2003; Lin and Zhang, 2005; Ivarson et al., 2013; Pathi et al., 2013). The proliferated embryogenic calli were sub-cultured in various media for induction of high frequency somatic embryogenesis. L-proline at different concentrations was used for further growth of somatic embryos. High frequency proliferation of embryogenic calli was obtainined on MS medium supplemented with 1.0 to 2.0 mg/l Kn and 2.0 to 3.0 mg/l 2,4-D and 200 mg/l L-proline in all the four varieties tested. In some cases, the secondary somatic embryos were developed in subsequent subculture. The percentage of embryogenic calli proliferation were 82.4, 83.7, 88.4 and 84.4 in variety Khandagiri, Udayagiri, Swarna and Pratikhya respectively on MS basal medium supplemented with 3.0 mg/l 2,4-D, 2.0 mg/l Kinetin and 200 mg/l L-proline (Table 2). Inclusion of higher concentration of L-proline (400 mg/l) in the induction medium, the growth of calli was reduced. At lower concentrations (50 to 100 mg/l) of L-proline did not show any significant differences on embryonic callus growth and embryo development. The embryogenic calli grown in L-proline containing medium became more yellowish white in colouration and fragile in nature (Figure 1B). Auxin-induced somatic embryogenesis in presence of proline is well documented (Chowdhry et al., 1993). Free proline was suggested to act as an osmoticum, a nitrogen storage pool and source of $NADP^{+}$, necessary for rapidly growing embryos.

The mediation of the cellular redox potential resulted from proline accumulation likely had a significant effect on the flux through redox– sensitive biochemical pathways like the pentose phosphate pathway (Ghanti et al., 2009).

Germination of somatic embryos

In addition to embryogenic callus formation, efficient regeneration also poses a major problem for transformation of *Indica* rice. The well developed group of embryos were transferred to various MS medium supplemented with growth regulators for germination. The medium devoid of growth regulators did not promote germination. The medium having 0.5 to 2.0 mg/l kinetin or BA along with 0.25 mg/l NAA and 50 mg/l adenine sulfate promote embryo germination. The germination frequency was varied in different genotypes. The maximum percentage of somatic embryo germination took place in medium supplemented with 2.0 mg/l kinetin, 0.25 mg/l

Table 2. Effect of different culture medium on proliferation of embryogenic calli of *O. sativa* varieties Khandagiri, Udayagiri, Swarna and Pratikhya after 8 weeks of subculture.

MS + growth regulator (mg/l)	Percentage (%) of embryogenic calli [mean (average) ± SE]*			
	Khandagiri	Udayagiri	Swarna	Pratikhya
0	**0**	**0**	**0**	**0**
MS+ 2,4-D 1.0 + Kn 1.0 + 50 mg/l L-proline	28.8 ± 0.9[a]	27.3 ± 0.8[a]	26.1 ± 0.6[a]	28.9 ± 0.7[a]
MS+2,4-D 2.0 + Kn 1.0 + 100 mg/l L-proline	37.9 ± 0.6[b]	35.7 ± 0.7[b]	35.3 ± 0.8[b]	37.6 ± 0.8[b]
MS+ 2,4-D 2.0 + Kn 2.0 + 150 mg/l L-proline	43.5 ± 1.0[c]	41.2 ± 1.1[c]	46.3 ± 1.2[c]	43.6± 0.9[c]
MS + 2,4-D 2.0 + Kn 2.0 + 200 mg/l L-proline	59.2 ± 0.8[e]	57.25 ± 1.7[e]	61.3 ± 1.0[e]	62.5 ± 1.3[e]
MS + 2,4-D 3.0 + Kn 2.0 + 200mg/l L-proline	82.4 ±1.4[g]	83.7 ±1.4[g]	88.4 ± 1.2[f]	84.4 ±1.0[f]
MS+2,4-D 3.0+ Kn 2.0 + 50 mg/l L-proline	62.9 ± 1.3[f]	62.8 ±1.2[f]	61.5 ± 1.1[e]	63.1±0.9[e]
MS + 2,4-D 3.0 + Kn 2.0 + 100mg/l L-proline	53.2 ± 1.8[d]	51.5 ± 1.7[d]	51.3 ± 1.7[d]	52.5 ±1.2[d]
MS + 2,4-D 2.0 + Kn 2.0 + 200mg/l L-proline	38.8 ± 0.7[b]	37.3 ± 1.0[b]	36.1 ±0.9[b]	38.9 ±0.7[b]

*20 replication/treatment; repeated thrice.
Means followed by the same letter within columns were not significantly different at P < 0.05.

Table 3. Effect of growth regulators on germination of somatic embryos of *O. sativa* varieties Khandagiri, Udayagiri, Swarna and Pratikhya after 4 weeks of culture.

MS + Growth regulators (mg/l)	Percentage (%) of somatic embryo germinated (mean ± SE)*			
	Khandagiri	Udayagiri	Swarna	Pratikhya
0	**0**	**0**	**0**	**0**
MS + Kn 0.5 + NAA 0.25 + Ads 50 mg/l	15.2 ± 0.7[a]	25.2 ± 1.0[a]	15.5±0.9[a]	13.7 ± 0.8[a]
MS + Kn 1.0+ NAA 0.25 + Ads 50 mg/l	33.8 ±0.9[b]	32.8 ± 0.7[b]	27.8±1.0[b]	26.3 ± 0.6[b]
MS + Kn 1.5 + NAA 0.25 + Ads 50 mg/l	57.2 ± 1.3[e]	48.7 ± 1.1[e]	48.2 ±1.3[d]	46.5 ± 1.2[c]
MS + Kn 2.0 + NAA 0.25 + Ads 50 mg/l	72.5 ±1.5[f]	67.5 ±0.6[g]	62.2±1.2[f]	58.5 ± 1.3[d]
MS + BA 1.0 + NAA 0.25 + Ads 50 mg/l	42.3 ± 1.6[c]	38.8 ±0.7[c]	36.6±1.0[c]	47.5 ± 1.5[c]
MS + BAP 2.0 + NAA 0.25 + Ads 50 mg/l	48.5 ± 1.1[d]	41 ±0.9[d]	45.4±1.2[d]	46.3 ± 1.2[c]
MS + BA 2.0 + Kn 1.0 + NAA 0.25 + Ads 50 mg/l	43.35 ±1.2[c]	57.8 ±1.8[f]	52.2±1.3[e]	62.7 ± 1.4[e]

*20 replication/treatment; repeated thrice.
Means followed by the same letter within columns were not significantly different at P < 0.05.

NAA and 50 mg/l adenine sulfate within 4 weeks of culture (Table 3). About 60 to 70% of embryos were germinated in different culture medium (Figure 1C and D). Further, the germinated embryos were developed into complete plantlets. With increase of NAA concentrations from 0.25 to 0.5 mg/l in the culture medium, the germination frequency was lower down. Extensive research has been conducted to improve the capacity of plantlet regeneration by manipulating the important factors within regeneration medium, such as carbohydrate source (Lee et al., 2002), nitrogen source (Grimes and Hodges, 1990), polyamines (Bajaj and Rajam, 1995, 1996), amino acids such as proline and tryptophan (Ozawa and Komamine, 1989; Chowdhry et al., 1993), and plant growth regulators (Kavi Kishor, 1987).

The medium supplemented with BA + NAA also promote embryo development and germination frequency but less than the Kn + NAA. Further, the germinated plantlets were transferred to sterile distilled water for a week and kept in the culture room for acclimatization (Figure 1E). After one week, the plants were planted in the pots and transferred to the greenhouse with 70% humidity. About 80% of plants survived in the greenhouse condition (Figure 1F).

Conclusion

Plant growth regulators and physiological status of the explants play a critical role in the control of growth and morphogenesis. It may be concluded that genotypic differences strongly influence on embryogenic callus formation and plant regeneration potential. The results suggest that embryonic explants are very good source material for efficient *in vitro* plant regeneration. The high frequency plant regeneration through somatic embryogenesis will be suitable for genetic transformation study of upland and lowland rice varieties.

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Figure 1. *In vitro* somatic embryogenesis of different varieties of rice (*O. Sativa* L.).
A. Induction of somatic embryogenesis of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 3.0 mg/l 2,4-D and 1.0 mg/l Kinetin (bar = 10 mm).
B. Induction of somatic embryogenesis of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 3.0 mg/l 2,4-D and 2.0 mg/l Kinetin and 200 mg/l L-proline (bar = 50 mm).
C. Germination of somatic embryos of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 2.0 mg/l Kinetin, 0.25 mg/l NAA and 50 mg/l adenine sulphate (bar = 25 mm).
D. Germination of somatic embryos of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 2.0 mg/l Kinetin, 0.25 mg/l NAA and 50 mg/l adenine sulphate (bar = 25 mm).
E. Somatic embryo derived plantlets were transferred to distilled water for primary hardening and acclimatization.
F. Germinated plantlets of *O. Sativa* varieties Khandagiri grown in the greenhouse (bar = 1.0 cm).

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Full Length Research Paper

Isolation and identification of a *Staphylococcus warneri* strain with anti-mycobacterial activity

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Tuberculosis is the principal cause of death from infection in the world. The resurgence of tuberculosis and the increase in mycobacterial infections, as well as multidrug-resistance of mycobacteria to available antibiotics, has incentivized research on new antimycobacterial agents. Therefore, research based on water and soil samples from the Moroccan biotopes, has led to the isolation of a bacterial strain capable of inhibiting mycobacterial growth (*Mycobacterium smegmatis* and *Mycobacterium aurum A*$^{+}$). The effect was due to an active substance secreted into the culture medium. Sequencing of the 16S rRNA gene identified the strain as belonging to the species *Staphylococcus warneri*. The active substance precipitated using ammonium sulfate, maintained its inhibitory properties, which were lost when treated with proteinase K. These results indicated that the active substance was protein. Study of the activity of the metabolite revealed its effect on *M. smegmatis* cell wall, facilitating genomic DNA extraction.

Key words: Tuberculosis, mycobacteria, anti-mycobacterial agents, *Staphylococcus warneri*, DNA extraction.

INTRODUCTION

Tuberculosis, caused by *Mycobacterium tuberculosis*, undeniably remains a menacing social disease (Musser, 1995; Berthet et al., 1999; Rossetti et al., 2002). Nevertheless, as of 1952, the onset of effective chemotherapy, followed by widespread Bacille de Calmette et Guérin (BCG) vaccination (Ann Ginsberg, 2002; Bonnaud, 1996), the discovery of effective antituberculosis agents and the improvement of living conditions, have led to a considerable decline in tuberculosis in industrialized nations. In fact, the rate of infection dropped from 200/100.000 inhabitants in 1900 to less than 10/100.000 inhabitants in 1980 (Rastogi et al., 2001; Jost et al., 2001).

However, in the early 90's, population mobility, economic and political migrations, together with ineffective tuberculosis control programs, where adequate treatment failed to reach many patients, gave rise to an increase in the global incidence of tuberculosis (Newton et al., 2000). These conditions also favored the onset of problematic multidrug-resistant strains (Jost et al., 2001). Furthermore, immunocompromised patients, especially those infected by the human immunodeficiency virus (HIV),

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Abbreviations: BCG, Bacille de Calmette et Guérin; **HIV,** human immunodeficiency virus; **WHO,** World Health Organization; **MDRTB,** multidrug-resistant tuberculosis bacilli; **XDRTB,** extensively drug-resistant tuberculosis; **NCBI,** National Center for Biotechnology Information;**BLAST,** Basic Alignment Search Tool.

are particularly vulnerable (Mohle-Boetani et al., 2002; Chin and Hopewell, 1996). Developing countries remain the most affected by tuberculosis with mortality rates close to 40% compared to 7% in the industrialized nations (Emile, 1996).

In spite of scientific progress, this disease continues to take its toll mainly in the poorer regions of the globe (Dharmarajan et al., 2007). According to the World Health Organization (WHO) 2010 report, more than two billion people, close to one third of the world's population, are contaminated with the tuberculosis bacillus. One out of ten infected individuals will develop active disease in the course of his or her lifetime. The risk is considerably higher for people living with HIV. WHO has estimated that between 2000 and 2020, close to one billion people will be newly infected and that 200 million of these will develop the disease, of these 35 million will die of tuberculosis, if adequate measures are not taken to improve disease control (WHO, 2005).

Resistance to antituberculosis agents is due to insufficient treatment in either duration or dosage. There are two forms of resistance, multidrug-resistance and extensive drug-resistance. In 2007, the estimate was of 500 000 tuberculosis cases due to multidrug-resistant tuberculosis bacilli (MDRTB); 85% of these were from 27 countries (of which 15 were from the European region). By the end of 2008, 55 countries and territories had reported at least one case of extensively drug-resistant tuberculosis (XDRTB) (WHO, 2009). In Morocco, 500 to 1.000 people die every year from tuberculosis. According to the latest reports from the Ministry of Health, the kingdom registered 25.500 new cases in 2007, corresponding to 82 cases for every 100.000 inhabitants. Seventy percent of these patients were between 15 and 45 years of age (Ben cheikh et al., 1996), and those populations from urban populated areas remain the most vulnerable.

The WHO, the Global Fund to Fight AIDS, Tuberculosis and Malaria and the Stop TB Partnership have appealed to world leaders to reinforce their commitment and increase their contributions so as to reach the objective of one million people diagnosed and treated against MDRTB between 2011 and 2015, implicating the necessity of developing new and effective antimycobacterial agents (Newton et al., 2000). The production of antimicrobial compounds is a phenomenon observed amongst an important number of, mainly Gram positive, bacteria (Sosunov et al., 2007). This corresponds to a defensive mechanism of these bacteria, through the production of antibiotics, organic acids, and lytic agents such as lysozymes. Furthermore, several types of protein exotoxins, and bacteriocins are produced (Mota et al., 2004). The purpose of this investigation is to search for new antimycobacterial substances from the Moroccan bacterial flora, that has not been extensively explored, that could improve control of mycobacterial infections bringing a solution to the problem caused by the emergence of strains resistant to the available antituberculous drugs.

MATERIALS AND METHODS

Bacterial strains

The tests were performed on non-pathogenic mycobacteria. These include the following:

**Mycobacterium aurum A$^+$*

A rapid growing scotochromogenic species, with a generation time of 6 h having a similar spectrum of drug susceptibility to *M. tuberculosis* (Chung et al., 1995). Accordingly, this strain has been proposed as a surrogate for antimycobacterial drug discovery (Chung et al., 1995).

**Mycobacterium smegmatis MC^2 155*

A thermophilic, nonpigmented species (cultures may turn yellow with time). This is a rapidly growing species having a generation time of 3 h. This species has been used as a model in antituberculosis drug discovery (Mitscher et al., 1998).

Isolation of a bacterial strain secreting a metabolite with antimycobacterial effect

Several samples (water, soil and water) were taken from different Moroccan ecological niches. The samples were kept under agitation for 2 h. The supernatant was recovered after sedimentation of the heavier particles. Serial dilutions (of 10^{-1} to 10^{-5}) were carried out in sterile Luria Bertoni (LB) broth (peptone: 10 g/l; yeast extract: 5 g/l; sodium chloride: 10 g/l). An aliquot of 70 µl of each dilution was spread on LB-agar previously inoculated with *M. aurum A*$^+$ or *M. smegmatis* cultures having an optical density at 600 nm (OD_{600nm}) of 0.3. After incubation at 37°C for 48 h, one colony surrounded by an inhibition zone was isolated.

To confirm that the antimycobacterial effect of this isolate was due to secreted substances, their cultures were filtered and the filtrate was tested on the mycobacteria using the well or diffusion method according to the following protocol: The bacterial strain was cultured in 50 ml of LB broth, for 48 h at 37°C with agitation; then centrifuged at 6000 rpm for 5 min to remove bacteria in suspension. The supernatant was recovered and filtered using 0.45 µm porosity filters. The filtrate obtained was tested by depositing 100 µl in wells cut out from solid media in plates previously inoculated with 100 µl of mycobacterial culture (*M. aurum* or *M. smegmatis*) at OD_{600nm} = 0.3. The plates were incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone was reported. The inhibition tests were repeated three times for each strain.

The control used was a culture filtrate from *Escherichia coli* prepared under the same conditions. 100 µl of this filtrate was deposited in the wells prepared in plates previously inoculated with mycobacterial culture (*M. aurum* or *M. smegmatis*) at OD_{600nm} = 0.3. The plates were incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone was reported.

Activity spectrum of the isolate

In order to get an idea on the spectrum of activity, the antimicrobial effect of the isolates under study was evaluated against the following bacteria: *Mycobacterium aurum A*$^+$, *Mycobacterium smegmatis MC^2 155*, *Escherichia coli DH5α*, *Bacillus subtilis* (EL Guendouzi et al., 2011), *Staphylococcus haemolyticus* (Hassi et al., 2007). The test was repeated three times for each strain. A few colonies from young cultures of the isolate were deposited at the center of a plate previously inoculated with 100 µl of microbial cul-

ture (for *E. coli, B. subtilis* and *S. haemolyticus* the $D.O_{600nm}$ was at 0.5, for *M. aurum* and *M. smegmatis* the $D.O_{600nm}$ was at 0.3). The plates were incubated at 37°C for 48 h. The presence or absence of an inhibition zone was then reported.

Identification of the isolate

The identification of the isolate was based on rRNA gene sequencing. This is the molecular approach more commonly used in bacterial phylogeny (Woese et al., 1990). It has permitted the constitution of important databases (Maidack et al., 1996; Van de Peer et al., 1999), and its use has led to the identification of several microorganisms from the environment including when culture was not successful (Relman et al., 1992; Strous et al., 1999). A 1 ml aliquot from a young LB broth culture of the isolate (24 hours) was transferred to a 1.5 ml microtube. The tube was centrifuged at 6000 rpm for 5 min. The pellet was resuspended in 100 µl of sterile distilled water, frozen at -20°C for 30 min, then heated at 95°C for 3 min. This thermolysis procedure was repeated twice in order to burst a large number of cells. After centifugation at 7000 rpm for 10 min, 2 µl of the supernatant (containing the DNA) were used in the amplification reaction (PCR) (Rodrigues Cavalcanti et al., 2007). For, amplification, universal primers, fD1 (5'AGAGTTTGATCCTGGCTCAG3') and Rs16 (5' TACGGCTACCTTGTTACGACTT 3'), used in the identification of bacteria by sequencing of 16S rDNA were selected (Weisberg et al., 1991). The reaction mix was prepared in a final reaction volume of 20 µl and contained: 4 µl of Taq buffer (5 x), 1,2 µl of $MgCl_2$ (25 mM), 4 µl of dNTPs (1 mM), 2 µl of fD1 (10 µM), 2 µl of Rs16 (10 µM), 0.2 µl of Taq polymerase (5 U/µl), 4,6 µl of pure H_2O and 2 µl of the DNA. The amplification conditions consisted of an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min 30 s; final extension was performed at 72°C for 10 min. The amplified fragment of approximately 1.5 Kb was visualized by electrophoresis using a 1% agarose gel. Two amplification controls were used, a negative control (T-) corresponds to a no template control, and a positive control (T+) corresponding to the 16S rDNA gene of *B. subtilis.*

Sequencing of the PCR products was carried out as previously described (EL Guendouzi et al., 2011). Sequences were compared to those from reference strains using the National Center for Biotechnology Information (NCBI) database and the Basic Alignment Search Tool (BLAST) N program. Similarity was determined according to sequence homology as previously described (EL Guendouzi et al., 2011; Hassi et al., 2012). Other than sequencing of the 16S rRNA gene, the molecular identification of the isolates under study was complemented using conventional preliminary tests: macroscopic observation, Gram stain and growth at 50°C (Meyer et al., 2004).

Precipitation of the active substance of the isolate by ammonium sulfate

Ammonium sulfate is frequently used to precipitate water soluble proteins. It is one of the most commonly used methods for the concentration of the active protein substances secreted by microorganisms in liquid culture (EL Guendouzi et al., 2011; Hassi et al., 2012). The precipitation protocol used was described for the purification and characterization of antimicrobial peptides (Subpeptin JM_4-A et JM_4-B), produced by *Bacillus subtilis* JM_4 (Shimei et al., 2005). Briefly, 100 ml of LB broth was inoculated with the selected strain and incubated at 37°C under agitation for 48 h. After incubation, it was centrifuged at 6000 rpm, at 4°C, for 5 min. The supernatant was recovered and 52.3 g of ammonium sulfate were progressively added under agitation. The mixture was left overnight under agitation at 4°C. The precipitate was centrifuged at 10000 rpm, at 10°C, for 20 min. The pellet was re-suspended in 200 µl of potassium phosphate buffer (KH_2PO_4, 50 mM, pH = 6). The suspension obtained was dialyzed against the same buffer at 4°C for 12 h. The dialysate was filtered using a 0.45 µm de porosity filter. The effect of the dialysate was then tested (3 x) against *M. aurum* and *M. smegmatis* using the well method.

The controls used in this experiment were the LB broth (with no inoculums) and the LB broth inoculated with *E. coli*. These controls were precipitated by ammonium sulfate, using same protocol for the precipitation of the active substance of the strain under study. The effect of the dialysate of these two controls was tested (three times) against *M. aurum* and *M. smegmatis* using the well method. The inhibition test was repeated three times.

Sensitivity of the isolate's protein precipitate to proteinase K

A 100 µl volume of the filtered dialysate was added to 40 µl of a proteinase K solution (1 mg/ml). The mixture was homogenized and incubated at 37°C for 3 h. The effect of the proteinase K treated dialysate, was tested (three times) against *M. aurum* and *M. smegmatis* using the well method (Hassi et al., 2007; EL Guendouzi et al., 2011). The control was a solution of proteinase K at the same concentration. The test was repeated three times.

Effect of the bioactive metabolite from the study isolate on the mycobacterial cell wall

In order to determine if contact with the bioactive metabolite secreted by the isolate resulted in lysis of the mycobacterial cell wall, its effect on the extraction of genomic DNA of *M. smegmatis* was studied as follows: A 3 ml volume of an overnight culture of *M. smegmatis* was centrifuged at 5000 rpm for 5 min. The bacterial pellet was resuspended in 400 µl of the bioactive metabolite, prepared using ammonium sulfate as described above, and recovered after the precipitation step in sterile distilled water instead of potassium phosphate buffer. The bacterial suspension was incubated at 37°C for 3 h and centrifuged at 5000 rpm for 5 min. The supernatant was then treated twice with phenol-chloroform. The DNA was precipitated using ethanol absolute, washed with 70% ethanol then dried, and finally dissolved in 20 µl of Tris-EDTA (TE) buffer (pH = 8) for electrophoretic analysis using agarose gel (1%). This experiment was repeated three times. Two controls were used; the first corresponded to *M. smegmatis* treated with the protein precipitate of the non-inoculated LB broth, and the second to the use of classical mycobacterial DNA extraction procedure (Houssaini-Iraqui et al., 1991).

RESULTS

Isolation of a bacterial strain secreting a metabolite with antimycobacterial effect

One bacterial strain was isolated from the samples analyzed. It exerted a growth inhibitory effect against the indicator mycobacterial strains (Figure 1).

The filtrate from the isolate exerted growth inhibition against the indicator mycobacterial strains as seen by the formation of inhibition zones (Table 1). The *E. coli* filtrate, used as control, did not present any antimycobacterial activity. The inhibitory effect of the active substance from each strain was relatively more important against *M. aurum* than *M. smegmatis*.

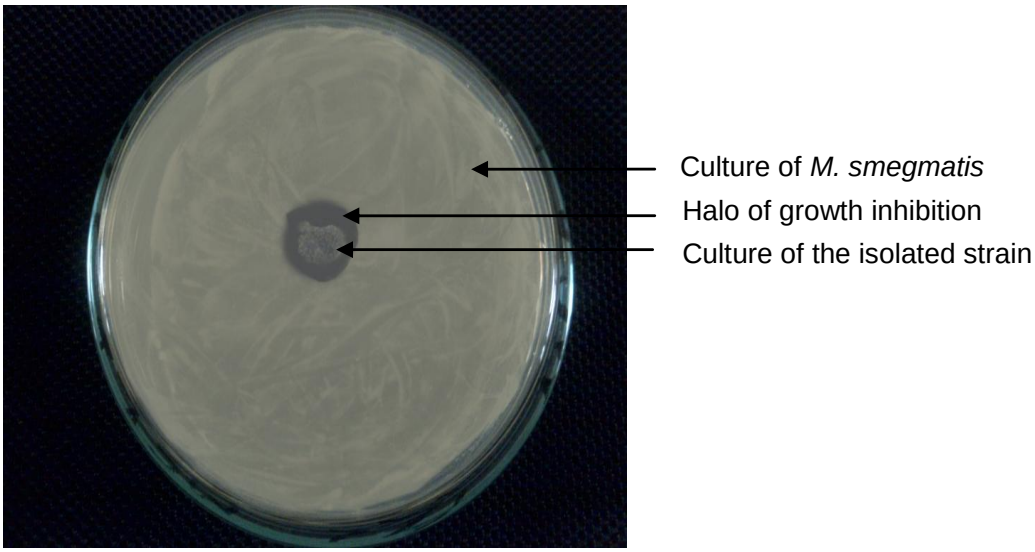

Figure 1. Antimycobacterial activity of the study isolate.

Table 1. Antimycobacterial effect of the filtrate from the study isolate.

Filtrate	Diameter of the inhibition zone (mm)	
	Effect against *MC²*	Effect against *A⁺*
Filtrate from the study isolate	23 ± 0,7	26 ± 0,5
Filtrate from *E. coli*	0	0

MC², *M. smegmatis* ; *A⁺*, *M. aurum*.

Table 2. Activity spectrum of the study isolate.

Bacterial species tested				
M. aurum **(mycobacteria)**	***M. smegmatis*** **(mycobacteria)**	***E. coli*** **(Gram⁻)**	***B. subtilis*** **(Gram⁺)**	***S. haemolyticus*** **(Gram⁺)**
+	+	+	+	+

+, Presence of an inhibition zone.

Activity spectrum of the isolate

Results of the activity spectrum of the isolate are shown in Table 2. These show that the isolate exerts an anti-microbial effect for all the bacterial strains tested.

Identification of the study isolate

In order to classify a microorganism within a genus or as a species, a number of criteria must be respected. The molecular definition of genus states that 16S rRNA sequence similarity should be superior or equal to 97%. Similarity superior to 99% indicates identical species; on the other hand, no identification results if the similarity is inferior to 97%. (Drancourt et al., 2000). Results of the sequences obtained using primers Rs16 and fD1, 762 bp and 551 bp respectively, were compared to those of the data base. According to the identification criteria described, results from the sequence analysis, indicated that the isolate corresponded to a strain of *Staphylococcus warneri* (Table 3). This result was also confirmed by the fact that the isolate was Gram+ and incapable of growth at 50°C.

Determination of the protein nature of the active substance of isolate *S. warneri*

Sensitivity of the active substance from S. warneri to proteinase K

The results obtained concerning the mycobacterial growth inhibitory effect of the protein extract from the study isolate are shown in Table 4. The protein extract from the study isolate exerted a growth inhibitory effect against mycobacteria showing an inhibition zone around the wells. This extract was inactivated upon treatment

Table 3. Identification of the study isolate.

Bacterial species showing a high degree of sequence similarity with the isolates under study	% of similarity obtained using primer Rs16	% of similarity obtained using primer fD1
Staphylococcus warneri (strain G72) (HQ407248)[a]	100%	100%
Staphylococcus warneri (strain LNP2) (GQ181035)[a]	100%	100%
Staphylococcus warneri (strain M-S-TSA 96) (JQ795875)[a]	100%	100%

[a], access number

Table 4. Sensitivity of the active substance from *S. warneri* to proteinase K.

Protein precipitate	Diameter of the inhibition zone (mm)			
	Precipitate not subject to Proteinase K treatment		Precipitate treated by Proteinase K	
	Effect against *MC²*	Effect against *A⁺*	Effect against *MC²*	Effect against *A⁺*
S. warneri	25 ± 1,4	28 ± 1,2	0	0
E. coli	0	0	-	-
LB broth	0	0	-	-

MC², *M. smegmatis*; *A⁺*, *M. aurum*; -, test not performed because the corresponding extract showed no biological effect.

with proteinase K (Table 4). A proteinase K solution, used as control, showed no antimycobacterial activity. No inhibition zones around the wells were observed for the study controls (LB broth and LB broth inoculated with *E. coli*).

Effect of the bioactive metabolite on the mycobacterial cell wall

The bioactive metabolite secreted by *S. warneri*, was tested for its capacity to lyse the cell wall of *M. smegmatis*. Results are shown in Figure 2. The protein extract from *S. warneri* allowed the extraction of DNA from *M. smegmatis* as efficiently as with the classic method. The control (non-inoculated LB broth culture protein precipitate) did not allow the extraction of *M. smegmatis* DNA.

DISCUSSION

The sample analyzed allowed the isolation of a bacterial strain of which the culture filtrate was capable of inhibiting mycobacterial growth due to the diffusion of an active substance produced on LB-agar. The inhibitory effect of the active substance of the study strain was relatively more accentuated against *M. aurum* than *M. smegmatis*. This difference could be due to the fact *M. aurum* has a longer generation time than *M. smegmatis*, which would allow for a more consequent diffusion of the inhibitory substance. Since the filtrate of this isolate was active against *M. aurum*, it is very probable that it would also be active against *M. tuberculosis* due to the fact that these two mycobacterial species have the same antibiotic susceptibility spectrum (Chung et al., 1995).

Results from the study of the activity spectrum of the isolate indicated that it exerted an antimicrobial effect against Gram positive bacteria (*B. subtilis*, *S. haemolyticus*), Gram negative bacteria (*E. coli*) and mycobacteria (*M. smegmatis*, *M. aurum*). Molecular analysis identified the isolate as a strain of *S. warneri*. This study shows for the first time that this species secretes an active substance against mycobacterial growth. Other studies have shown that the genus *Staphylococcus* includes several species (*S. haemolyticus, S. aureus*) known for their production of antibacterial substances (Frenette et al., 1984; Beaudet et al., 1982; Hassi et al., 2007). Verdon et al. (2008) isolated a strain of *S. warneri* that inhibited the growth of *Bacillus megaterium* and *Legionella pneumophila*.

This study reports for the first time a strain of *S. warneri* capable of inhibiting the proliferation of mycobacteria. The *S. warneri* extract was proteic as the ammonium sulfate preparation used is known to precipitate proteins (Shimei et al., 2005). The protein extract showed a growth inhibitory effect against mycobacteria; this activity is lost in the presence of proteinase K, which corroborates the protein nature of the active substance from *S. warneri*. Previous investigations have shown that *S. aureus* secretes a protein (Aureocin A53) that exerts a bactericidal effect against lactic bacteria, *Listeria monocytogenes* (Oliveira et al., 1998a), and several other strains distant from *S. aureus* responsible for bovine mastitis (Oliveira et al., 1998b). Hassi et al. (2007) also showed that *S. haemolyticus* produced a protein that inhibited mycobacterial growth. The protein extract from *S. warneri* was capable of extracting mycobacterial DNA

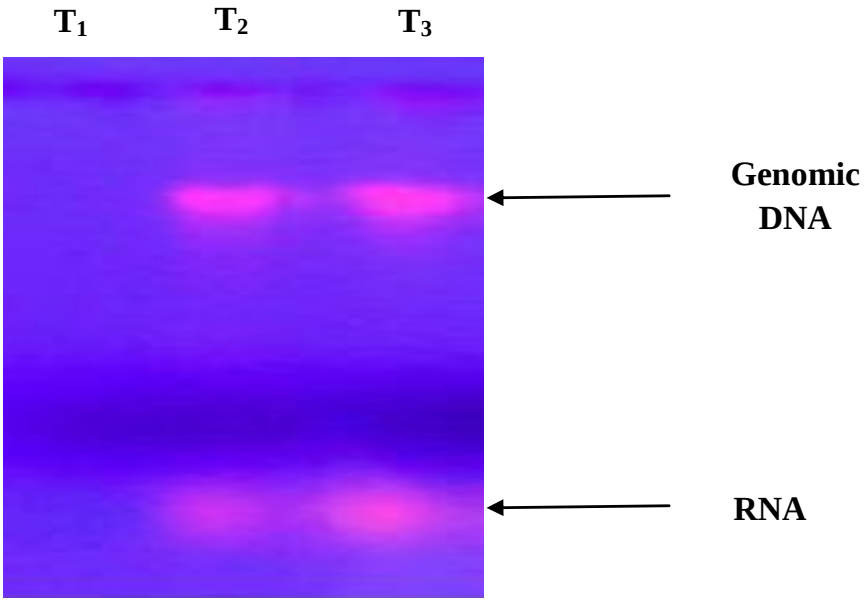

Figure 2. Extraction of genomic DNA from *M. smegmatis* by the bioactive metabolyte from *S. warneri*. T_1, Extract using the protein precipitate from non-inoculated LB broth; T_2, extract from *M. smegmatis* prepared using a conventional DNA extraction method (Houssaini-Iraqui et al., 1991); T_3, extract from *M. smegmatis* prepared using the *S. warneri* extract in replacement of the lysis agent in the conventional method.

and RNA, in the absence of conventional cell lysing agents (lysozyme, SDS, proteinase K). This shows that the active substance acts upon the cell wall of *M. smegmatis*. This result suggests that the growth inhibition of mycobacteria by this strain may be due to cell wall lysis.

Conclusion

This investigation focused on a bacterial strain isolated from a Moroccan biotope identified as *S. warneri*. This isolate secreted an active substance of protein nature that inhibited the growth of mycobacteria as well as Gram positive and negative bacteria. This active substance was capable of extracting DNA and RNA from *M. smegmatis* and therefore, may be useful in the extraction of the mycobacterial genome in molecular engineering. Since mycobacteria infect macrophages, the effect of the active substance may be tested *ex vivo*. Finally, this effect will also be tested against pathogenic bacteria, namely those responsible for nosocomial infections.

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Full Length Research Paper

Effect of partially purified fumonisins on cellular immune response in experimental murine paracoccidioidomycosis

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Fumonisins are mycotoxins produced mainly by *Fusarium verticillioides*, which can modulate the immune response. Paracoccidioidomycosis (PCM), caused by the fungus *Paracoccodioides brasiliensis* (Pb), is one of the most important systemic mycoses in Latin America. The aim of this study was to evaluate the effect of the partially purified fumonisins on cellular immune response in mice infected with Pb. Four groups of male BALB/c mice were used. Groups PB and PB/FB were inoculated i.v. with 1×10^5 Pb yeast cells and, after 28 days, groups FB and PB/FB were inoculated (s.c.) with partially purified fumonisin B_1 from *F. verticillioides* (5 × 2.25 mg FB1/kg body weight). After 7 days, cellular immune response was evaluated by delayed-type hypersensitivity (DTH) and lymphoproliferative assays (LA) using spleen cells. Nitric oxide (NO) production by spleen cells was also evaluated. The specific LA response to Pb antigen was higher in group PB than in FB and CTR groups ($p < 0.05$) but not significant with PB/FB. The DTH response was higher in infected than non infected groups ($p<0.05$) but also no significantly with PB and PB/FB groups. The lyphoproliferative response to ConA was decreased in FB or PB/FB in relation to CTR ($p<0.05$) but not with PB/FB and also a reduction of NO levels was observed in fumonisin treated in relation to control group FB1/kg ($p<0.05$). In conclusion, fumonisin B_1 or other components of *F. verticillioides* extracts significantly suppress the unspecific cellular immune response and the NO production by splenocytes from *P. brasiliensis* infected or not infected BALB/c mice.

Key words: Fumonisin, *Paracoccodioides brasiliensis*, lymphoproliferative assay, nitric oxide.

INTRODUCTION

Fumonisins are a group of mycotoxins produced by the plant pathogen *Fusarium verticillioides* and found predominantly in maize (Shephard et al., 1996). The extent of contamination of raw corn with fumonisins varies with geographic location, agricultural and storage practices and the vulnerability of the plants to fungal invasion

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during all phases of growth, storage and processing (Shelby et al., 1994). Brazil produces and consumes very large quantities of maize in a variety of forms, and Ono et al. (2000, 2001) reported fumonisin contamination in 98% of analyzed samples. Paracoccidioidomycosis (PCM) is a granulomatous disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis* (Pb). The mycosis is endemic in Latin America and the majority of cases are reported in Brazil, where it is the 8th commonest cause of mortality among infectious diseases (Coutinho et al., 2002). PCM affects preferentially male farm workers (Coutinho et al., 2002; Franco et al.,1989). Airborne fungal propagules, consisting of conidia or hyphal fragments, begin the infection and undergo con-version to the yeast phase, the infective stage of PCM, in the lungs, progressing to haematogenic or lymphatic dissemination to the liver, spleen, skin and mucosa (McEwen et al., 1987). The disease may be developed in multiple forms, ranging from benign and localized (unifocal) to severe and disseminated (multifocal), depending on the host immune response (Marques et al., 2002). Fumonisin B_1 (FB_1) and their analogs are secondary toxic metabolic produced, mainly by *F. verticillioides* and *F. proliferatum* in maize (Leslie, 1996). The ingestion of FB_1 is related to many pathologies in humans as well as mice, rats, equines and swines (Sydenham et al., 1990; Ueno et al., 1997; Howard et al., 2001; Gelderblom et al., 1991; Marasas et al., 1988; Colvin and Harrison, 1992; Vizcarra-Olvera et al., 2012, Khan et al., 2012). The FB_1 can modulate the cellular or humoral immune response, affecting the antigen presenting cells, B and T lymphocytes and macrophages (Liu et al., 2002; Taranu et al., 2006; Martinova and Merrill, 1995; Devriendt, et al., 2009, Grenier et al., 2011, Bracarense et al., 2012, Burel et al., 2013). Fumonisin B_1 (FB_1) and their analogs are secon-dary toxic metabolic produced, mainly by *F. verticillioides* and *F. proliferatum* in maize (Leslie, 1996). The ingestion of FB_1 is related to much pathology in humans as well as mice, rats, equines and swines (Sydenham et al., 1990; Ueno et al., 1997; Howard et al., 2001; Gelderblom et al., 1991; Marasas et al., 1988; Colvin and Harrison, 1992). The FB_1 can modulate the cellular or humoral immune response, affecting the B and T lymphocytes activities and can also disrupt effector function of macrophages (Liu et al., 2002; Taranu et al., 2006; Martinova and Merrill, 1995). Due to chronicicity of PCM and its preva-lence in Brazil, there is a possibility of the concomitant contamination of fumonisin and Pb, mainly, maize is used as a basic feeding for the local poor community who are involved greatly in agriculture and the illness also affects the agricultural and poor population. In a previus experi-mental study, it was observed significantly increased the specific antibody response in male Swiss mice infected with Pb by FB_1 or other components of *F. verticillioides* extracts (Itano et al., 2008).

Considering the cellular immune response as the main defense mechanism against Pb (Silva et al., 1995). The aim of this study was to evaluate the effect of the partially purified fumonisins on cellular immune response in mice infected with Pb-infected BALB/c mice.

MATERIALS AND METHODS

Fumonisin production and determination

F. verticillioides 103F (isolated from feed involved in animal intoxication in 1991 at Paraná State, Brazil) was grown on potato dextrose agar (PDA) slants at 25°C for 7 days. An aliquot of conidia suspension (10^6 conidia mL^{-1}) was inoculated into three 500 ml Erlenmeyer flasks containing 100 g ground maize adjusted to 42% moisture content and autoclaved twice for 30 min. The culture was incubated at 25°C for 14 days, extracted twice by shaking with ethyl acetate (1 L/kg) and filtered. Next, the residue was extracted once with 1 L/kg methanol: water (3:1, v/v) and twice with 1.5 L/kg of the same solvent. The dried extract was dissolved in 200 ml methanol: water (1:3, v/v) at 50°C and partitioned three times with 100 ml chloroform. The aqueous phase was dried at 50°C under vacuum (Cawood et al., 1991). This residue was dissolved in 20 ml methanol: water (1:3, v/v), and partially purified by passing successively through columns of Amberlite XAD-2 (100 g, 2.0 × 60 cm, Supelco, 10357-EA), silica gel (120 g, 2.0 × 60 cm, Sigma, 70 to 230 mesh, S2509), silica gel (120 g, 2.0 × 60 cm, Sigma, 70 to 230 mesh, S2509) and reversed-phase C18 (50 g, 3.0 × 30 cm, Acros, S2425) columns, using methanol, chloroform: methanol: acetic acid (6:3:1, v/v/v), chloroform: methanol: water: acetic acid (55:36:8:1, v/v/v/v) and a linear gradient from methanol: water (1:1, v/v) to methanol: water (4:1, v/v) as the mobile phases, respectively.

Fumonisins were determined by high-performance liquid chromatography (HPLC) (Shephard et al., 1990) with some modifications (Ueno et al., 1993). An aliquot of partially purified extract (6 ml) was filtered through a 0.45-μm syringe filter (Millipore, Windham, US) and suitably diluted with acetonitrile: water (1:1, v/v). After derivatization with 200-μL of *o*-phthaldialdehyde (OPA) reagent, the sample was injected into the HPLC system within 1 min. Fumonisins were analyzed by a reversed-phase, isocratic system (Shimadzu LC-10 AD pump and RF-10A XL fluorescence detector), using a Shim-pack CLC-ODS (M) column (4.6 × 250 mm, Shimadzu). Fluorescence excitation and emission wavelengths were 335 and 450 nm, respectively. The eluent was CH_3OH: 0.1 M NaH_2PO_4 (80:20, v/v), adjusted to pH 3.3 with H_3PO_4 solution, flowing at 1 ml/min. The detection limits for the fumonisins FB_1 and FB_2 were 27.5 and 35.3 ng/g, respectively. FB_1 and FB_2 levels in the partially purified extract were 152.1 and 53.34 mg/mL, respectively. The sample was diluted in sterile PBS to a concentration of 22.5 mg FB_1/ml, this partially purified fumonisin B_1 (PPF) was used for animal treating.

P. brasiliensis

P. brasiliensis strain 18 (Pb 18) was cultured on Sabouraud agar (Micromed, Rio de Janeiro, RJ, Brazil), and maintained at 35°C, subculturing at 5 day intervals. The yeast cells were collected in sterile saline, filtered through cotton and gauze layers, and the concentration was adjusted to 1×10^6 cells/ml.

Experimental design

Male BALB/c mice (n = 24), 4 to 5 weeks old (20 to 25 g), provided by the animal facilities of Londrina State University (UEL), were kept in a 12:12 h light-dark cycle at 25°C, with free access to sterilized commercial mouse food (Nuvital, Curitiba, Brazil) and water. Four groups of 6 mice were used: infected (PB), treated (FB),

infected and treated (PB/FB) and uninfected and untreated as control (CTR). Groups PB and PB/FB were inoculated (i.v.) with 1 × 10^5 yeast cells (Pb 18) and, 28 days later, groups FB and PB/FB were inoculated (s.c.) with PPF from *F. verticillioides* (5 × 2.25 mg FB1/kg body weight/day), as described by Johnson and Sharma (2001). The control group was inoculated with sterile PBS. Plasma samples and spleens cells were taken 7 days after PPF inoculation. The procedures and experimental design were approved by the Animal Research Ethics Committee of the UEL.

Exoantigen (exoAg)

A lyophilized exoAg was prepared from a yeast-phase culture of Pb strain B-339 according to Camargo et al. (1989).

Lymphoproliferation assay

Spleen cells from infected and normal mice were removed aseptically and teased. The erythrocytes were lysed with tris-ammonium chloride, and the cell suspension was washed three times in RPMI medium. For lymphoproliferative assays, 100 µl of the cells were cultured in triplicate wells at a concentration of 1 × 10^6 cells/ml in RPMI 1640 containing L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA), 10% fetal calf serum (Gibco, USA) in 96-well flat-bottom culture plates. ExoAg or concanavalin A (Con) (Sigma-Aldrich, St. Louis, Missouri, USA) was then added to each well at concentrations of 50 and 10 µg/ml, respectively and as control with RPMI medium. The cells were cultured for 3 (ConA) or 5 (exoAg) days at 37°C with 5% CO_2 and were pulsed with 1 µCi of (^{3}H) thymidine 18 h before harvesting on glass filter strips. The radioactivity was determined by a liquid scintillation counter (Beckman LS 6.800). The 'stimulation index' was calculated as the triplicate of stimulated cells (cells + ExoAg or ConA) divided by the cell control (cell + RPMI).

Delayed-type hypersensitivity assay (DTH)

The DTH reactions were performed in all groups of animals. Mice were inoculated with exoAg (10 µg) and sterile PBS in right and left footpad respectively. The footpad thickness was measured at 24 h after inoculation with caliper (Mitotoyo, Tokyo, Japan). The increase in thickness was calculated, as in the formula ($A = D^2/4$, = 3.14).

Measurement of nitric oxide (NO)

The concentration of NO in spleens cells was measured by a microplate Griess assay. Spleen cells (100 µL) prepared as described earlier, were cultured with lipopolysaccharides (LPS), (7 µg) during 24 h at 37°C. The supernatants were collected and 50 µL of these samples were transferred to wells of 96-well flat bottom microtiter plate and incubated with an equal volume of the Griess reagent for 10 min at room temperature. The absorbance at 550 nm was determined with a Titertek Multiskan apparatus (Multiskan EX, Uniscience – Labsystems, Helsinki, Finland). The NO concentration was determined using a standard curve determined with different concentrations of $NaNO_2$.

Statistical analysis

The data were analysed by using a Tukey-Kramer ANOVA test for comparisons and declaring significance at $p<0.05$.

RESULTS

Proliferation response of spleens cells to exoAg and to ConA

During the present investigation, higher proliferation response to exoAg in infected groups (PB: 2.04 ± 0.71; PB/FB: 1.55 ± 0.26) in relation to not infected (FB: 0.87 ± 0.15; CTR; 1.10 ± 0.23) was observed (Figure 1). The decreased proliferation response in PB/FB was not statistically significant in relation to PB group. CTR × PB ($p<0.01$), FB × PB ($p<0.001$) and FB × PB/FB ($p<0.05$). Figure 2 shows decreased proliferation response to ConA in FB treated groups, both in infected or not infected (FB: 0.12 ± 0.08 and PB/FB: 0.17 ± 0.24) in relation to not treated (PB: 1.52 ± 0.28 and CTR:1.95 ± 0.41). CTR × FB and PB/FB, $p < 0.001$.

DTH assay

Results regarding the 'delayed type hypersensitivity' in the infected groups showed higher inflammatory response than control not infected group (PB: 1.59 ± 0.19; PB/FB: 1.49 ± 0.09, FB: 0.98 ± 0.08; CTR: 1.10 ± 0.16;), $p < 0.001$, but these results are similar between PB and PB/FB, $p > 0.05$ (Figure 3).

NO concentration in culture supernatants of spleens cells stimulated with LPS

During the present study, 'nitrous oxide' mount was measured in the supernatants of spleens stimulated with LPS and it was detected decreased level in FB treated or infected with Pb or infected and treated with PPF in relation to control group ($p < 0.001$), but similar between FB treated or infected with Pb or infected and treated with PPF ($p > 0.05$) (Figure 4).

DISCUSSION

In PCM, the cellular immune response represents the main defense mechanism against Pb and in this study, it was observed an induction of specific cellular response in infected mice both by DTH and LA assays. However, it was not observed significant supression in simultaneous infection with Pb and contaminated with PPF from *F. verticillioides* in experimental PCM in male BALB/c mice. However, there were a significant supression of response to ConA in mice treated with PPF in relation to the normal control without treatment with PPF, suggesting that the FB_1 in our conditions of experiment does not affect the specific cellular immune response but can suppress in specific immune response. Our results are partially in accordance with the data of Theumer et al. (2002), which

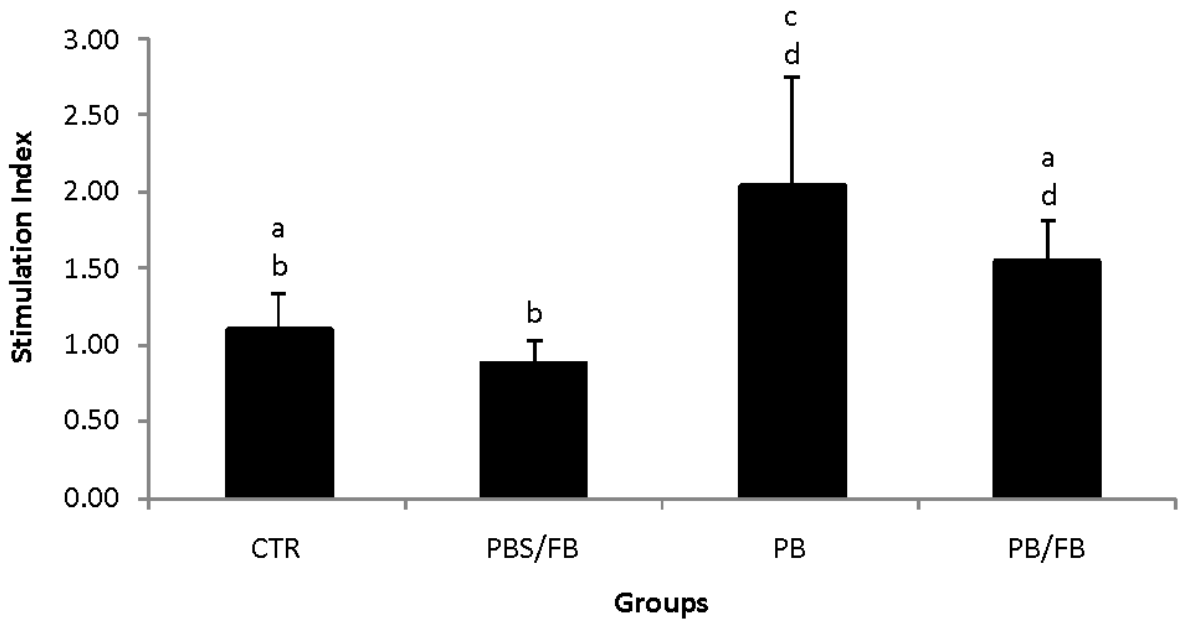

Figure 1. Proliferation response of spleens cells to exoAg. Spleen cells (1 × 105 cells/ml) were cultured with *P. brasiliensis* exoAg (50 µg/ml) for 5 days, pulsed with 1 µCi and the stimulation index calculated as the triplicate of stimulated cells divided by the control not stimulated cells. A higher proliferation response to exoAg was observed in infected groups in relation to not infected. PB/FB = BALB/c mice infected with *P. brasiliensis* (1 x 105 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB1/kg body weight/day); PB = only infected; FB = only treated with PPF and CTR = inoculated with sterile PBS. Different letters indicate significant differences between groups ($p<0.05$).

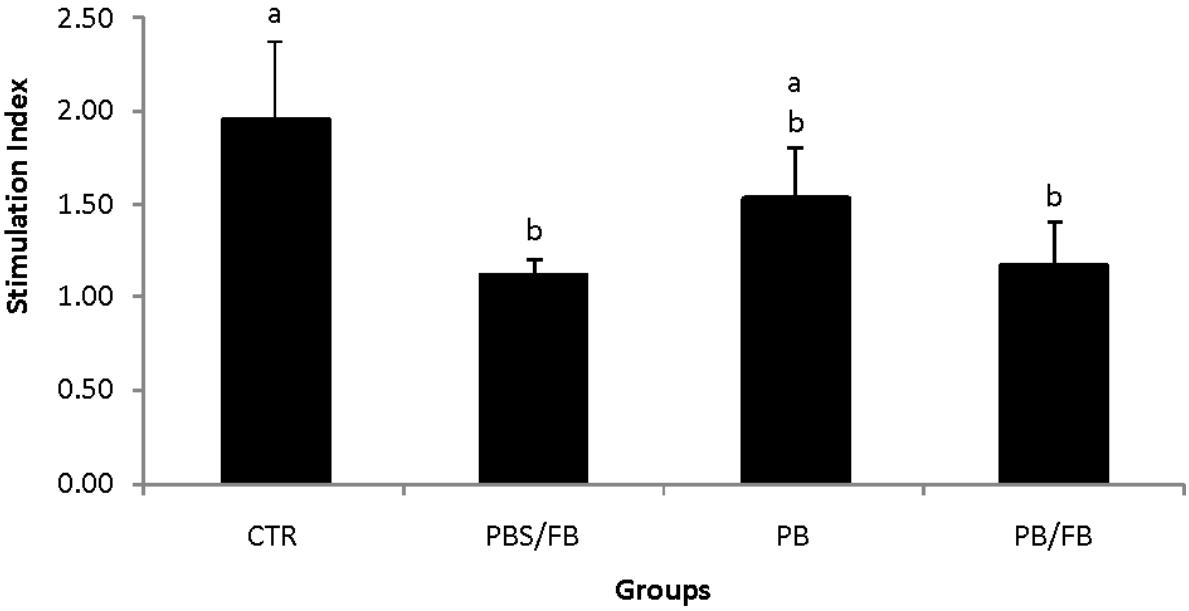

Figure 2. Stimulation of cellular proliferation with ConA. Spleen cells (1 × 10^5 cells) were cultured with ConA (10 µg/ml) for 72 h, pulsed with 1 µCi and the stimulation index (SI) calculated as the triplicate of stimulated cells divided by the control not stimulated cells. PB/FB = BALB/c mice infected with *P. brasiliensis* (1 x 10^5 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB_1/kg body weight/day); PB = only infected; FB = only treated with PPF and CTR = inoculated with sterile PBS. SI were more elevated in CTR and Pb groups than PBS/FB and PB/FB groups; however, significant results were found only between CTR and PBS/FB, and between PBS and Pb/FB. Different letters indicate significant differences between groups ($p<0.05$).

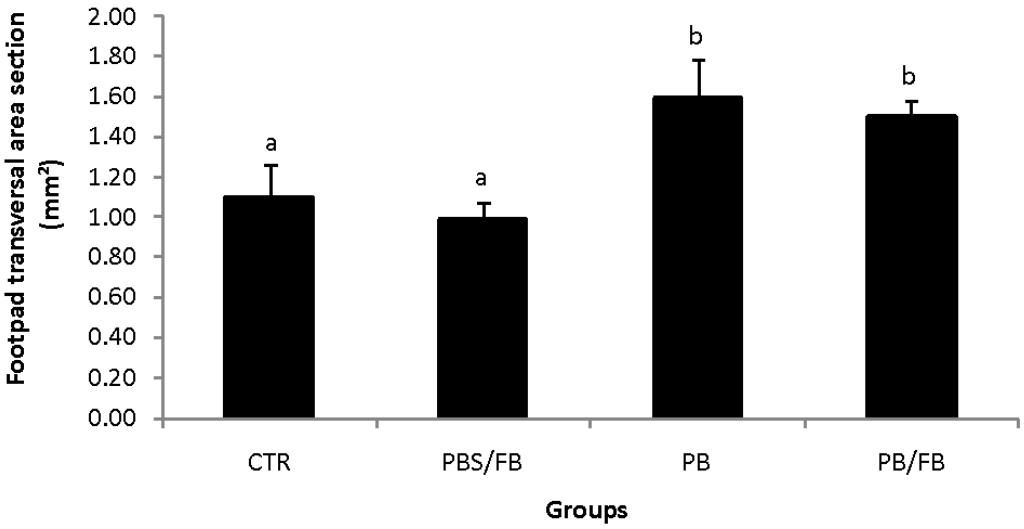

Figure 3. DTH assay. BALB/c mice were infected with *P. brasiliensis* (1 x 105 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB1/kg body weight/day) (PB/FB) or only infected (PB) or only treated with PPF (FB) or inoculated with sterile PBS (CTR). ExoAg from *P. brasiliensis* were injected intra-footpad 24 h before measurement of the footpad response. Different letters indicate significant differences between groups ($p<0.05$).

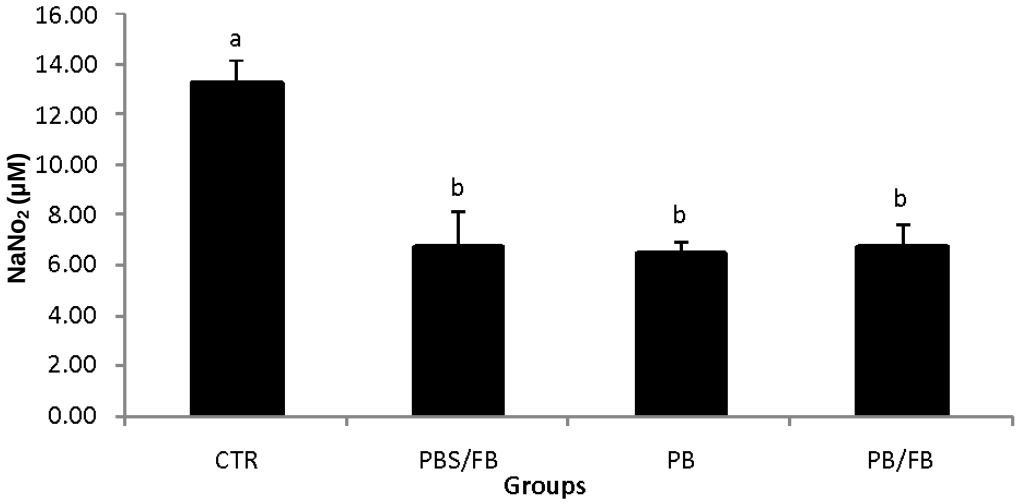

Figure 4. NO determination. Spleen cells were stimulated with LPS to determine the release of NO in the culture medium. PB/FB = BALB/c mice infected with *P. brasiliensis* (1 x 105 yeast cells, 28 days) and treated with PPF from *F. verticillioides* (5 x 2.25 mg FB1/kg body weight/day); PB = only infected; FB = only treated with PPF and CTR = inoculated with sterile PBS. Inhibition of NO was seen in groups infected and treated with PPF. Different letters indicate significant differences between groups ($p<0.05$).

they had observed no significant effect on mitogen-induced proliferation of spleen cells in mice treated with FB_1 and stimulated with phytohemaglutinin mitogen (PHA). The inflammatory reaction is a non-specific response and Gonzalez et al. (2000) reported that the NO produced by spleen macrophages has crucial role in *P. brasiliensis* defense. Our NO analysis demonstrated decreased levels when spleen macrophages from groups of infected or PPF treated or infected/treated mice stimulated with LPS in relation to control not treated and not infected group, suggesting that both PPF and Pb can modulate the NO production by spleen macrophages, but not with synergic

effect. Also, Popi et al. (2004) reported the decreased NO production by peritoneal macrophages incubated with gp43 and stimulated with zymozan.

The infection was confirmed by histologic analysis of lungs and liver, where no significant difference between the infected and infected/treated group was observed (data not shown), suggesting that in the conditions of the experiment the mycotoxin did not affect the gravity of the illness. All groups of animals had the same initial healthy appearance, which they maintained throughout the period of the study. This finding is in accordance with the results of evaluation of the specific cellular immune response, the main protective response against the Pb (Silva et al., 1995; Calich and Kashino, 1998; Kashino et al., 2000). In Bhandari et al. (2001) work, it was reported that FB_1 modulates the IFN-g, a Th1 marker cytokine in BALB/c mice according to the sex, with elevated expression (male) or down-modulation (female). The lack of change in specific cellular immune response in BALB/c mice (male) infected and treated with partially purified fumonisin could be due to the balance between FB_1 (Bhandari et al., 2001) and immunossupressive Pb antigens actions (Benard et al., 1997; Cacere et al., 2002 and Rigobello et al., 2013), that require further study.

The literature data has shown the low proliferation of spleenocytes to some Pb antigens (Benard et al., 1997), that induce apoptosis and sugested that it may be one of the mechanisms leading to hyporesponsiveness (Cacere et al., 2002). The relevance of this study stems from the fact that PCM is the 8th most common cause of death from predominantly chronic or recurring infectious and parasitic diseases in Brazil. It is believed that about 10 million people are infected with the fungus, and 2% of them may develop the disease (McEwen et al., 1995). Thus, any factor that leads to modulation of the immune response, such as the presence of mycotoxin, could contribute to the development of the disease, increase its severity or control the disease. For the best understanding about the action of the FB1 in the course of the experimental PCM, there are necessary additional studies using purified FB_1 in diverse concentrations/times of incubation/mouse isogenic lines by using both sex. We concluded for the results that FB or other components of *F. verticillioides* extracts does not affect specific cellular immune response in experimental PCM in male BALB/c mice, but can affect non-specific response, in the conditions of the work.

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Full Length Research Paper

Pharmacognostic and phytochemical evaluation of the *Solanun sisymbriifolium* leaf

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Solanum sisymbriifolium **Lam. (Solanaceae) is an important medicinal herb in Ayurvedic medicine. In the present investigation, the detailed pharmacognostic study of *S. sisymbriifolium* leaf was carried out to lay down the standards which could be useful in future experimental studies. The study included macroscopy, microscopy, preliminary phytochemical screening and physicochemical evaluation. These observations will help in the Pharmacognostical identification and standardization of the drug in the crude form and also to distinguish the drug from its adulteration.**

Key words: *Solanum sisymbriifolium*, pharmacognosy, microscopy.

INTRODUCTION

Herbal drugs play an important role in health care programs especially in developing countries. Such herbal drugs are promising choice over modern synthetic drugs, as they show minimum/ no side effects and are considered to be safe (Gokhale, 1979; Shankar and Ved, 2003). Generally, herbal formulations involve use of fresh or dried plant parts. Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers 'all' plant parts to be potential sources of medicinal substances (Mukherjee, 2002). However, a key obstacle, which has hindered the acceptance of the alternative medicines in the developed countries, is the lack of documentation and stringent quality control. There is a need for documentation of research work carried out on traditional medicines (Dahanukar et al., 2000). With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine. The process of standardization can be achieved by stepwise pharmacognostic and phytochemical studies (Ozarkar, 2005). These studies help in identification and authentication of the plant material.

Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy. Simple pharmacognostic techniques used in standardization of plant material include its morphological, anatomical and biochemical characteristics (Anonymous, 1998). *Solanum sisymbriifolium* Lam. (Solanaceae) is commonly known as sticky nightshade, the fire-and-ice plant, Litchi tomato or Morelle de Balbis. It is a perennial herb that has been used as a traditional medicine possessing diuretic and antihypertensive properties in Paraguay. In Argentina, the roots of the plant were traditionally used as diuretic, analgesic, contraceptive, antisyphilitic and hepatoprotective, while the aerial parts of the plant were used in treatment of diarrhea, infections of respiratory and urinary tracts. The flowers are used in India as analgesic and the leaves are used as febrifuge in Peru (Ibarrola et al., 1996; Ferro et al., 2005). The solasodine, the steroidal saponin isolated from the plant was found very potent for the treatment of neurological disorders (Chauhan et al., 2011). Literature details of morphology, phytoconstituents, medicinal properties and uses of *S. sisymbriifolium* are very scarce;

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therefore, in the present study, pharmacognostic and phytochemical standards of the leaves of *S. sisymbriifolium* WEre studied.

These standards are of utmost importance not only in finding out genuity, but also in the detection of adulterants in marketed drug (Johanson, 1940).

MATERIALS AND METHODS

Collection of the plant material

Fresh leaves of *S. sisymbriifolium* were collected from Saurashtra University Campus, Rajkot, Gujarat (India). The sample was authenticated for its botanical identity by Botanist Botanical Survey of India, Rajasthan and voucher specimen deposited in herbarium of the institute. Dried leaves were made into powder.

Morphological evaluation

The macroscopic characters such as size, shape, surface, margin, colour, odour, taste etc. were studied for morphological investigation (Kokoski et al., 1958; Brain, 1975; Khandelwal, 1998; Kokate et al., 1999; Anonymous, 2001).

Microscopic evaluation

For microscopical studies, free hand section of leaf were cut, cleared and stained with saffranine according to the prescribed method (Kokoski et al., 1958; Brain, 1975; Khandelwal, 1998; Kokate et al., 1999; Anonymous, 2001). The results were registered by botanical illustration and photos taken by means of the digital light microscope fitted with 1/3" CCD camera imaging accessory with Scoptek image 2000 image analysis software.

Histochemical colour reactions

The different histochemical colour reactions were performed on the leaf transverse sections to differentiate the different cell compositions and identification (Trease and Evans, 1986).

Physicochemical evaluation

The foreign matter, ash values, extractive values and foaming index were performed according to the officinal methods prescribed in Indian pharmacopeia and the WHO guidelines on quality control methods for medicinal plants materials (Anonymous, 1996).

Phytochemical screening

The preliminary phytochemical tests for dried leaves powder were also carried out according to the standard procedures described by Kokate (1986) and Horborne (1998). All the reagents used were of analytical grade obtained from Fine Chemicals Ltd., Mumbai, India.

Thin layer chromatography (TLC)

Ethanolic extract of *S. sisymbriifolium* leaves was prepared by using Soxhlet extraction method. The extract was filtered and concentrated on rotary evaporator. The concentrated extract was spotted on a normal phase plate previously activated at 110°C for 2 h, using a capillary tube. The plate was developed using mobile phase of toluene:ethylacetate (9:1). Further, the TLC was sprayed with 10% methanolic sulphuric acid and Dragendorff's reagent. The Retardation factor (R_f) was determined using this formula:

$$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

RESULTS

Macroscopic features

Phyllotaxy: Alternate.
Shape: Ovate-oblong, deeply pinnatisect or pinnatifid many prickles.
Size: Leaf blade – 10 to 15 cm in length and 6 to 10 cm in width.
Petioles: 1.5 to 5 cm, spiny.
Surface: sparsely stellate-hairy above.
Margin: Sinuate, lobes rounded.
Venation: Reticulate (Figures 1 and 2).

Microscopic features

Epidermis

In transection (Figure 3), the blade epidermis was single-layered (Figure 5A). The epidermis shows presence of anisocytic stomata (Figure 5B), sharp pointed unicellular type (Figure 5C) and multicellular collapsed type (Figure 5D) trichomes occur predominantly on leaf epidermal cell surface.

Mesophyll

The mesophyll was dorsiventral, consisting of one layer of palisade parenchyma (Figure 5A) and four strata of spongy parenchyma (Figure 5F). The midrib region (Figures 3 and 4), in transverse section, is biconvex. The epidermis is uniseriate and has multicellular trichomes similar to the blade. They are seldom unicellular too. Adjacent to the epidermis, angular collenchymas (Figure 3) occur, comprising approximately four to six rows on the dorsal side and six to eight on the ventral one. The calcium oxalate prisms are found in some of the spongy parenchymatous cells (Idioblasts).

Conducting tissue

Compactly arranged endarch and collateral type vascular bundles (Figure 5E) are found, which are embedded in the ground parenchyma region (Figure 5F).

Histochemical color reactions

The results of histochemical color reactions performed

Figure 1. Entire plant of *S. sisymbriifolium.*

Figure 2. Compound leaf of *S. sisymbriifolium.*

on transverse section and dry powder of leaf were given in Table 1.

Physicochemical evaluation

The moisture content, ash values likes (total ash, acid insoluble ash, water soluble ash), water soluble extractive, methanol soluble extractive and foaming index of stem powder were evaluated. The data obtained are shown in Table 2.

Qualitative phytochemical screening

The extracts and powder drug were subjected to preliminary phytochemical screening for the presence of type of phytoconstituets. The extracts and powder were found to contain alkaloids, terpenoids, saponins, sterols, carbohydrates and amino acids as shown in Table 3.

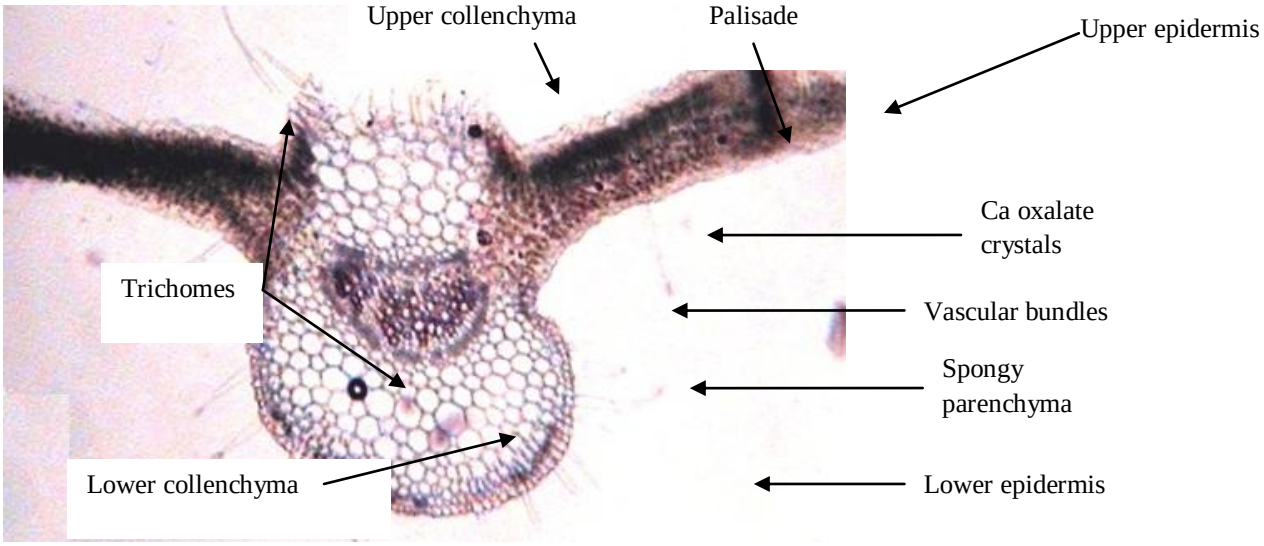

Figure 3. Transverse section of *S. sisymbriifolium* leaf (4X).

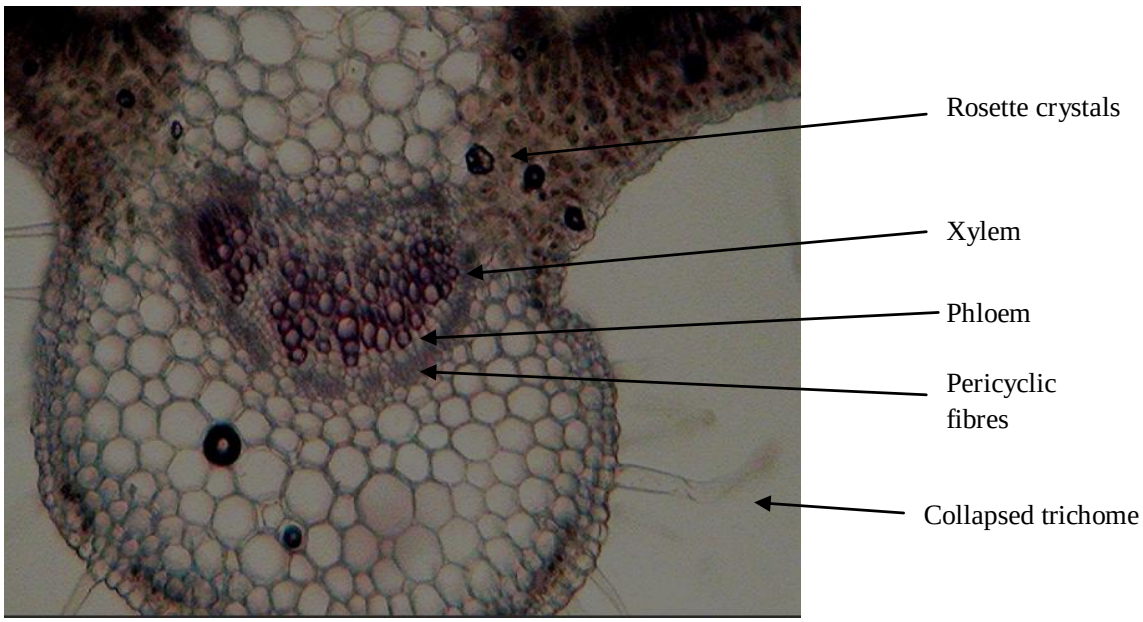

Figure 4. Transverse section of *S. sisymbriifolium* leaf showing leaf blade region (10X).

Thin layer chromatography profile of ethanolic extract of *S. siymbriifolium* leaves

Six different compounds were separated when sprayed with 10% methanolic H_2SO_4 (Figure 6A, Table 4); whereas, when sprayed with Dragendorff's reagent, two different spots were examined (Figure 6B, Table 5).

DISCUSSION

In the present study, the pharmacognostic and phytochemical standards for the leaves of *S. sisymbriifolium* were shown for the first time. Morphological and anatomical studies of the leaf will enable the identification of the crude drug. Various diagnostic characters were identified in the leaf like single layered palisade tissue; anisocytic type of stomata; uni- and multicellular collapsed trichomes; endarch and collateral type vascular bundles. These characters might be a very important tool to identify or to authenticate the drug for future reference. The information obtained from physicochemical evaluation will be useful in finding out the genuity of the drug. In that respect, various physicochemical parameters like moisture content, loss on drying, ash values, and extractive values

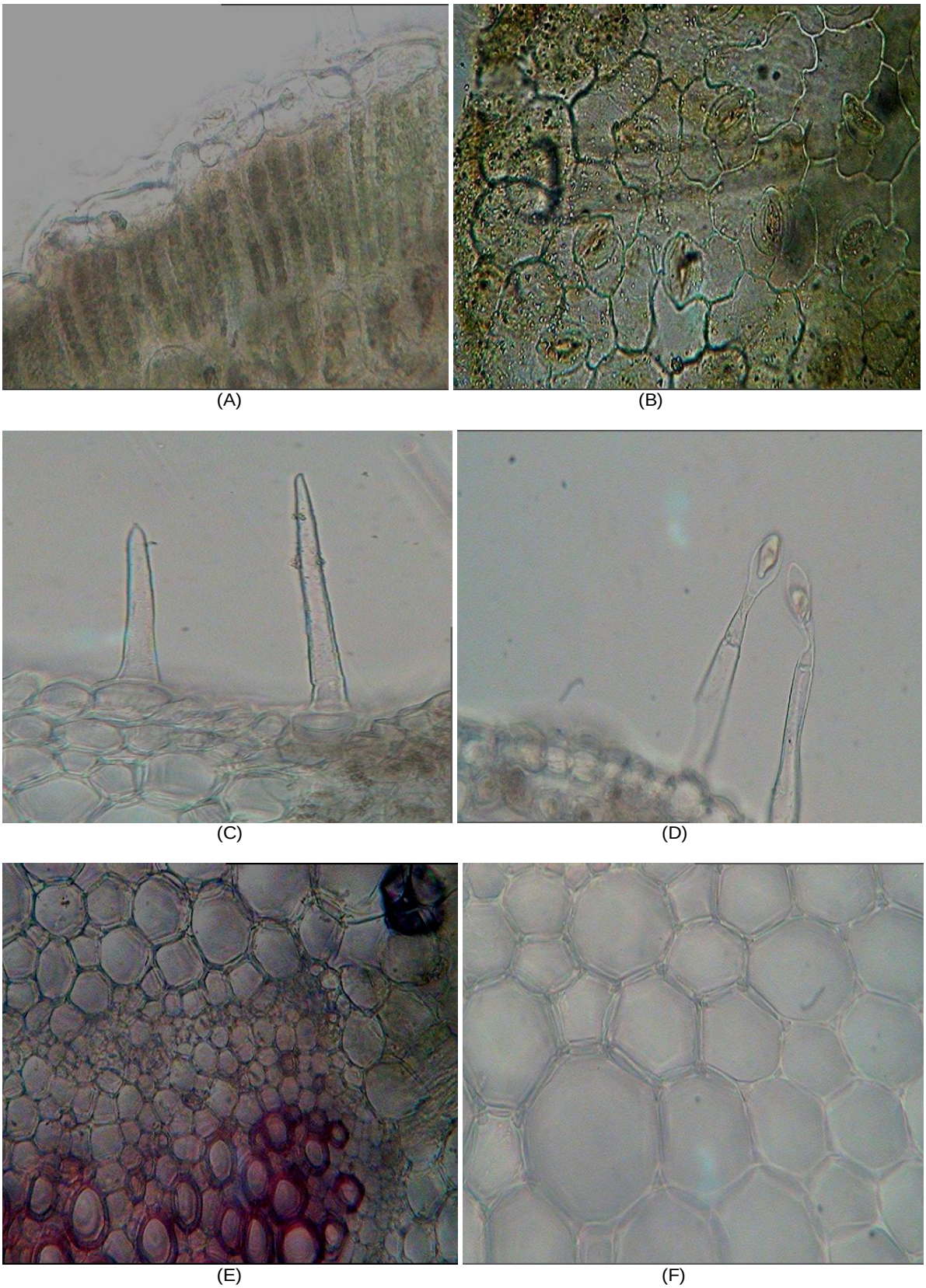

Figure 5. Magnified view of transection of *S*. *sisymbriifolium* leaf showing various characters (40X). (A). Palisade layer (40X), (B) anisocytic stomata in surface preparation (40X), (C) pointed unicellular trichomes (40X) , (D) collapsed glandular trichomes (40X), (E) vascular bundles, (F) spongy parenchyma.

Table 1. Histochemical color reactions of *S. sisymbriifolium* leaf powder.

Reagent	Constituent	Color	Histological zone	Degree of intensity
Phloroglucinol + HCl	Lignin	Pink	Xylem, sclerenchyma	+++
Aniline SO_4 + H_2SO4	Lignin	yellow	Xylem	++
Weak Iodine solution	Starch	-	-	-
H_2SO_4	Calcium oxalate	Needles	Mesophyll and midrib parenchyma	+
$SbCl_3$	Steroids/triterpenoids	Reddish pink	Mesophyll	++
Dragendroff's reagent	Alkaloids	Cream	Spongy paranchyma	+++

Table 2. Physicochemical parameters of *S. sisymbriifolium* leaf powder.

Parameter	Values
Ash values	
Total ash	6.00% w/w
Acid insoluble ash	1.00% w/w
Water soluble ash	0.5% w/w
Extractive values	
Ethanol soluble extractive	34 .00% w/w
Water soluble extractive	15.00% w/w
Moisture content or water content	
Loss on drying at 110°C	9.5% w/w
Foaming index	
Foaming index of entire powder	<100

Table 3. Qualitative phytochemical screening of successive extracts of *S. sisymbriifolium* leaves.

Test	P. E.	Benzene	Chloroform	E. A.	Methanol	Water	95% ethanol
Carbohydrates	–	–	+++	+++	+++	+++	+++
Alkaloids	-	-	++	++	++	++	++
Protein	–	–	+	+	+	+	+
Fats and oils	+	+	+	+	+	+	+
Terpenoid/steroid	–	++	++	++	++	++	++
Tannins	–	–	–	–	–	–	–
Glycosides	–	–	–	+	+	++	++
Flavonoids	–	–	–	–	–	–	–

P. E., Petroleum ether; E. A., ethyl acetate; + present; - absent.

were also determined; those can be used as reliable aid for detecting adulteration in the drugs. Also, for identification of allied species as well as adulterants, such parameters are useful. Plants show their biological activity through their secondary metabolites, those are actually the biologically active constituents of the plant. Therefore, the quality as well as quantity of such phytoconstituents must be evaluated during standardization of the plant drug.

In the present study, preliminary screening was performed

Table 4. R_f values for thin layer chromatography sprayed with methanolic sulphuric acid.

R_f	Colour
0.23	Brown
0.29	Yellowish pink
0.34	Pink
0.40	Light pink
0.71	Yellow
0.85	Reddish yellow

Table 5. R_f values for thin layer chromatography sprayed with Dragendorff's reagent.

R_f	Colour
0.58	Yellowish orange
0.85	Yellowish orange

by conducting qualitative chemical tests and the results of screening showed the presence of alkaloids, terpenoids (steroids) and glycosides, as the major class of phyto-constituents. TLC analysis was carried out with the leaf extract that showed the presence of seven components in the extract. These simple but reliable standards will be useful to a lay person in using the drug as a home remedy. Also, the manufacturers can utilize them for identification and selection of the raw material for drug production.

Conclusion

Establishing standards is an integral part of establishing the correct identity and quality of a crude drug. Before any drug can be included in the pharmacopoeia, these standards must be established. The majority of the information on the identity, purity and quality of the plant material can be obtained from its macroscopy, microscopy, physiochemical and phytochemical parameters. As there is no record on pharmacognostical and phytochemical work on *S. sisymbriifolium* leaf, the present work is undertaken to produce some pharmacognostical and phytochemical standards.

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African Journal of Biotechnology

Full Length Research Paper

Genotypic profiling of coding region of leptin gene and their association studies on reproductive and milk production traits in Sahiwal and Frieswal cattle of India

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Leptin gene has its role in appetite, metabolism, growth and milk production in cattle. Single nucleotide polymorphisms (SNPs) in leptin gene in different cattle breeds have been reported and subsequently associated with their production performance. The objective of this study was to evaluate the association of genetic differences in the bovine leptin gene with milk production, reproduction, milk constituents in Sahiwal and Frieswal cattle of India. In total, one hundred and seventy six cows were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to screen the presence of three SNPs in leptin gene. The testing of Hardy- Weinberg equilibrium for the three SNPs of within Frieswal and Sahiwal population indicated that the polymorphism site in the populations fitted with Hardy-Weinberg equilibrium (P > 0.05) except for C/*BspEI*/T and C/*NruI*/T position in Sahiwal. Polymorphism C/*NruI*/T have significant association with age at first service and age at first calving and heterozygotes have more prolonged age at fist service and age at first calving. For milk protein, C/*BspEI*/T and C/*HphI*/T was found to have significant effect. For lactose and SNF, C/*HphI*/T polymorphism has found to be significant. In case of combined genotyping, genotype CTCTCC (713.00±167.99 days) was found to have noticeable higher age at first service and age at first calving. But milk production higher first lactation yield was noted for CCCCCT (3987.00±337.86 kg).

Key words: Leptin gene, polymorphism, Frieswal, Sahiwal, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), combined genotype.

INTRODUCTION

Remarkable progress has been achieved in milk production since 1980, due to the intense selection of animals based on the production performance. But this resulted in the declining trend in various non-yield traits like reproductive performance which in turn resulted in the low economic output of the dairy farmers. Identification of single nucleotide polymorphisms (SNP) opened new vistas in animal breeding as these methods are quite cheaper and resulted in direct genotyping for candidate genes using polymerase chain reaction (PCR) (Karen et al., 1998; Mataves et al, 2003).

Leptin is a 167 amino acid or 16 kDa polypeptide,

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Abbreviations: WBC, White blood cells; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **SNP,** single nucleotide polymorphisms.

Table 1. Details of the primers.

SNPs	Primer sequence	Product size (bp)	Annealing temperature (°C)	Reference
C/*BspEI*/T	F5ATGGGCTGTGGACCCCTGTATC 3' R 5' TGGTGTCATCCTGGACCTTCC 3'	94	60	Haegeman et al., 2000
C/*NruI*/T	F 5' CAAGATGGACCAGACATTCG 3' R 5'CTGGACTTTGGGAAGAGAGG 3'	317	58	Buchanan et al. 2002
C/*HphI*/T	F 5' GGGAAGGGCAGAAAGATAG 3' R 5' TGGCAGACTGTTGAGGATC 3'	331	54	Lagonigro et al., 2003

which is synthesized predominantly in the adipose tissue. It is involved in the growth and metabolism and plays a crucial role in the regulation of feed intake, energy balance, fertility, milk production and immune functions (Singh et al., 2012; Blache et al., 2000; Chilliard et al., 2005; Liefers et al., 2002; Nkrumah et al., 2005). The bovine leptin gene has been mapped to chromosome 4 (Stone et al., 1996; Pomp et al., 1997). It consists of three exons and out of which only two exons are translated into the proteins (He et al., 1995).

Several polymorphisms in this gene have been found which have significant role in production, reproduction, milk constituents, and growth traits and carcass characteristics (Lien et al., 1997; Haegeman et al., 2000; Buchanan et al., 2002; Lagonigro et al., 2003; Kulig and Kmei, 2009). So the objective of the present work was formulated to distinguish the allelic variation of leptin gene coding exons (exon 2, 3) among Frieswal (Holstein Friesan X Sahiwal) and an indigenous breed viz. Sahiwal of Indian origin. It is also aimed to associate the effect of individual SNP effect on the production and reproduction traits as well as milk constituents. Since genotypic effect of one SNP may be influenced by other SNPs and the genotype combination effect is a reflection of interactions of multiple SNPs, the study was designed to identify the association of haplotypes of leptin gene coding exons (exon 2, 3) with the milk production and reproductive traits.

MATERIALS AND METHODS

DNA isolation and genotyping of animals

Blood samples were collected randomly from 126 Frieswal and 50 Sahiwal cows of Indian origin maintained at Military Farm, Meerut, Uttar Pradesh, India under the same management regimen. Genomic DNA was isolated from white blood cells (WBC) pellet using standard phenol chloroform extraction method (Sambrook and Russel, 2001). The PCR-restriction fragment length polymorphism (RFLP) technique was used to screen the DNA polymorphisms of the leptin gene. Two regions in exon 3 (317 and 331 bp) and one region in exon 2 (94 bp) of leptin gene were amplified. Amplification of the desired leptin gene fragments was performed with published primer pairs (Table 1) (Haegeman et al., 2000; Buchanan et al., 2002; Lagonigro et al., 2003). PCR were performed using genomic DNA template (approximately 100 ng) in a final reaction volume of 25 µl containing 1X PCR buffer (Sigma Aldrich, India), 1.5 mM $Mgcl_2$ (Sigma Aldrich), 200 µM dNTPs (Sigma Aldrich, India), 0.5 µM of each primer and 1 U Taq DNA polymerase (NEB, India). Initial denaturation for 5 min at 94°C followed by 35 cycles of 94°C (30 s), variable annealing temperature (30 s), 72°C (30 s) and a final extension at 72°C for 10 min. The PCR products were isolated and verified by agarose gel electrophoresis methods using 1.5% agarose gel with ethidium bromide for 20 min and visualized under UV trans-illuminator and Gel Documentation System (AlphaImager EP, USA). The restriction digestion was carried out in a final volume of 15 µl reaction. The PCR products for each sample was digested for 4 h at 37°C with 8 U of restriction enzyme BspEI (Haegeman et al., 2000) for exon 2 SNP and 10 U of restriction enzymes that is, NruI (Buchanan et al., 2002) and HphI (Lagonigro et al., 2003) for two SNPs at exon 3 regions. Digested products were separated in horizontal gel electrophoresis using 2.5% agarose gel. Digested fragments' size was estimated by comparing them against DNA ladder (low molecular weight ladder for exon 2 and 2-log DNA ladder for both regions of exon 3).

Statistical analysis

For each breed, calculation of allele and genotypes frequencies was based on direct counting. The Chi-square ($\chi 2$) analysis was performed to test whether the genotype distributions obtained were in accordance with the Hardy-Weinberg equilibrium. Allele frequencies between breeds were compared by Fisher's exact test. Analysis of associations between the genotypes of SNPs reproduction and production, were carried out with the GLM procedure, using SPSS software by the following model:

$$Y_{ijklm}=\mu+G_i+B_j+Y_k+S_l+e_{ijklm}$$

Where, Y_{ijklm} is the observed value; μ is the overall mean; G_i is the effect of genotype ; B_j is the effect of season; Y_k is the year of calving; S_l is the effect of season of calving, and e_{ijklm} is the random error. Values of $P < 0.05$ were considered to be significant.

In case of reproduction traits like age at first service and age at first calving, year and season of birth are considered in place of year and season of calving. But for milk constituents, the effect of breed is excluded as only one breed is under consideration and the model is:

$$Y_{ijkl}=\mu+G_i+Y_j+S_k+e_{ijkl}$$

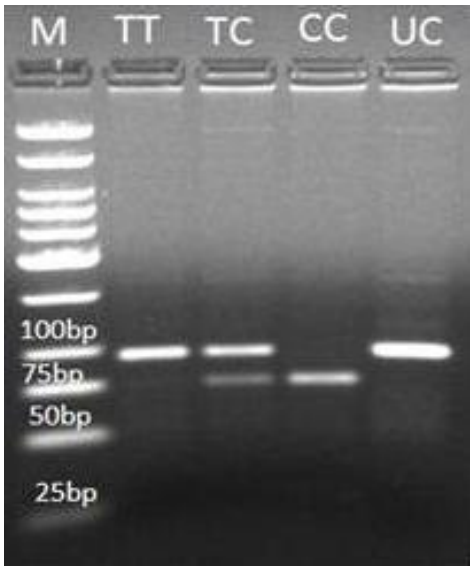

Figure 1. A 2.5% agarose gel displaying *BspEI* digestion of an amplified portion (94 bp) of leptin gene exon 2 (C/*BspEI*/T) of animals with genotypes TT, TC and CC. M, Low molecular weight DNA ladder; UC- uncut.

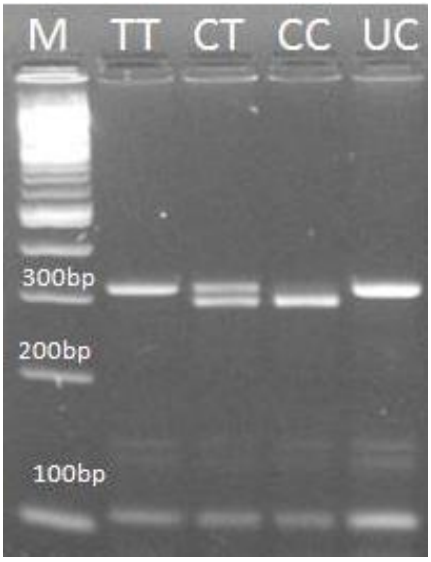

Figure 2. A 2.5% agarose gel displaying *Nru*Idigestion of an amplified portion (317 bp) of leptin gene exon 3 (C/*Nru*I/T) of animals with genotypes TT, CT and CC. M, 2-log DNA ladder; UC, uncut.

RESULTS AND DISCUSSION

Identification and genotyping of SNPs

Three SNP were identified in the bovine leptin gene using PCR-RFLP method. The PCR product of fragment 1 of exon 2 was digested with the BspEI enzyme. The three possible genotypes were defined by three distinct banding patterns: CC (75 and 19 fragments), CT (94, 75 and 19 fragments) and TT (94 fragment) as shown in Figure 1. This is in confirmation with the study of Konfortov et al. (1999) and Buchanan et al. (2002) who described a cytosine (C) to thymine (T) substitution (C—T substitution) in exon 2 of the leptin gene of *Bos taurus and its cross*breds. For the polymorphism of C/*Nru*I/T at exon 3, three digestion patterns were found in the leptin gene among Frieswal. Three genotypes were found as shown in Figure 2; an intact 317 bp fragment as TT genotype, 297 and 20 bp as CC and 317, 297 and 20 bp as TC genotype after digesting with *Nru*I (Buchanan et al., 2002). Similar pattern was observed in in Golpayegani, Najdi, Sarabi and Sistani by Ali Asghar et al. (2010). However, Nassiry et al. (2008) in Golpayegani and Choudhary et al. (2005) in Hariana, Sahiwal, Gir and Nimari cattle couldnot detect TT genotypes. The PCR product of Fragment 3 was digested with HphI enzyme (Lagonigro et al., 2003), and identified three genotypes which were CC (331 bp fragments), CT (331, 311 and 20 bp fragments) and TT (311 and 20 bp fragments) as shown in Figure 3. DNA sequencing analysis confirmed a C/T transition which resulted in the Alanine to Valine (A59V) change in the secreted protein. The genotypic and allelic frequencies of the leptin gene in 176 cattle are presented in Table 2. The testing of Hardy- Weinberg equilibrium for the three SNPs within Frieswal population indicated that the polymorphism site in the populations fitted with Hardy-Weinberg equilibrium ($P > 0.05$). But it has been found that within Sahiwal breed, polymorphisms at C/*BspEI*/T and C/*Nru*I/T position showed significant departures from Hardy Weinberg equilibrium as shown in Table 2. Fisher's exact test revealed that two breeds differ significantly. From the results of Fisher's exact test, it is clear that frequency of genotpes between two breeds differ significantly as shown in Table 2. The frequency of T allele was found to be comparatively lower in Frieswal crossbred. This is in confirmation with the reports of Konfortov et al. (1999) and Choudhary et al. (2005).

Association of the leptin gene polymorphisms with production and reproduction traits

The breed, year and season of calving are found to be significantly associated with production traits like first lactation milk yield and peak yield. Least squares means of these traits with respect to three SNPs are given in Table 3. The three SNP providing genotypes were not significant predictors of the traits used as first lactation milk yield and peak yield. In the present study, none of the polymorphism was found to have a significant effect

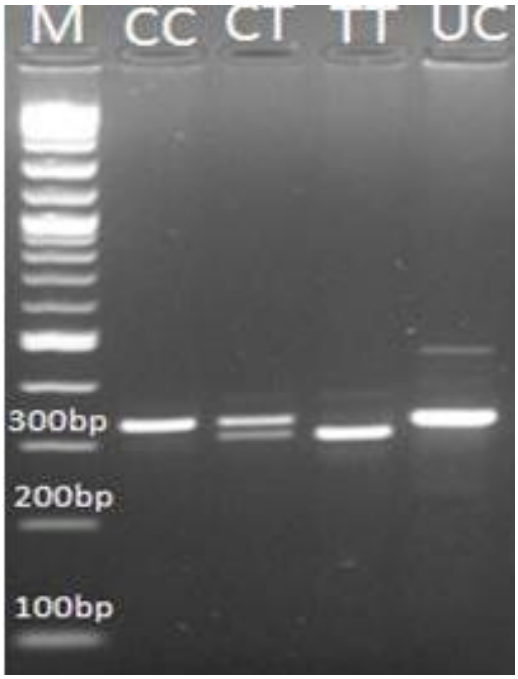

Figure 3. A 2.5% agarose gel displaying *HphI* digestion of an amplified portion (331bp) of leptin gene exon 3 (C/*HphI*/T) of Animals with genotypes CC, CT and TT. M, 2-log DNA ladder; UC, uncut.

Table 2. Gene and Genotype frequencies of three regions of leptin gene after PCR-RFLP in Frieswal and Sahiwal cattle.

Polymorphism	Breed	CC	CT	TT	C	T	Hardy Weinberg equilibrium χ2 test	Fishers exact test
C/*BspEI*/T	Frieswal	0.38 (49)	0.51 (63)	0.11 (14)	0.64	0.36	0.88^{ns}	p<0 .001
	Sahiwal	0.08 (4)	0.88 (42)	0.04 (2)	0.52	0.48	27.22**	
	Total	0.30 (53)	0.60 (105)	0.09 (16)	0.61	0.39	12.13**	
C/*NruI*/T	Frieswal	0.27 (34)	0.51 (64)	0.22 (28)	0.52	0.48	0.04^{ns}	p<0 .001
	Sahiwal	0.41 (21)	0.55 (28)	0.04 (2)	0.69	0.31	3.86*	
	Total	0.31 (55)	0.52 (92)	0.17 (30)	0.57	0.43	0.65^{ns}	
C/*HphI*/T	Frieswal	0.58 (73)	0.38 (48)	0.04 (5)	0.77	0.23	0.71^{ns}	p<0 .001
	Sahiwal	0.96 (48)	0.04 (2)	0 (0)	0.98	0.02	0.02^{ns}	
	Total	0.69 (121)	0.28 (50)	0.03 (5)	0.83	0.17	0.0^{ns}	

** P < 0.001; * P < 0.05; ns-non-significant.

on production. This is in confirmation with the study of Madeja et al. (2004) and Leifers et al. (2002). On the contrary, many of the researchers could establish a significant association between leptin gene polymorphism and milk production traits (Veerkamp et al., 2000; Buchanan et al., 2002; Sadeghi et al., 2008; Dandapat et al., 2009).

In case of reproductive traits like age at first service and age at first calving, year of birth of animals is found to have a significant effect. Least square means of age at first service and age at first calving are presented in Table 3 with respect to SNPs. Heterozygotes have more prolonged age at fist service and age at first calving when compared with both homozygotes in cases of three polymorphisms studied and for which C/*NruI*/T is found to be significant. Contrary to the results, Dandapat et al. (2009) and Moussavi et al. (2006) observed non-

Table 3. Least squares mean and standard errors for production and reproduction traits of different Leptin genotypes.

SNP	Genotypes	Age at first service	Age at first calving	First lactation milk yield	Peak yield
	CC	638.14±22.91	925.67±21.79	2585.06±219.62	11.76±0.83
C/*BspEI*/T	CT	678.56±14.62	955.44±13.90	2687.06±178.88	13.00±0.67
	TT	651.87±43.60	940.49±41.46	2113.34±456.72	9.75±1.72
	CC	631.40±19.43^{b}	916.57±18.44^{b}	2756.86±196.34	12.74±0.76
C/*NruI*/T	CT	692.47±16.24^{a}	970.87±15.42^{a}	2538.27±183.37	12.35±0.71
	TT	670.73±27.85^{ab}	940.42±26.44^{ab}	2922.35±302.81	13.50±1.17
	CC	669.04±14.57	948.43±13.79	2631.79±170.11	12.68±0.65
C/*HphI*/T	CT	671.44±22.26	952.24±21.07	2748.67±240.65	12.30±0.92
	TT	572.99±64.90	854.39±61.43	2115.38±522.71	9.55±2.00

Mean values with the different superscript lower case letters in the same mutational site and column denote significant difference, P < 0.05.

Table 4. Least squares mean and standard errors for milk constituents of different Leptin genotypes in Sahiwal cattle.

SNP	Genotypes	Fat (Year)	Protein	Lactose	SNF
	CC	4.14±0.06	3.05±0.02^{a}	4.62±0.03	8.53±0.04
C/*BspEI*/T	CT	4.03±0.05	2.99±0.02^{b}	4.58±0.02	8.50±0.03
	TT	3.99±0.09	3.07±0.04^{a}	4.56±0.05	8.48±0.06
	CC	4.09±0.05	3.02±0.02	4.57±0.03	8.49±0.03
C/*NruI*/T	CT	4.02±0.06	3.01±0.02	4.60±0.03	8.52±0.03
	TT	4.06±0.08	3.00±0.03	4.60±0.04	8.48±0.05
	CC	4.02±0.05	2.99±0.02^{b}	4.55±0.02^{b}	8.46±0.03^{b}
C/*HphI*/T	CT	4.12±0.05	3.04±0.02^{a}	4.64±0.02^{a}	8.56±0.03^{a}
	TT	4.05±0.14	3.03±0.05^{ab}	4.56±0.06^{ab}	8.50±0.08^{ab}

Mean values with the different superscript lower case letters in the same mutational site and column denote significant difference at P < 0.05.

significant association in reproduction traits.

Association of the leptin gene polymorphisms with milk constituents in Frieswal cattle

Least square means of various milk constituents like fat, protein, lactose and SNF with respect to three SNPs are presented in Table 4. Year and season of calving is found to have a significant effect on the milk constituents. Among the polymorphisms, none of the SNPs was found to have significant association between fat content in milk. But for protein, C/*BspEI*/T and C/*HphI*/T was found to have significant effect. In C/*BspEI*/T polymorphism, heterozygotes are found to have significantly lower protein in milk when compared to both homozygotes. On the contrary C/*HphI*/T polymorphism, CT and TT genotypes have found to be higher protein in comparison to CC genotypes. For lactose and SNF, C/*HphI*/T polymorphism was found to be significant. As in the case of protein, homozygote dominant genotypes (CC) are found to have lower lactose and SNF content in milk in comparison to both genotypes. This is in confirmation with the findings of Leifers et al. (2002) who could establish a significant association between per cent of lactose in milk.

Association studies with combined genotypes

The genotype effect of one SNP may be influenced by other SNPs and the genotype combination effect is a reflection of interactions of multiple SNPs. Therefore, the analysis of genotype combination is superior to the analysis of one single SNP. So we have made an attempt to study the association between combined genotypes and the traits. In this work, 16 combined genotypes consisting

Table 5. Mean±SE of age at first service, age at first calving, first lactation milk yield and peak yield with respect to combined genotypes.

Combined genotype	n	Age at first service	Age at first calving	First lactation milk yield	Peak yield
CCCCCC	5	643.57±157.71	928.14±156.38	2245.00± 659.63	11.20±3.19
CCCCCT	3	577.20± 42.71	863.20± 44.57	3987.00± 337.86	15.67±1.53
CCCTCC	6	661.64±115.00	945.73±113.16	2421.67±1501.82	12.67±5.75
CCCTCT	4	613.82±127.14	896.18±130.15	2036.00±1151.46	12.25±4.19
CCCTTT	2	559.67± 72.42	844.00± 84.66	2031.00± 79.20	10.50±0.71
CCTTCC	6	589.38± 93.32	876.00± 95.22	2727.33±747.48	12.17±5.00
CCTTTT	1	506.00±0.00	791.00±0.00	2446.00±0.00	11.00±0.00
CTCCCC	24	640.14±131.19	922.69±132.69	2265.29± 811.25	11.13±3.71
CTCCCT	1	515.25± 28.25	822.00± 66.30	2130.00±0.00	10.00±0.00
CTCTCC	25	713.00±167.98	983.44±171.49	2087.20±1076.81	10.76±3.99
CTCTCT	7	685.44±151.94	966.50±152.27	2551.00± 356.91	12.00±1.15
CTTTCC	4	669.30± 89.04	950.70± 94.82	2928.75±1603.71	16.00±7.12
CTTTCT	3	695.80±205.96	902.80±143.08	2592.67± 195.37	14.33±1.53
TTCCCC	1	531.00±0.00	822.00±0.00	1715.00±0.00	10.00±0.00
TTCTCC	3	671.43±122.04	955.71±120.65	2068.00± 128.85	9.33±1.15
TTTTCC	1	496.00±0.00	778.00±0.00	3471.00± 0.00	17.00±0.00

of three SNPs were identified in Frieswal cattle, whereas 8 combined genotypes in Sahiwal. The frequencies of some of the combined genotypes were very low. So no statistical analysis was taken up in the case of combined genotypes. The Mean ± SE of age at first service, age at first calving, first lactation milk yield and peak of all the combined haplotypes are presented in Table 5. The combined genotype CTCTCC (713.00 ± 167.99 days) were found to have noticeable higher age at first service, followed by CTTTCT (695.80±205.95 days) and CTCTCT (685.44±151.94 days) in age at first service. But for age at first calving, CTCTCC (983.44±171.49) is followed by CTCTCT (966.50 ± 152.27) and TTCTCC (955.71 ± 120.653 day). But milk production was higher first before lactation yield was noted for CCCCCT (3987.00±337.86 kg).

In conclusion, the present study suggests that single nucleotide polymorphisms in leptin gene can be ideal markers for reproductive traits and milk constituents like fat, protein, lactose and SNF. Findings of this study in relation with combined genotypes need to be carried out in a large population before suggesting the haplotype pairs to be convincing molecular markers. So leptin gene is an ideal candidate gene that may assist in marker assisted selection for production as well as reproduction which is the need of the time.

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