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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

Assessment of genetic diversity among sugarcane cultivars using novel microsatellite markers

Manish Dev Sharma[1,3], Upma Dobhal[1], Prashant Singh[1], Shailender Kumar[1], A. K. Gaur[1], S. P. Singh[2], A. S. Jeena[2], Eapen P Koshy[3] and S. Kumar[1]*

[1]Molecular Biology and Genetic Engineering College of Basic Science and Humanities, G. B. Pant University of Agriculture and Technology Pantnagar, Uttrakhand, India.
[2]College of Agriculture, G. B. Pant University of Agriculture and Technology Pantnagar, Uttarakhand, India.
[3]Sam Higginbottom Institute of Agriculture, Technology and Science, Allahabad, India.

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Genetic diversity based on the characterization of genetic makeup, using molecular markers is of utmost importance for breeders in crop improvement programme. A total of 26 microsatellite primers were used to determine the genetic diversity among 40 sugarcane genotypes including their parents. The polymerase chain reaction (PCR) products were examined for both size and polymorphism using these primers. Overall alleles are amplified with an average of 2.3 per locus in this study. Of the total 26 simple sequence repeat (SSR) markers, only 10 (38.4%) displayed polymorphism, with polymorphism index contents (PIC) values ranging from 0.15 to 0.67. The observed homozygosity (Ho) and gene diversity (Nei's) for individual loci varied from 0.0000 to 0.277 and 0.129 to 0.473, respectively. Shannon's informative index (I) was found to be highest (0.661) in SKM04 while the lowest was 0.252 in SKM01 SSR loci with an average of 0.524. Fixation index was also calculated which was in the range of -0.074 to 1.00. A genetic relationship among cultivars and parental genotype was also analyzed by cluster analysis using unweighted pair group method with arithmetic mean (UPGMA), the average-linkage method, with the similarity matrix as input data. The genetic relationship and genetic diversity among the cultivars depicted from this study can be used to select the parents in sugarcane breeding programme.

Key words: Sugarcane, microsatellite, genetic diversity, SSR.

INTRODUCTION

Sugarcane (*Saccharum* spp.) is an important cash crop in the tropical areas that is cultivated for its stalks, which accumulate sucrose. It contributes 60% of the raw sugar produced worldwide, the remaining 40% coming from sugar beet (Grivett and Arruda, 2002). Modern sugarcane varieties that are cultivated for sugar production are complex interspecific hybrids (*Saccharum* spec. hybrid), that have arisen through intensive selective breeding of species within the *Saccharum* complex, primarily involveing crosses between the species *Saccharum officinarum*

*Corresponding author. E-mail: malik.sundip@gmail.com. Tel: + 91-9410168458. Fax: 91-5944-233473.

L. and *Saccharum spontaneum* L. (Sajjad and Khan, 2009). Considering the current needs of cane industry, it is imperative to breed high sugar producing and disease resistant varieties with other desirable traits such as high tillering ability and high ratooning capacity. The success of sugarcane breeding program lies in the proper choice of rich and genetically diverse parents. On the flip side, though, genetic base of sugarcane varieties appears to be narrow and at present, this is reflected in slow progress in sugarcane improvement. This could possibly reflect the use of same and over exhausted genotypes as germplasm in the repeated sugarcane breeding programme. Regular selection of genetically diverse parents for crossing programme is therefore a crucial factor to enhance the efficiency of genetic improvement in sugarcane (Kanwar et al., 2009). The genetically diverse parents may be selected on the basis of diverse geographical distribution of the genotypes, information on agronomic characters or diversity analysis through molecular markers (Melchinger, 1998).

The study of genetic diversity is a key for successful breeding programme and it helps in inserting desirable characters into any genotype through crossing of diverse parents. Diversity naturally exists for different characters, to be used in breeding programme and the screening of genotype for these desirable characters is important to develop improved genotypes. Parameters which are considered to be useful for screening the genetic diversity are morphological, physiological and molecular. Although, morphological (agronomic) parameters are simple to use but they are time consuming and expensive and inaccurate, most of these parameters are being affected by the environment. Physiological characters are also influenced by external environment. However, molecular parameters are reliable, fast, and cost effective and are not under the influence of environment. Hence, techniques which measure the genetic diversity without the influence of environmental factors hold the key for successful breeding program. Thus, molecular marker offers an efficient measure of genetic diversity on the basis of genetic characteristics.

The use of molecular markers allows the assessment of genetic diversity at DNA level. Different molecular markers such as restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNAs (RAPDs), sequence tagged sites (STS), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) or microsatellites, single nucleotide polymorphisms (SNPs) etc. have been developed and applied to wide range of crop species. These markers widely used for increasing the understanding of genetic and taxonomic complexity of various agricultural crops. The desirable attributes of these markers encouraged their development (Cordeiro et al., 2000) and utilization to achieve important agronomic traits in sugarcane (Rossi et al., 2003; Aitken et al., 2005). Among the PCR-based markers, microsatellites or SSR markers has proved to be the most powerful tool for diversity analysis in molecular breeding due to their abundant genetic distribution, high reproducibility, multi allelic nature co-dominant inheritance and cross transferability to closely related genera (Powell et al., 1996; Gupta and Varshney, 2000; Pan, 2006; Gupta and Prashad, 2009; Kalia et al., 2011; Yu et al., 2011). SSR markers can be developed either from the DNA sequence information available in the databases or by screening of genomic DNA libraries enriched for different repeat motifs including sequence based methods (Gupta and Varshney, 2000; Zane et al., 2002; Jones et al., 2002; Kalia et al., 2011).

In recent, SSR markers have been extensively used in genetic diversity study of many plants like maize (Yao et al., 2008; Selvi et al., 2003) and rice (Kibria et al., 2009), foxtail millet (Gupta et al., 2012) including sugarcane (Pandey et al., 2011). The present investigation aims for genetic diversity analysis of 40 important genotypes of sugarcane and polymorphic information content for 26 newly developed SSR markers for their subsequent use in molecular marker studies.

MATERIALS AND METHODS

Plant material

Sugarcane genotypes used in the present investigation were available in Norman E. Borloug Crop Research Centre, G. B. Pant University of Agriculture and Technology, Pantnagar, India. A total of 40 genotypes were used in the present investigation including 34 F_1 generation genotypes and 6 of their parents genotypes namely BO91, Co 0238, CoPant 99214, CoS 767, Co Pant 90223, CoS 8436.

Development and characterization of simple sequence repeat (SSR)

Genomic DNA of ISH 100 was extracted from fresh leaves, using CTAB method (Hoisington et al., 1994) and send to Genetic Identification Service Inc. (GIS, Chatsworth, CA) for the construction of microsatellite enriched library. Total genomic DNA was digested using *Eco*R1. The recombinant plasmids were produced by ligating restriction fragments from *Saccharum* DNA into the *Hin*d III site of pUC19 plasmid. The fragments were enriched for microsatellite motifs CA, GA, ATG and TAG prior to ligation. Ligated products were introduced into *E. coli* strain DH5α (ElectroMaxJ, Invitrogen) by electroporation. 2 μl of ligation mix was used for each of the libraries. After transformation and recovery incubation in SOC broth (Invitrogen), glycerol was added to a level of 20% of the final volume. Libraries were stored at approximately -70^{O}C. To isolate colonies for sequencing, cells from the glycerol stock were spread on X-gal/IPTG/ampicillin-LB agar plates. Sterilized toothpicks were used to transfer white colonies from the spread stock plates onto a X-gal/IPTG/ampicillin LB plate. The plate was incubated overnight, and colonies were selected from this plate. Plasmid DNA was isolated from the cultures using miniprep spin kit (Qiagen, Germany). Plasmids were sequenced by ABI 377 automated DNA sequencers (Applied Biosystems, Foster city, (CA)). Sequenced data from the clones containing SSRs were analyzed for primer selection and primers were designed from

Table 1. List of sugarcane genotypes were used for genetic diversity analysis.

Genotype code	Name of genotypes
C1,C2,C8-C12, C34	CoS 8436 X CoPant 97222
C3-C7	CoSe 92423 X CoS 767
C13-C16	Co 0238 X CoPant 97222
C17	CoPant 90223 X Bo 91
C18,C19,	CoS 8436 X CoPant 99214
C20-C28,C33	CoSe 92423 X CoS 8436
C29-C32	CoPant 99214 X CoS 8436
C35	Bo 91
C36	Co 0238
C37	CoPant 99214
C38	CoS 767
C39	CoPant 90223
C40	CoS 8436

flanking regions surrounding the SSR motif with Designer PCR ver. 1.03 (Table 2). All the primer pairs were first screened on DNA of sugarcane species and commercial varieties. The polymorphic primer pair with non-specific amplifications and too faint products was discarded using high throughput touchdown and gradient PCR.

DNA extraction and amplification

The genomic DNA was extracted from young leaves of each of the genotypes using modified CTAB method (Hoisington et al. 1994). 500 mg leaves from different sugarcane genotypes were separately grinded to fine powder in liquid nitrogen using pre-chilled mortar pestle and transferred to 25 ml sterilized tube containing 10 ml pre warmed CTAB buffer [2% (w/v) CTAB, 20 mM EDTA, 1.4 M NaCl, 0.5% sodium bisulfite, 100 mM Tris-HCl (pH 8.0) and 0.2% (v/v) β-mercaptoethanol] and purified by RNase treatment. The quantity of isolated DNA was determined spectrophotometrically; visualized on 0.8% agarose gel stained with ethidium bromide and a final concentration of 80 ng was used for PCR. For PCR amplification, 25 µl of reaction mixture containing 80 ng DNA, 1X Taq buffer, 1.5 mM $MgCl_2$, 10 pmoles each of forward and reverse primers, 0.2 mM dNTPs and 1 U of Taq DNA polymerase. A total of 26 SSR primer pairs were used to determine the diversity among sugarcane genotypes used in the present investigation. PCR amplification was carried out using the PCR profile; denaturation at 94°C for 40 s followed by annealing temperature (Ta) for 40 s at range of 54 to 58°C (according to the sequence of the specific primer) and extension at 72°C for 30 s and final extension at 72°C for 7 min in Thermal Cycler (Applied biosystem, USA). The amplified products were electrophoresized in 2.5% agarose gels in 1X TAE buffer and visualized by staining the gels in 0.5 µg/ml ethidium bromide and captured under UV light in gel documentation system (Avegene, Taiwan).

Data analysis and genetic diversity estimation

For determining the genetic relationships among genotype used in the present investigation, the profile of SSR markers were scored on the basis of their band size, either present (1) or absent (0) for each SSR loci. Polymorphic information content (PIC) was used to determine allele diversity at each locus and was calculated according to Roldan-Ruiz et al. (2001) as:

$$PIC_i = 2f_i(1 - f_i)$$

Where, f_i is the frequency of the amplified allele (Band present) and $(1 - f_i)$ is the frequency of null allele (band absent) of marker *i.*

The genetic similarities among the accession were calculated according to Jaccard's coefficient (Jaccard, 1908) using NTSYS-pc software package version 2.10d. A phylogenetic tree was constructed using UPGMA method. The observed heterozygosity (H_0), Nei's average gene diversity (Nei, 1973), fixation index (F_{IS}) and Shannon's informative index (*I*) were calculated using POPGENE 1.32 software (Yeh and Boyle, 1997).

RESULTS

In the present investigation, 26 SSR primers were designed and tested against 40 (6 parental and 34 of F_1 generation) genotypes of sugarcane to determine their genetic diversity (Table 1). PCR amplification with SSR primers revealed that 16 developed markers were monomorphic and 10 were polymorphic. The identified polymorphic SSR markers were further used to estimate the genetic diversity among the genotypes studied

Genetic diversity

The analysis of polymorphic markers (38.5%) in this study was carried out using tools described in material and methods section. A total of 22 alleles were found, averaging 2.3 alleles per locus varying from two (SKM01, SKM02, SKM05, SKM06, SKM07, SKM08, SKM09, SKM10), to three (SKM02, SKM03). Substantial variations in allelic polymorphism were also observed and the size range from 147 to 320 bp (Figure 1, Table 3). The PIC value extended from 0.15 (SKM03) to 0.67 (SKM07) with the mean of 0.34. Generally, PIC values increased proportionally with increasing heterozygosity at a locus.

The observed heterozygosity (Ho) for individual loci varied from 0.0000 to 0.277 with an average of 0.163 per locus. The expected heterozygosities or gene diversity (Nei's) ranged from 0.129 to 0.473 with an average of 0.336 per loci.

Fixation index (F_{IS}), a measure of genetic diversity was calculated, which is ranging from (-) 0.074 to 0.570 with an average of 0.515, positive value of fixation index represent excess of observed homozygotes where as negative value is demonstrating extra heterozygotes. The Shannon's informative index (I) of loci varied from 0.252 to 0.661 with the mean of 0.524 per locus. The data for PIC value, observed heterozygosity (Ho), expected heterozygosities or gene diversity (Nei's), Fixation index (F_{IS}), Shannon's informative index (I) of loci is shown in Table 2.

The genetic distance of genotypes under study on the basis of the current study was constructed according to Nei (1978), and relationships between genotypes were displayed graphically in the form of dendrogram (Figure 2).

Table 2. List of all 26 SSR primers with their Tm and PUID No.

Primer	Forward sequence	Reverse sequence	Tm (^{0}C)	PUID No
SKM01	TATGGAGAGAGCAACCTATCA	GACGGAAGATTGGGATTC	56.9	28374217
SKM02	GGCCTTCGATTAACCGAT	ACAGGACGCTGCTTCTTG	57.7	28374215
SKM03	CCTATCGAATTGTGCTACTC	GCATGTGTATTGTGTTAGAGAA	54.8	28374210
SKM04	TTATTTGTCCAACCTGCTTCTG	CATGGATGCTTTTGCGTTAG	56.9	28374225
SKM05	ACCACCACCACTTTGTCTT	GGATTGCTAAAGCATTGGT	57.3	28374233
SKM06	ACCACCACCACTTTGTCTT	CGTGAGAAGGTAGGGAAACA	56.7	28374240
SKM07	CCAAACCACATTGTAGCAG	CTTCTTGTCATCATCACTTGAG	56.6	28374252
SKM08	TTATCCCTTTCGTTCAGTAGAG	ATTTTGCGTAGGGTCTGAG	57.3	28374222
SKM09	GGTGGCTAACAGACAGGG	TTGCTGCCGAGAGTCATA	56.9	28374220
SKM10	GCGCCTATTTAATACCAGA	CTTTCCCTATACCCATGATAG	56.0	28374247
SKM11	TCAAAGTGGCTACAGAATAGGT	CAGCAAGGTTCCAAGTACC	56.7	28374214
SKM12	AGTTCCCTGTACTTGTCTACCA	TTGGTCGCTTAAAGTCAATC	56.4	28374204
SKM13	GGATACAAAGGAGAGCACAAC	CGAGGAATCAGTTCACCC	57.5	28374209
SKM14	CCCAGTAAGCTGTTGTTGC	TCTGCGATGTAACCCTATTTC	56.0	28374237
SKM15	CACCCAGCAGTTATTGGA	CAGCAATCAAGTGTTCACTG	56.6	28374228
SKM16	AATGGTTCACTGCATGATATG	GGACGACTTAAAGTTCTTGTGA	57.7	28374235
SKM17	GCTTTGAATGCCACACTC	CACCGTGCTAGTGAGGAC	56.1	28374249
SKM18	GAGGGTGTTGGAGACCAT	ATCCCAATTCAATCCGTC	56.2	28374250
SKM19	GGCTTCTTGTTGATAGCAATG	AGAGGGGCAAGTTTGAGAA	56.2	28374251
SKM20	GAGGTGATGAGTCCATACC	CCTTGAATACGGTGGTCT	56.4	28374244
SKM21	TGAGAACTTGATGGAGTATCTC	GAGCACTCACTTGATTAGTAGC	55.3	28374241
SKM22	CCCAAACTAACCCACATG	CACAACCTCTGCAAAGTGT	56.3	28374242
SKM23	ACCGTCATCGTCCACTAC	TGGAAGACCATGAGGATC	56.6	28374243
SKM24	GCTGAGGTGATGATGACA	GGAGAGCACAAAAGATAACTC	56.0	28374245
SKM25	CTTTGGTTTGTTGTAGCATATC	GAGGCCAAGATGACATTC	56.8	28374246
SKM26	AAGGGAAGAGCAGGAGAG	CGGGAGGTCAAAATGTTA	56.8	28374248

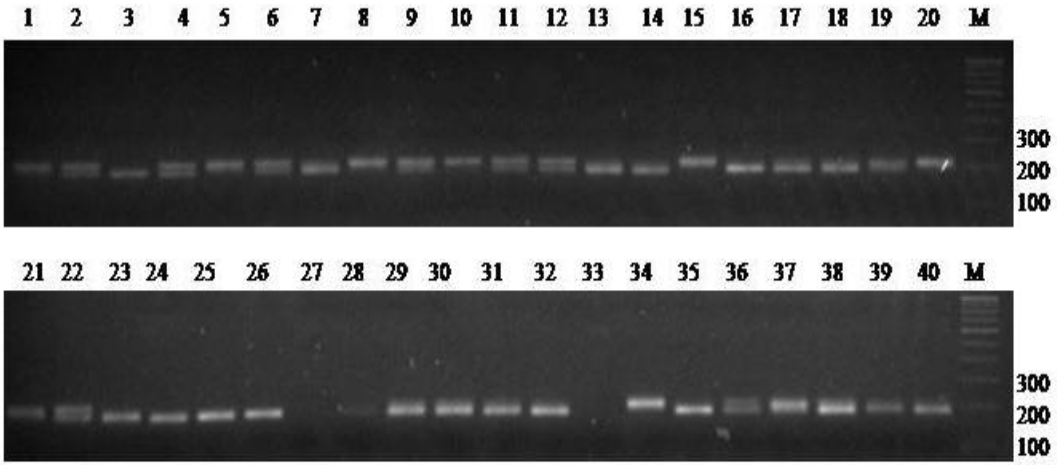

Figure 1. Molecular profiting of 40 genotypes of sugarcane with SSR primer SKM10 as given in table 1 and 3 with 100-bp DNA ladder.

Genetic relationship

Genetic similarity was minimum (0.13) between C1 and C40 genotypes and maximum (1.00) between C20 and C24/C22 and C27 genotypes calculated via genetic similarity matrix. The UPGMA clustering method based on Nei's (1978), unbiased genetic distance classified all the sugarcane accessions into two major groups (I and II)

Table 3. Summary of genetic diversity of 40 sugarcane genotypes using 10 microsatellite markers.

Marker	SSR motif	N_A	Allele size range (bp)	H_o	Nei	F_{Is}	I	PIC
SKM01	$(CA)_{25}$	2	210-243	0.138	0.129	-0.074	0.252	0.19
SKM02	$(GA)_{17}$	3	150-256	0.277	0.387	0.282	0.649	0.26
SKM03	$(CA)_{16}$	3	147-320	0.128	0.210	0.391	0.436	0.15
SKM04	$(CT)_{22}$	2	149-200	0.250	0.468	0.466	0.661	0.43
SKM05	$(CT)_{25}$	2	287-300	0.000	0.438	1.000	0.630	0.44
SKM06	$(CAT)_{13}$	2	200-220	0.000	0.142	1.000	0.271	0.15
SKM07	$(AGA)_7$	3	150-180	0.256	0.473	0.458	0.660	0.67
SKM08	$(CT)_{26}$	2	296-320	0.250	0.334	0.327	0.517	0.29
SKM09	$(CT)_{16}$	2	180-200	0.171	0.382	0.551	0.570	0.22
SKM10	$(CAT)_{11}$	2	200-220	0.840	0.399	0.539	0.589	0.40
Average		2.3		0.163	0.336	0.515	0.524	0.34
Std. Dev.				0.099	0.129		0.155	

Number of allele (N_A); Observed Heterozygosity (Ho); Nei's average gene diversity (Nei); fixation index (Fis); Shannon's informative index (I); polymorphic information content (PIC).

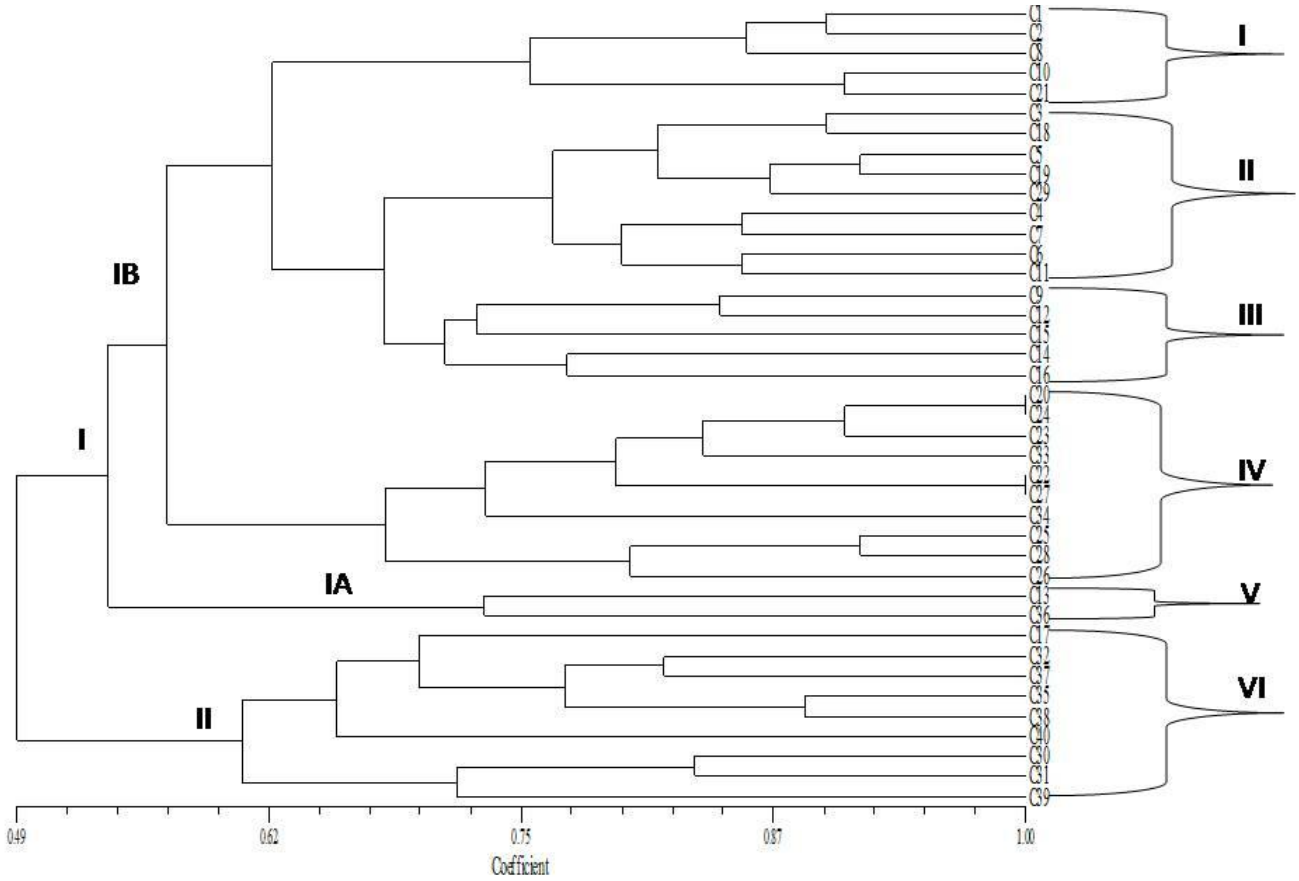

Figure 2. Dendrogram showing the relationship among 40 sugarcane genotype based on 10 SSR primer pairs.

comprising six clusters (Figure 2). The first major group consisted of two subgroup IA and IB with 31 genotypes. The subgroup IA was further subdivided into five clusters, first comprising five genotypes (C1, C2, C8, C10 and C21) of common origin with the only exception being C21. The Second cluster comprised of nine genotypes

(C3, C18, C5, C19, C29, C4, C7, C6 and C11), third cluster consisted of five genotypes (C9, C12, C15, C14 and C16), fourth cluster included maximum number of genotypes (C20, C24, C23, C33, C22, C27, C34, C25, C28, and C26) and the fifth cluster represented only two genotypes (C13 and C36), respectively. The clusters II and III included in Subgroup IB exhibited maximum similarity of 69% and showed 63% similarity with cluster I of same subgroup. Clusters I, II and III showed 55 and 50% similarity with clusters IV and V of subgroup IA, respectively. The group II included a single cluster with remaining 9 genotypes (C17, C32, C37, C35, C38, C40, C30, C31 and C39). Out of nine genotypes included in this group, five were parental genotypes. The single cluster included in this group exhibited minimum similarity of 49% with the clusters included in group I.

DISCUSSION

The identification of varieties based on molecular markers may be important to establish distinctness, uniformity and stability of protected cultivars (Swapna et al., 2010). Among the various molecular markers, SSR markers have evolved as a boon to breeders as they have revolutionized the crop breeding, by their higher efficiency and uses in assessment of genetic variability, characterization of germplasm, estimation of genetic distance between population, inbreeds and breeding material, detection of monogenic and quantitative trait (Tommasini et al., 2002; Hoxha et al., 2004; Gupta et al., 2012; Yepuri, 2013). Microsatellite variability is widely used to infer levels of genetic diversity in natural population; also its mutation rate is very high.

In the present study, SSR markers were developed from microsatellite library derived from DNA of inter specific hybrid (ISH 100). These genomic SSR markers were further used to study the genetic diversity and genetic relationship among 40 genotypes of sugarcane including their parents. Ten (10) out of 26 SSR primers were found polymorphic when screened. These polymorphic SSR primers were used for genetic analysis of sugarcane cultivars. The highest PIC value obtained was 0.67 for SKM07 and lowest as 0.15 for SKM06. Microsatellite markers have revealed high PIC values in other studies of sugarcane (Cordeiro et al., 2003; Pinto et al., 2006; Oliveira et al., 2009; Chen et al., 2009) which suggests the suitability of SSR markers for the diversity analysis. In present study, we have obtained a broad range of PIC values, indicative of the presence of unique alleles in some accession which facilitates their differentiation from another. The average number of alleles obtained per locus was 2.3, which closely resembles the previous reports from foxtail millet (Gupta et al. 2012) in which the number of alleles per locus was 2.2, sorghum (Brown et al. 1996) the number of alleles per locus was 2.3, for ground nut it was 2.3 (Gautami et al. 2009). Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level (Weir, 1989). Selection of appropriate genetic distance involved in any study requires extremely careful consideration of the evolutionary history of the populations involved and the specific goals of the study. The gene diversity is a measure of genetic distance ranged from 0.129 to 0.473 in the present investigation with an average of 0.336 which indicates significant distance amongst the alleles using SSR markers, which is supported by previous reports on various molecular markers from sugarcane (Cordeiro et al., 2003; Pinto et al., 2004; Pan, 2006; Zhang et al., 2008; Chen et al., 2009; Pan, 2010).

Genetic similarity coefficient was carefully observed for deducing relationships between the studied varieties. The similarity coefficient 1.0 was observed in C20 and C24, C22 and C27 having same parents. The similarity coefficient 0.90 was observed between C1 and C8, C23 and C24, C20 and C23 genotypes exhibiting their similar lineages, while C1 and C40 genotype exhibit the lowest similarity 0.13 which reflects differences in lineage involved. The molecular results obtained in present study are represented and which agree with the possible evolutionary course of sugarcane genotypes. The UPGMA cluster analysis of 40 genotypes in the present study produced meaningful grouping based on pedigree or geographical origin of the accessions. The grouping pattern of 40 genotypes are based on their genetic similarity pattern which showed that sugarcane genotypes from same geographical regions tend to cluster together which may be the result of similar evolutionary relationship. The five out of six parental genotype, C35, C37, C38, C39 and C40 were clustered together representing their possible convergent evolution. Genotypes lying on similar clan were representatives of northwest origin (in Indian context) while one parental genotype, C36 which formed cluster in another group represented another region (Southern) origin. The grouping according to the place of origin is supported by previous findings (Zhang et al., 2004; Chen et al., 2009; Singh et al., 2010). Sugarcane cultivars are aneupo-lyploid hybrids that have the most complex genomes of any crop plant with chromosome number in excess of 100 (D'Hont et al., 1996). Interestingly, clones derived from the parental genotypes represented distant relation with their parents, may be due to the complex genetic makeup of sugarcane genome. The genotypes of same parental origin tend to remain closer as depicted from the dendrogram. The similarity Jaccard's coefficient values among 40 genotypes ranged from 0.13 to 1.00 with the highest value of 1.00 exhibited between genotypes C20 and C24, C22 and C27. The lowest similarity coefficient 0.13 was depicted in between genotype C1 and C40, showing their distant homology.

Thus in the present study, SSR markers was found to be the useful tool for genetic diversity analysis in sugar-

cane genotypes. These SSR markers can further be utilized to facilitate marker assisted selection as well as genetic analysis of other sugarcane cultivars and wild grasses related to sugarcane. The genetic relationship among various genotypes depicted from this study can be used to select the better genotypes as germplasm in breeding programmes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Molecular and phenotypic characterization of shigatoxigenic *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) from piglets and infants associated with diarrhoea in Mizoram, India

Jubeda Begum[1,2], T.K. Dutta[2], Rajesh Chandra[2], Parimal Roy Choudhary[2], Zomuankima Varte[2] and Molalegne Bitew[1]

[1]Division of Bacteriology and Mycology, IVRI, Izatnagar, Bareilly, 243122 India.
[2]Department of Microbiology, C.V. Sc & A.H. Selesih, Aizawl, C.A.U. India.

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Limited information is available on shiga toxin producing *Escherichia coli* (STEC) in pigs and infants from Aizawl, Mizoram and North Eastern region of India. This cross sectional study was conducted on faecal samples from pigs and infants to detect and characterize of STEC and enteropathogenic *E. coli* (EPEC). Serogrouping, molecular and phenotypic characterizations were done by standard molecular and cytotoxic assays. Out of 48 *E. coli* strains isolated from 320 diarrhoeic fecal samples of piglets, 44 belonged to 18 different serogroups, 3 (6.25%) were untypeable (UT) and 1(2.08%) was recorded as rough strain (R). Similarly, out of 17 *E. coli* strains isolated from 264 diarrhoeic fecal samples of infants, 16 belonged to O60 (94.1%) and 1(5.88%) was untypeable. Virulence genes (*stx*1, *stx*2, *eaeA* and *hlyA*) were detected by multiplex PCR assay. A total of 1260 *E. coli* were isolated from piglets (720) and infants (540) from 584 faecal samples. All together, 5.16% (65) *E. coli* isolates were found to be positive for at least one virulence gene (6.66% piglets and 3.15% infants). Out of the virulent gene positive *E. coli* 3.17% (32 from piglets and eight from infants) and 1.98% (16 from piglets and nine from infants) were recorded as STEC and EPEC, respectively. On the other hand, from the total 2.14% stx_2 positive isolates, 16 and 11 were positive for stx_{2e} and stx_{2c} subtypes, respectively. Similarly, from the 4.04% eaeA positive isolates, 1.19% (15) were positive for *bfpA* gene, of which 1.67% (12) were piglets and 0.60% (3) were infants. All the isolates were exhibited varying degree of CPE on vero cell lines. In conclusion, STEC and EPEC seem to be associated with diarrhoea in piglets and infants in Mizoram. In piglets STEC strains represent as a major cause of diarrhoea while EPEC strains represent as major cause of diarrhoea in infants in North Eastern region of the India.

Key words: Enteropathogenic *E. coli* (EPEC), infants; piglets, shigatoxigenic *E. coli* (STEC), vero cell cytotoxicity.

INTRODUCTION

Pathogenic *Escherichia coli* are one of the most important groups of bacteria causing diarrhoea and extraintestinal infections in human and animals (Levine, 1987). Major diseases caused by *E. coli* are piglet diarrhoea (Fair brother et al., 2000), oedema disease (Chen et al., 2004) and postweaning diarrhoea in pigs

(Choi et al., 2001). In human, it causes infant diarrhoea, thrombotic thrombocytopenia purpurea (Paton and Paton, 2005), haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Nataro and Kaper, 1998; Leelaporn et al., 2003; Paton and Paton, 2005; Zweifel et al., 2006). Shigatoxin producing *E. coli* (STEC), also known as verotoxin producing *E. coli* (VTEC), comprises a serologically diverse group of pathogens that cause disease in humans and animals (Barman et al., 2008). STEC constitute one of the most important causes of foodborne diseases worldwide (Wani et al., 2004). The common features of STEC are the production of shigatoxins (Stx) that are considered to be the major virulence factors. Two major groups of Stxs are named as stx_1 and stx_2; where the former one is nearly identical to the toxin of *Shigella dysenteriae* type 1. stx_2 shares less than 60% amino acid sequence with stx_1 (Barman et al., 2008).

EPEC isolates are defined as an intimin containing diarrhoeagenic *E. coli* that possess the ability to form attaching and effecting lesions on intestinal cells and that do not possess genes coding for Shiga toxins (Kaper, 1996; Bhat et al., 2008). EPEC isolates are leading cause of diarrhoea, especially among infants in the developing world (Bhat et al., 2008).

Diarrhoeagenic *Escherichia coli* (STEC and EPEC) are responsible for an estimated 300,000 to 500,000 death annually in children under the age of five years (Fleckenstein et al., 2010). STEC and EPEC are commonly recovered from the faeces of food producing animals and pose threats to health of humans and livestock.

The majority of the populations in north eastern region of India are traditionally dependent on pig rearing for their livelihood; and there are no taboos for consumption of meat. It is a common feature to observe that each and every rural family maintains a backyard piggery unit in this region.

The density of pig population in North eastern region of India is highest in the country with Mizoram contributing 2.18×10^4 out of total 3.879×10^5 pig population in North eastern region of India (Statistics of animal population in NE India, 2003).

There is limited information available on incidence of STEC and EPEC in piglets and infants in India and particularly in North Eastern Region of India. The present study was undertaken with the objectives to study the level of STEC and EPEC in the piglets and infants in Mizorem state, to investigate the association of STEC and EPEC with diarrhoea of piglets and infants.

MATERIALS AND METHODS

Sampling and isolation of *E. coli* isolates

A total of 584 faecal samples were collected from 320 piglets (20 to60 days old) and 264 infants (2 to 24 months old) with the history of diarrhoea from different districts of Mizoram during June, 2010 to April, 2011. Fecal samples were inoculated on Sorbitol MacConkey's agar (HiMedia, Mumbai, India) plates and incubated at 37°C for 24 h. Five randomly selected pink colored colonies were picked up from each plate and were again sub cultured on to Eosin Methylene Blue agar (HiMedia, Mumbai, India) plates to observe the characteristics metallic sheen of *E. coli*. The well separated single colonies were picked up on nutrient agar slants as pure culture and subjected to standard morphological and biochemical tests.

Serogrouping

All the *E. coli* isolates were serotyped on the basis of their "O" & "H" antigen. Somatic (O) typing was done at National *Salmonella* and *Escherichia* Center, Central Research Institute, Kasauli H.P. 173204 (India) while flagellar (H) typing was carried out by agglutination test using specific flagellar antisera (Denka Seiken, Japan) as per manufacturer's instruction with suitable modifications. Specific antisera were used for flagellar typing.

Preparation of bacterial DNA lysates

Each E. *coli* isolates were inoculated in 5 ml Luria Bertani (LB) (HiMedia, Mumbai, India) broth and incubated at 37°C overnight under constant shaking. After incubation, 1 ml of the bacterial broth culture was taken in a sterile microcentrifuge tube and pelleted at 8000 rpm for 10 min at 4°C. The bacterial pellet was washed thrice with sterile normal saline solution (NSS) (0.85% NaCl) and centrifuged at 8000 rpm for 10 min at 4°C and finally resuspended in 300 µl nuclease free sterile water (NFW). Bacterial suspension was boiled for 10 min in a boiling water bath followed by immediate chilling for 10 min. The bacterial lysate was centrifuged again at 5000 rpm for 5 min to sediment the cell debris and the supernatant was used as template DNA for PCR assay.

Detection of virulence genes for STEC and EPEC by multiplex PCR

A multiplex PCR was carried out using four sets of oligonucleotide primers for stx_1, stx_2, *eaeA* and *hlyA* genes as per the method described by Paton and Paton (1998) with suitable modifications. The detail of the primers used in this study is depicted in Table 1. The multiplex PCR mixture of 25 µl contained 2.5 µl of 10X PCR buffer with $MgCl_2$, 1 µl each primer within the four primer sets at a concentration of 250 nM, 2 µl of 10 mM each of dNTPs, 0.2 µl of 5.0 U of *Taq* DNA polymerase and 4.0 µl of template DNA and the rest is NFW (8.3 µl) to make up volume of 25 µl. The PCR reaction was performed in a thermal cycler (Thermo Electron, Germany) using following cycling conditions: initial denaturation at 94°C for 5 min

*Corresponding author. E-mail: jubedavet@gmail.com. Tel: +91 9457012172.

Abbreviations: HC, Haemorrhagic colitis; **HUS,** haemolytic uraemic syndrome, **STEC,** shigatoxigenic *E. coli*; **EPEC,** enteropathogenic *E. coli*.

Table 1. Details of the oligonucleotide primers used in the present study.

Primer name	Sequences (5`- 3`)	Expected amplicon size (bp)	References
stx_1 F	ATAAATCGCCATTCGTTGACTAC	180	Paton and Paton (1998)
stx_1 R	AGAACGCCCACTGAGATCATC		
stx_2 F	GGCACTGTCTGAAACTGCTCC	255	Paton and Paton (1998)
stx_2 R	TCGCCAGTTAATCTGACATTCTG		
stx_2c F	GCGGTTTTATTTGCATTAGT	124	Paton and Paton (2005)
stx_2cR	AGTACTCTTTTCCGGCCACT		
stx_2d F	GGTAAAATTGAGTTCTCTAAGTAT	175	Paton and Paton (2005)
stx_2d R	CAGCAAATCCTGAACCTGACG		
stx_2e F	ATGAAGTGTATATTGTTAAAGTGGA	303	Paton and Paton (2005)
stx_2e R	AGCCACATATAAATTATTTCGT		
stx_2f F	TGTCTTCAGCATCTTATGCAG	150	Paton and Paton (2005)
stx_2f R	CATGATTAATTACTGAAACAGAAAC		
eaeA F	GACCCGGCACAAGCATAAGC	384	Paton and Paton (1998)
eaeA R	CCACCTGCAGCAACAAGAGG		
hlyA F	GCATCATCAAGCGTACGTTCC	534	Paton and Paton (1998)
hlyA R	AATGAGCCAAGCTGGTTAGCT		
bfpA F	GATTGAATCTGCTCTGGATTGA	426	Wieler et al. (1996)
bfpA R	GGATTACTGTCCTCACATAT		

F = Forward primer; R= reverse primer.

followed by 30 cycles of denaturation at 94°C for 45 s, primer annealing at 65°C for 45 s and extension at 72°C for 45 s, followed by a final extension at 72°C for 5 min. The amplified PCR products were analysed by agarose gel electrophoresis (1.5% agarose in 1X TAE) at 80 V/cm for 45 min and stained with ethidium bromide (0.5 µg/ml).

The products were visualized under ultraviolet transilluminator and documented by a gel documentation system (AlphaImager). A known molecular weight marker (100 bp DNA ladder) was used for each run to compare the amplicon size. The PCR reaction was subtypes (*stx_2c, stx_2d, stx_2e and stx_2f*) encoding genes using specific primer sets as described by Wang et al. (2002) with suitable modifications. The PCR reaction was carried out in a 0.2 ml thin wall PCR tubes using the reaction mixture as described above for each isolates with a final volume of 25 µl except the concentration of each primer was 500 nM. PCR was carried out in a thermal cycler and the cycling condition consisted of initial denaturation at 95°C for 8 min followed by 30 cycles of amplification with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, ending with a final extension at 72°C for 7 min. The amplified PCR products were analyzed by agarose gel electrophoresis (1.5% agarose in 1X TAE) at 80 V/cm for 45 min and the gels were then stained with ethidium (0.5 µg/ml) and data was documented with gel documentation system (AlphaImager). A 100 bp DNA ladder was used as marker for band interpretation (Jomezadeh et al., 2009).

PCR for detection of bfpA gene

All the *eaeA* positive isolates were further subjected to PCR for the detection of *bfpA* gene (426 bp) using *bfpA* specific primer as described by Wieler et al. (1996) with suitable modifications. PCR reaction was carried out in a 0.2 ml thin wall PCR tubes with the final volume of 25 µl reaction mixture with the 2.5 µl of 10X PCR buffer (with $MgCl_2$), 2.0 µl dNTP's (10 mM), 1 µl of bfpA forward and reverse primer (500 nM), 0.2 µl of Taq polymerase (5 U/µl), 4 µl of DNA lysate and the rest is NFW (14.3 µl) to make up volume of 25 µl. PCR assay was carried out in a thermal cycler with the cycling condition consisting of initial denaturation step (94°C for 5 min) and followed by 30 cycles of amplification with denaturation at 94°C for 1 min, primer annealing at 57°C for 45 s, extension at 72°C for 1.2 min and a final extension at 72°C for 5 min. The amplified PCR products were analyzed by agarose gel electrophoresis (1.5% agarose in 1X TAE) at 80 V/cm for 45 min and stained with ethidium bromide (0.5 µg/ml). The products were visualized with UV trans-illuminator and imaged with gel documentation system (AlphaImager). A known molecular weight marker (100 bp DNA ladder) was also used for each run to compare the amplicon size (Rugeles et al., 2010). All the PCR were performed three times to record the repeatability of the result. Samples with repeated result in all the three occasions were recorded as either positive or negative.

Multiplex PCR for subtyping of Stx_2 encoding genes using specific primers

All the stx_2 positive STEC isolates were further tested for their

performed three times to ensure the repeatability of the techniques and to make sure that strains were correctly assigned to respective patterns.

Phenotypic characterization of STEC

All the STEC isolates were further characterized phenotypically by observing the cytotoxic effect on vero cell line as per the method described by OIE Terrestrial Manual (2008) as well as El Sayed Zaki and El-Adrosy (2007) with suitable modifications. Vero cell line was procured from NCCS, Pune and maintained by periodic sub culturing in laboratory.

Table 2. Virulence genes profile of *E.coli* isolated from diarrhoeic piglets.

Serogroup	Number of isolates	*stx₁*	*stx₂*	*eaeA*	*hlyA*
O9	2	-	+	-	-
O20	2	-	-	+	-
O24	2	+	-	-	-
O59	1	-	+	+	+
O60	1	-	+	+	-
O85	8	-	-	+	+
O100	3	-	+	-	+
O103	3	+	+	+	+
O112	6	-	-	+	+
O113	3	+	+	-	+
O116	2	+	-	-	+
O118	2	+	-	-	-
O119	2	-	+	+	+
O123	3	-	-	+	-
O137	2	-	+	+	-
O152	2	-	+	+	+
UT	3	-	-	+	+
Rough	1	+	-	-	+
Total	48	13	19	34	35

+ = Positive; - = Negative; UT = untypable.

Procedure for vero cell cytotoxicity assay

Colony sweeps of the STEC isolates were inoculated in Penassay broth (antibiotic medium 3 supplemented with bacitracin) (HiMedia, Mumbai, India). After inoculation, the isolates were allowed to grow by overnight incubation at 37°C and centrifuged the fluid cultures at 10,000 x *g* for 10 min. Supernatants obtained after centrifugation were filtered through 0.2 µm pore size membrane filters. The cell monolayer was washed once with the maintenance medium (DMEM, Hyclone) and volumes (50 µl) of the filtrates were applied to confluent vero cell monolayers (in 12 well plate). Cytopathic effects were evaluated after 72 h of incubation at 37°C maintained with a constant supply of 5% CO_2. Rounding of 50% or more of the cells as well as the cell sheets becoming disintegrated with blackening were considered as positive.

Data management and analysis

Data collected during sampling and laboratory analysis were entered and stored in MS Excel spreadsheet. Data were thoroughly scrutinized for errors and proper coding before subjected to statistical analysis and analyzed using SPSS soft ware version 17.0.

RESULTS

Out of 48 *E. coli* strains isolated from 320 diarrhoeic fecal samples of piglets, 44 belonged to 18 different sero-groups, 3 (6.25%) were untypeable (UT) and 1(2.08%) was recorded as rough strain (R) (Table 2). Out of total 18 somatic groups, 0.85 was found in highest number (8; 16.6%) followed by O112(6; 12.5%), O103(3; 6.25%), O123(3; 6.25%), O100(3; 6.25%), O113(3; 6.25%), O9(2; 4.16%), O24(2; 4.16%), O20(2; 4.16%), O119(2; 4.16%), O137(2; 4.16%), O116(2; 4.16%), O152(2; 4.16%), O118(2; 4.16%), O60(1; 2.08%) and O59(1; 2.08%). Similarly, out of 17 *E. coli* strains isolated from 264 diarrhoeic fecal samples of infants, 16 belonged to O60 (94.1%) and 1(5.88%) was untypeable (Table 3). Out of total 65 *E. coli* strains isolated from both piglets and infants, 50 were subjected to flagellar typing (40 from piglets and 10 from infants) after checking their motility test by hanging drop method and Craiege's tube experiment. 15 E. *coli* isolates (8 from piglets and 7 from infants) were found to be non- motile and were recorded as H^- serotype. Four flagellar types were recorded in infants: H2 (3, 17.6%; typical EPEC), H27 (5, 29.41%; atypical EPEC), H21 (2; 11.76%) and H^- (41.2%). Similarly, 6 flagellar types were recorded in piglets: H4 (10, 25%), H6 (8, 20%), H10 (6, 15%), H19 (9, 22.5%), H40 (7, 17.5%) and H- (16.7%).

A total of 1260 *E. coli* colonies were isolated from the 584 faecal samples. Of 1260 *E. coli* isolates (720 from piglets and 540 from infants) tested for four virulence genes (*stx₁, stx₂, eae*A and *hly*A), 5.16% (65) carried at least one virulence gene. 6.67% (48) from piglets were classified under 11 genotypic profiles (Table 2, Figure 1). It was also found that 0.55% (4), 0.27% (2), 0.83% (6), 0.41% (3), 0.41% (3), 0.69% (5), 0.69% (5), 1.52% (11), 0.41% (3), 0.41% (3) and 0.41% (3) were carried in the pattern of *stx₁* only, *stx₂* only, *stx₁* + *stx₂, stx₂* + *eaeA* , *stx₂* + *hlyA, stx₂* +*eaeA* + *hlyA, eaeA* only, *eaeA* + *hlyA*,

Table 3. Virulence genes profile of *E. coli* isolated from diarrhoeic infants.

Serogroup	Number of isolates	*stx*$_1$	*stx*$_2$	*eaeA*	*hlyA*
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
O60	1	-	+	+	-
O60	1	-	-	+	-
UT	1	-	-	+	-
Total	17	0	8	17	0

+ = Positive; - = Negative; UT = untypable.

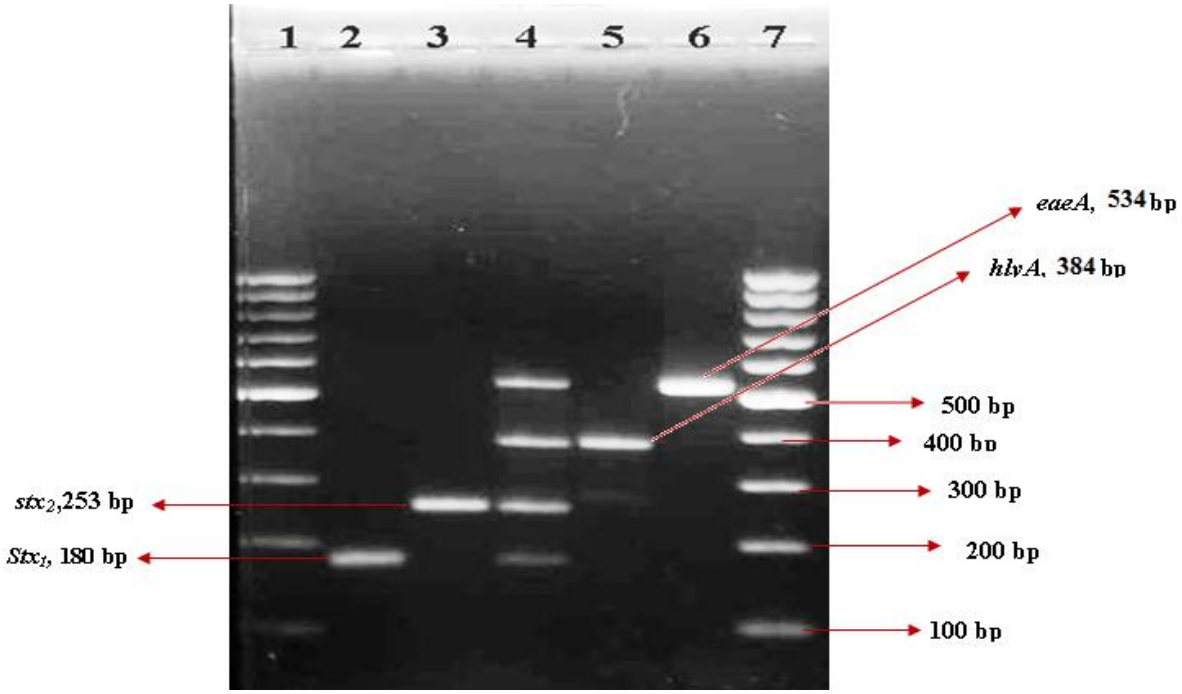

Figure 1. Multiplex PCR analysis of *E. coli* isolates for four virulence genes (*stx*$_1$, *stx*$_2$, *eaeA* and *hlyA*). Lane 1 and 7: 100 bp DNA ladder, Lane 2: *E. coli* isolate positive for *stx1* (180 bp), Lane 3: *E. coli* isolate positive for *stx2* (255 bp), Lane 4: positive JK1 control, Lane 5: *E. coli* isolate positive for *eaeA* (384 bp), Lane 6: *E. coli* isolate positive for *hlyA* (534).

stx$_1$ + *stx*$_2$ + *eaeA* + *hlyA*, *stx*$_1$ + *hlyA* and *stx*$_1$ + *stx*$_2$ + *hlyA*, respectively (Table 4). Of the total 2.14% (27) *stx*$_2$ positive *E. coli* isolates (19 from piglets and 8 from infants) subjected to subtyping by using specific primers (*stx*$_2$*c*, *stx*$_2$*d*, *stx*$_2$*e* and *stx*$_2$*f*), 1.80% (13) and 0.83% (6) from a total of 2.64% (19) were found to be positive for

Table 4. Distribution of virulence genes in *E. coli* isolated from piglets from different districts of Mizoram.

Virulent gene	No. of *E. coli* isolates and their percentage
stx$_1$ only	4 (0.55%)
stx$_2$ only	2 (0.27%)
stx$_1$ and *stx*$_2$	6 (0.83%)
stx$_2$ and *eaeA*	3 (0.41%)
stx$_2$ and *hlyA*	3 (0.41%)
stx$_2$, *eaeA* and *hlyA*	5 (0.69%)
eaeA only	5 (0.69%)
eaeA and hlyA	11(1.52%)
stx$_1$, *stx*$_2$, *eaeA,* and *hlyA*	3 (0.41%)
stx$_1$ and *hlyA*	3 (0.41%)
stx$_1$, *stx*$_2$, and *hlyA*	3 (0.41%)
Total	48 (6.67%)
STEC	32 (4.44%)
EPEC	16 (2.22%)

Similarly, out of the 3.14% (17) *E. coli* isolated from infants, 1.48% (8) were STEC and 1.66% (9) were EPEC. All the isolates were categorized into 2 genotypic profiles (Tables 3 and 5), where 1.48% (8) and 1.66% (9) were carried in *stx*$_2$ + *eaeA* and *eaeA* only, respectively.

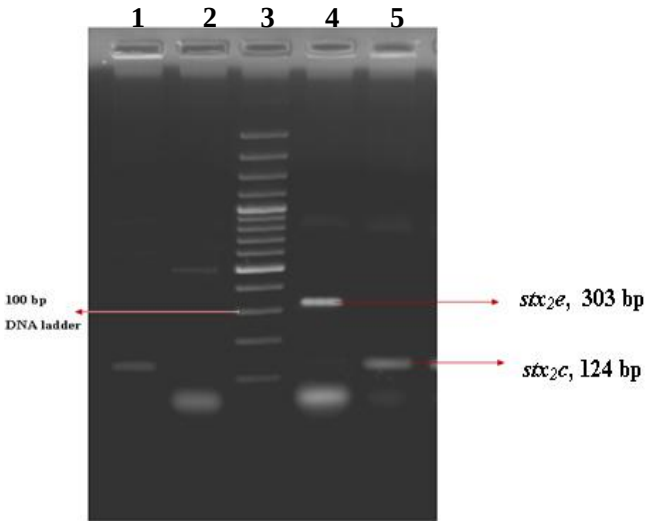

Figure 2. Subtyping of *stx*$_2$ positive *E. coli* isolates by PCR using different subtypes (stx$_2$c, stx$_2$d, stx$_2$e and stx$_2$f) specific primers. Lane 1: *E. coli* isolate positive for stx$_2$c (124 bp), Lane 2: *E. coli* isolate negative for stx$_2$ subtypes, Lane 3: 100 bp marker of DNA ladder, Lane 4 : *E. coli* isolate positive for stx$_2$e (303 bp), Lane 5: *E. coli* isolate positive for stx$_2$c (124 bp).

stx$_2$*e* and *stx*$_2$*c* subtype, respectively in piglets (Figure 2 and Table 5). Similarly, out of the total 1.48% (8) *stx*$_2$ positive *E. coli* isolates from infants, 0.93% (5) and 0.56% (3) were positive for *stx*$_2$*c* and *stx*$_2$*e* subtypes, respectively. Of the total 4.04% (51) *eaeA* positive E. *coli* isolated (34 from piglets and 17 from infants), 29.41% (15)

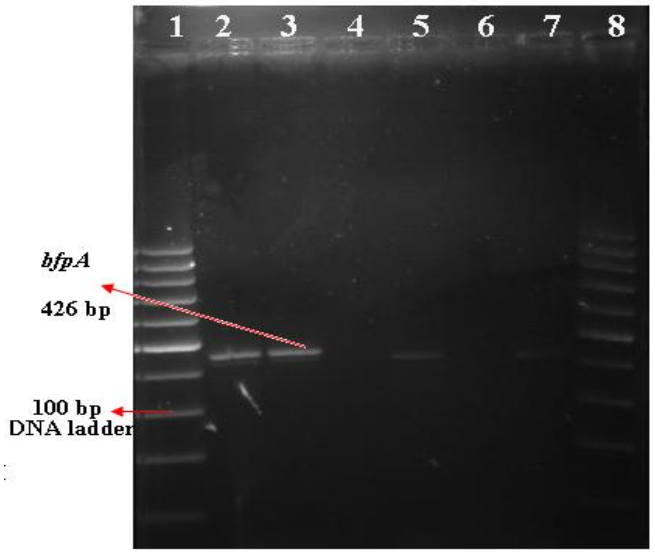

Figure 3. bfpA specific PCR for detection of *bfpA* gene in *eaeA* positive *E. coli* isolated from piglets and infants. Lane 1: 100 bp DNA ladder; Lane 2: *E. coli* isolate positive for *bfpA* (426 bp); Lane 3: *E. coli* isolate positive for *bfpA* (426 bp); Lane 4: *E. coli* isolate negative for *bfpA*; Lane 5: *E. coli* isolate positive for *bfpA* (426 bp); Lane 6: *E. coli* isolate negative for *bfpA;* Lane 7: *E. coli* isolate positive for *bfpA* (426 bp); Lane 8: 100 bp DNA ladder.

Table 5. Distribution of virulence genes in *E. coli* isolated from infants in different hospitals of Aizawl, Mizoram.

Virulent gene	No. of *E. coli* isolated
stx$_1$ only	-
stx$_2$ only	-
stx$_1$ and *stx*$_2$	-
stx$_2$ and *eaeA*	8 (1.48%)
eaeA only	9 (1.66%)
Total	17 (3.14%)
STEC	8 (1.48%)
EPEC	9 (1.66%)

(15) *E. coli* isolates were found to be positive for *bfpA* gene with an amplicon size of 426 bp, of which, 23.53% (12) were from piglets and 5.88% (3) were from infants (Figure 3).

A total of 40 STEC isolates (8 from infants and 32 from piglets) were subjected to vero cell cytotoxicity assay. All the isolates exhibited characteristic cytotoxic effect (Figure 4a to d).

DISCUSSION

Pathogenic *E. coli* are one of the most important groups of bacteria causing diarrhoea and extraintestinal infections in human and animals (Levine, 1987). The

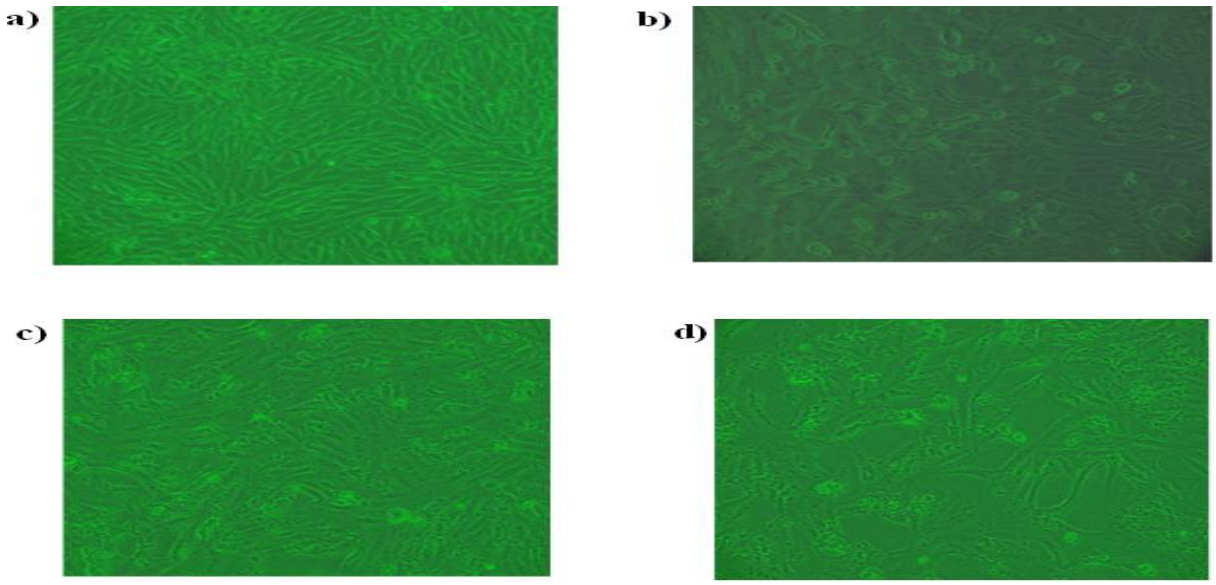

Figure 4. a) Confluent monolayer of healthy vero cells; **b)** CPE in verocells after 12 h of incubation with filtrate extracts of STEC isolated from piglets and infants (200X); **c)** CPE in vero cells after 24 h of incubation (200X); **d)** CPE in vero cells after 48 h of incubation (200X).

present study identifies different types of serotypes of *E. coli*. Remarkably large number of O85, 18.2% (8) serogroup recorded from piglets in the present study may be an indication of potential newly emerging pathogenic serogroup associated with diarrhoea in piglets in this region of India. It may pose threat to human infections in future as pigs are one of the major reservoirs of STEC. Most of the oedema disease causing STEC in pigs belongs to serogroup O8, O138, O139, O141, O147 and UT (Parma et al., 2000; Helgerson et al., 2006; Barman et al., 2008). Most of the diarrhoea causing STEC and EPEC in piglets belong to the serogroups O8, O9, O64, O101, O149, O162 (Parma et al., 2000), while most of the post weaning diarrhoea (PWD) causing STEC and EPEC in pigs belong to the serogroups O8, O138, O141, O147, O149 and O157 (Frydendahl, 2002), but Chen et al. (2004) reported that O9, O11, O20, O32, O91, O101, O107, O115, O116 and O131 as some of the novel serogroups emerged as PWD causing pathogens on pigs in some regions of China.

In case of infants, the predominant serotype recorded in this study was 060 and 94.11% (16) followed by UT, 5.88% (1). In case of infants, Pandey et al. (2003) reported O86 and O138 serogroups as pathogenic EPEC detected from patients with acute diarrhea at Kolkata, India. Rivero et al. (2010) reported O157, O145, O126, O121, O111, O118 serogroups of STEC and EPEC isolated from infants suffering from diarrhea in Argentina. In the present study O60 (n =16) was recorded as only valid serogroup. Till date, no valid data is available related with infant diarrhoea in this region of India. Although, the sample size is too low, but with the available data from the present study, O60 may be considered as the newly emerging pathogenic *E. coli* responsible for diarrhoea in infants in Mizoram. O60 serogroup was detected from both piglets 2.08% (1) and infants 94.11% (16), which suggests that there might be possibility of transmission of infection between piglets and infants. But it will be too early to mention that, common pathogenic *E. coli* are interchanging their host in this region of India. A further large scale study is required to put any valid conclusion in this aspect.

The serogrouping based on H antigen is in line with different authors throughout the world. Rivero et al. (2010) reported flagellar types H7, H^-, H11, H19 and H2 in diarrhoeic infants, whereas Ge et al. (2002) reported flgellar types H11, H7 and NM in diarrhoeic infants. A wide range of of flagellar types in diarrhoeic and non diarrhoeic infants were also reported by various others workers (Rehua-Mangia et al., 2004; Bettelheim et al., 2003; Jenkins et al., 2003). Schierack et al. (2006) recorded the prevalence of flagellar types H6, H^-, H10 and H4 in pigs.

The present study clearly indicates that the prevalence of stx_2 gene in STEC isolates were higher than stx_1 gene. This finding is in line with the works of different authors in India and international who have got higher prevalence of stx_2 gene in piglets with and without diarrhoea (Kataria, 2009; Parma et al., 2000; Helgerson et al., 2006). Therefore, presence of higher number of stx_2 (22/48) than stx_1 (16/48) indicate the magnitude of virulence in both pig and human and also indicating the possibility of transmission of such organisms between pig and human beings due to close association. In case of piglets, the

present study found that 18.75% (9) of STEC isolates harbored both *stx*$_1$ and *stx*$_2$ genes, which is higher than the result obtained by Kataria (2009) in piglets in the same region. Bhat et al. (2008) recorded 41.71% STEC harboring both *stx*$_1$ and *stx*$_2$ genes in lambs but not in piglets. These variations might be because of the species differences as well as health condition of the animals, from where samples were being collected. In the present study, out of 16 EPEC isolates, 68.75% (11) carried both *eaeA* and *hlyA* genes, while 31.25% (5) carried only *eaeA* gene. Kataria (2009) reported 80.95% (17) EPEC isolates carried *eaeA* and *hlyA* and 19.08% (4) EPEC isolates carried only *eaeA* gene.

Ghosh and Ali (2010) reported a total of 17 typical and atypical EPEC from 396 children with and without diarrhoea. The present study also supports the opinion that Shiga toxin-producing *E. coli* does not pose a major threat to human health in India. Prevalence of *eaeA* gene was higher than *stx*$_2$ gene in infant isolates in the present study. Enteropathogenic *E. coli* (EPEC) was first recognized as a cause of infantile diarrhoea in the 1940s, and was associated with outbreaks in hospitals and nurseries in most of the developing countries (Trabulsi et al., 2002; Blanco et al., 2006b; Aslani and Alikhani, 2009).

STEC and EPEC are commonly recovered from the faeces of food producing animals and pose threats to health of humans and livestock (Bhat et al., 2008; Nataro and Kaper, 1998). STEC is an emerging pathogen, which is recognized as an important cause of human sporadic and epidemic diarrhoea, hemorrhagic colitis and HUS (Gavin and Thomson, 2004). EPEC isolates are leading cause of diarrhoea, especially among infants in the developing world (Bhat et al., 2008). In India, there is a paucity of information on STEC and it has not been identified as a significant etiological agent of diarrhoea for humans (Wani et al., 2004). Till date, sporadic reports are available on isolation, identification and characterization of STEC in human and animals (Wani et al., 2004; Barman et al., 2008). Information on prevalence of STEC and EPEC in pigs is rare in India. Only few reports are available on association of STEC and EPEC in pigs and till date no reports are available for infants in this region (Mizoram) of the country. In Mizoram, pork is the major source of animal protein and people of this hilly state rear about 5 to 10 pigs near their residence and share the common source of water. Therefore, chances of getting infection of STEC and EPEC through contaminated water are very high.

Till date only a few variants of stx$_1$ and more than 20 variants of stx$_2$ have been reported (Gourmelon et al., 2006; Hussain et al., 2007). Bastian et al. (1998) reported that stx$_2$e production is specific to strains pathogenic for pigs. Many studies showed that *stx*$_2$ and *eaeA* of *E. coli* were associated with oedema disease in pigs (Wani et al., 2006). Barman et al. (2008) revealed that *stx*$_2$*e* bearing haemolytic *E. coli* was the prime cause of oedema disease in pigs in Assam state of India.

Schierack et al. (2006) reported higher prevalence of *stx*$_2$*e* positive *E. coli* isolates obtained from diarrhoeic piglet than from clinically healthy piglets. Wang et al. (2002) reported various subtypes of stx$_2$ with stx$_2$c being the highest prevalent subtype in human. Some stx variants such as stx$_2$c and stx$_2$d act are associated with an increased risk of developing HC and/or HUS (Paton et al., 1998; Friedrich et al., 2002). Wani et al. (2006) reported stx$_1$c as major prevalent subtype in calves and lambs. Zweifel et al. (2006) reported that stx$_2$e producing *E. coli* isolates from human and pigs differ in their virulence profiles and interactions with intestinal epithelial cells.

Blanco et al. (2006a) detected 5.2% (105) atypical EPEC strains (*eae*+ and *bfp*-) and 0.2% (5) typical EPEC (*eae*+ and *bfp*+) in 2015 patients investigated in Spain. Wani et al. (2006) recorded 23 *E. coli* isolates as EPEC carrying *eaeA* gene out of 326 *E. coli* isolates. Of the 23 EPEC isolates, majorities (78.26%) were recorded as atypical and 21.73% (5) were recorded as typical EPEC. Dhanashree and Mallya (2008) in Manglore, India reported that out of 110 *eaeA* positive *E. coli* isolates only 1 which was found to be positive for *bfpA*. Ghosh and Ali (2010) in Delhi, India recorded a total of 17 typical and atypical EPEC from 396 children with and without diarrhoea. Typical EPEC strains possess the bundle-forming pilus (*bfpA*) gene and have been almost exclusively isolated from humans and are well recognized as a cause of gastroenteritis in infants (Nataro and Kaper, 1998). Meanwhile, atypical EPEC do not harbor the *bfpA* gene and have been isolated from different animal species (Nataro and Kaper, 1998; Trabulsi et al., 2002; Corte´s et al., 2005).

Rivero et al. (2010) reported the confirmation of VT production in children associated with acute diarrhoea in Argentina by cytotoxicity assay on vero cell monolayers. Similar kind of observation was also reported by other worker (Leelaporn et al., 2003). The profound cytotoxicity of Stx to vero cells remains the 'gold standard' for confirmation of putative Stx-producing isolates (Paton and Paton, 1998; 2005; Cermelli et al., 2002). While the test is sensitive, it is not available in most routine diagnostic laboratories because it is labour intensive and results can take three to four days after the cell culture is inoculated. Therefore, molecular techniques such as PCR should be preferred over cytotoxicity assay for detection of STEC in future studies, as PCR is sensitive, specific and can provide effective results within few hours, but for effective confirmation of STEC, both PCR and cytotoxicity assay should be preferred.

Conclusion

In conclusion, the present study found out that STEC strains were found to be a major cause of diarrhoea in piglets while EPEC strains in infants in Mizoram state of India. The presence of higher number of *stx*$_2$ (22/48) than

stx$_1$ (16/48) indicate the highest magnitude of virulence in both pig and human and also indicating the possibility of transmission of such organisms between pig and human beings due to close association. Both PCR and Cyto-toxicity assays are required for confirmation of STEC diagnosis.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Construction of intergeneric conjugal transfer for molecular genetic studies of *Streptomyces mobaraensis* producing transglutaminase

Jae-Young Park and Sun-Uk Choi*

Department of Food Science and Biotechnology, Kyungnam University, Changwon 631-701, South Korea.

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To facilitate molecular studies of *Streptomyces mobaraensis* producing transglutaminase, an effective transformation method was established via intergeneric conjugal transfer using *Escherichia coli* ET12567 harboring the ØC31-derived integration vector, pSET152. The highest frequency was attained on ISP4 medium containing 20 mM $MgCl_2$, using 2.5 × 10^8 *E. coli* as donor and spore treated with heat treatment at 35°C for 10 min as host. The *attB* integration site in the *S. mobaraensis* genome was detected as a single *attB* site within an open reading frame coding for a pirin homolog. Its sequence showed the highest degree of homology with *S. aureofaciens*.

Key words: *Streptomyces mobaraensis*, conjugal transfer, integration site, exoconjugant, transglutaminase.

INTRODUCTION

Transglutaminase (TGase, EC 2.3.2.13) catalyzes an acyl transfer reaction between the primary grade amine and protein or γ-carboxyamide group of peptide-bound glutaminyl residues (Lorand and Conrad, 1984). TGases are used in food processing material, specifically to gel protein-rich foods after the formation of cross-links, and are present in most animal tissues and body fluids. TGases have also been discovered in microorganisms including *Bacillus subtilis* (Kobayashi et al., 1996), *Candida albicans* (Ruiz-Herrera et al., 1995), *Escherichia coli* (Schmidt et al., 1998), *Physarum polycephalum* (Klein et al., 1992), and actinobacteria (Duran et al., 1998). Among them, TGases of actinobacteria have several advantages as they are extracellular enzymes, calcium-independent, and can be produced by general fermentation at low cost (Yokoyama et al., 2004). Although *Streptomyces mobaraensis* is well known producer in actinobacteria, many approaches for increasing production of TGases have been restricted to the mutation of *S. mobaraensis*, $MgCl_2$ stress, and high level expression in heterogeneous host (Date et al., 2004; Yokoyama et al., 2010; Zhang et al., 2012),

*Corresponding author. E-mail: suchoi@kyungnam.ac.kr. Tel: +82-55-249-2258. Fax: +82-505-999-2171.

Abbreviations: ORF, Open reading frame; ***int*,** integrase gene; **ATTPR**, *attP* right; **ATTPL**, *attP* left; **ISP,** international *streptomyces* project medium; **AS-1,** amino acid soluble starch medium; **MS,** mannitol soya flour medium.

because actinobacteria has strong restriction barriers, absence of an efficient transformation system, and the inherent instability of recombinants (MacNeil, 1988; Baltz, 1998). The protoplast technique has generally been used for actinobacteria transformation. However, it suffers from low efficiency and minimal application (Matsushima and Baltz, 1996; Voeykova et al., 1998). Therefore, there has been considerable interest in the use of intergeneric conjugation as a means of transferring single-stranded DNA (Flett et al., 1997; Stegmann et al., 2001). In the present study, to promote the molecular genetic study of *S. mobaraensis*, which is well known as a representative strain for TGases production, we established an efficient transformation procedure using intergeneric conjugation as a means of transferring single-stranded DNA (Stegmann et al., 2001).

METHODOLOGY

The method used for the intergeneric conjugation was the bacteriophage ϕC31 *att*/*int* system, integrase-mediated site-specific integration, conjugally transferring a single-stranded DNA from *E. coli* into *S. mobaraensis*. In addition, the *attB* site in *S. mobaraensis* chromosome integrated with the *attP* site of a site-specific integration vector was cloned from several exconjugants of *S. mobaraensis*, and its properties were identified through sequencing and alignment.

Conjugal transfer of *S. mobaraensis*

S. mobaraensis ATCC29032 was used as a recipient and *E. coli* strain XL10-Gold (Stratagene, La Jolla, CA) was used as the general cloning host. The methylation-deficient *E. coli* strain ET12567 (*dam-13*: *Tn9*, *dcm-6*, *hsdM*, *hsdS*) containing pUZ8002, a derivative of RK2 with a defective *oriT* (*aph*), was employed as the donor in intergeneric conjugations. The site-specific integration vector, pSET152 (5.7 kb), harbors ØC31 *int*, *attP*, and *oriT* of RK2, as well as an aparamycin-resistant gene for selection in actinobacteria and *E. coli* (Bierman et al., 1992). This plasmid does not carry out the replicative functions of the actinobacteria plasmid, and can only be maintained in recipient strains in its chromosomally integrated state. The early intergeneric conjugation was carried out in accordance with the basic transconjugation protocol developed by Kieser et al. (2000). *E. coli* donor cells (0.5 mL; 2.5×10^6 - 2.5×10^8 cells) were added to the spores (1×10^4 - 1×10^6), and the mixtures were spread on MS plates containing 0 - 50 mM $MgCl_2$. For confirming the chromosomal integration of pSET152, the exconjugants were analyzed via PCR and Southern-blot hybridization.

RESULTS AND DISCUSSION

Confirmation of parameters and the *attB* site affecting efficiency of conjugal transfer

As the medium were used, it profoundly affects the successful conjugal transfer of actinobacteria, four representative media (AS-1, ISP2, ISP4, and MS) for the conjugal transfer in actinobacteria were tested to select a medium promoting the conjugal transfer of *S. mobaraensis* (Bierman et al., 1992; Flett et al., 1997; Choi et al., 2004). Of the four media, no exconjugants were obtained with AS-1 and ISP2, although AS-1 has been frequently employed in the conjugal transfer of streptomycetes and ISP2 also was the base media used for the growth and spore formation of *S. mobaraensis*. ISP4 and MS were suitable for the conjugation of *S. mobaraensis*; the transconjugation efficiencies of ISP4 and MS were 3.4×10^{-4} and 8.1×10^{-5}, respectively. ISP4 and MS could be employed for the conjugation of *S. mobaraensis*. However, ISP4 medium was selected as the most appropriate medium in the present work and was employed in all subsequent experiments due to its 4.2-fold higher conjugation efficiency than that observed in the MS medium. Recipient spores employed for conjugation are often subjected to heat treatments before being mixed with *E. coli* donor cells for conjugal transfer in actinobacteria (Kieser et al., 2000), because the heat treatment promotes spore germination to increase efficient conjugation (Mazodier et al., 1989) and may be effective in temporarily reducing the restriction barrier (Bailey and Winstanley, 1986). To determine the appropriate temperature for spore germination, the heat tolerance of *S. mobaraensis* spores was first examined in a temperature range between 25 (control) to 60°C. As shown in Figure 1, although the spores incubated at 55°C for 10 min quickly lost viability, viability of spore was the highest by the heat treatment at 45°C as it increased spore germination, and was maintained over 100% until 50°C, appeared to be more tolerance to temperature than other actinobacteria. Based on this result, the heat treatment of spores for conjugal transfer was performed below 50°C. The transconjugation frequency was increased by the heat treatment of spores until 45°C compared with control (Figure 1). Specifically, because heat treatment at 35°C gave the highest conjugation efficiency, it was selected as the most suitable temperature for the heat treatment of spores. Since $MgCl_2$ added to the medium for conjugal transfer affects the conjugation efficiency (Kieser et al., 2000) and its optimal concentration differs with strains (Choi et al., 2004), the optimal concentration of $MgCl_2$ added to the ISP4 medium used for conjugation of *S. mobaraensis* was surveyed. When 0 ~ 50 mM $MgCl_2$ was added to ISP4 medium, 20 mM yielded the highest efficiency for *S. mobaraensis*, and its conjugation efficiency was 3.5-fold higher than that of 10 mM, although all concentration of added $MgCl_2$ increased the transconjugation frequency (data not shown). The mixing ratio of the number of recipient spores and *E. coli* donor cells is important in actinobacteria conjugation (Choi et al., 2004). In the conjugal transfer of *Kitasatospora setae*, a standard number of *E. coli* donor cells (2.5×10^7) yielded no exconjugants, but transconjugation was possible with a further increase in the number of *E. coli* donor cells ($\geq 1.25 \times 10^8$) (Choi et al., 2004). As shown in Table 1,

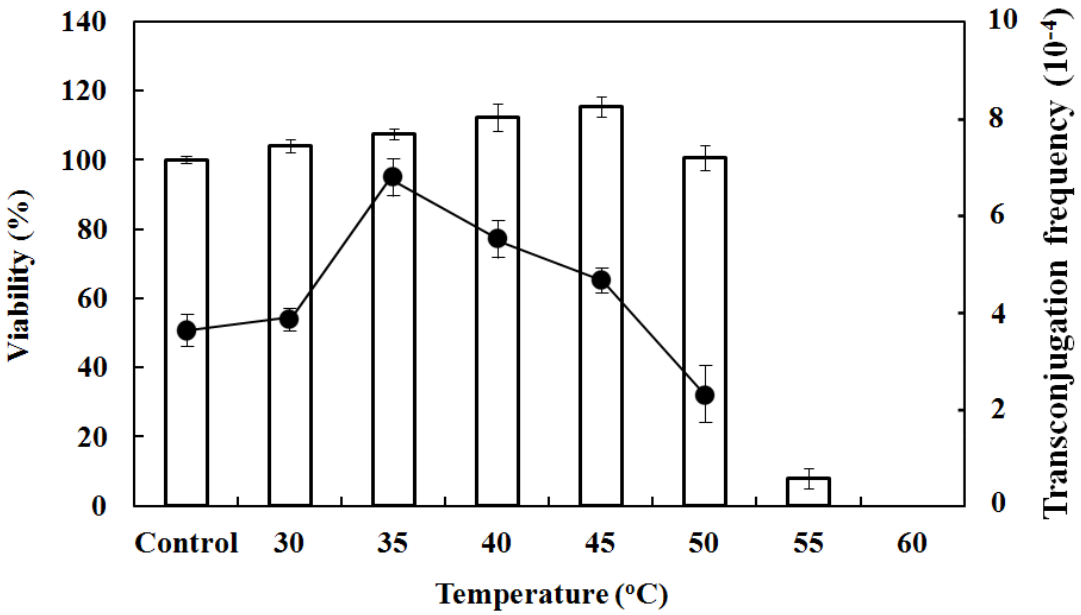

Figure 1. Effects of temperature on the viability of *S. mobaraensis* spores (bar graph) and the heat treatment of spores on transconjugation efficiency (line graph). For measuring spore viability after the heat treatment of spores, spores (1×10^3) that were incubated for 10 min at the temperatures indicated were resuspended with 0.5 mL of 2 × YT broth (1% yeast extract, 1.6% tryptone, 0.5% NaCl) at room temperature (25°C). Viability was determined by counting colonies on ISP2 medium after 12 days of incubation at 28°C. Control means no heat treatment (25°C). For measuring transconjugation efficiency after the heat treatment of spores, a culture of the donor *E. coli* ET12567/pUZ8002 harboring pSET152 was grown to an OD_{600} of 0.4 in the presence of 50 mg/L aparamycin, 25 mg/L chloramphenicol, and 50 mg/L kanamycin. To remove the antibiotics, the cells were washed twice in an equal volume of LB and resuspended in 0.1 volume of LB. *E. coli* (2.5×10^7) were added to the resuspended spores, and the mixtures were spread on ISP4 plates containing 10 mM $MgCl_2$. The conjugation plates were incubated for 16 - 18 h at 28°C and overlaid with 1.5 mL water containing 0.5 mg of nalidixic acid and 1 mg of aparamycin. The plates were subsequently incubated for 12 day at 28°C. The results represent SD of three experiments (n=3 per experiment).

Table 1. Effects of the number of donor *E. coli* on the number of recipient spores for transconjugation efficiency.

Number of recipient spores	Transconjugation frequency[a]		
	2.5×10^6 *E. coli*	2.5×10^7 *E. coli*	2.5×10^8 *E. coli*
1×10^4	0	4.5×10^{-4}	1.3×10^{-2}
1×10^5	1.1×10^{-4}	3.5×10^{-4}	2.2×10^{-3}
1×10^6	1.3×10^{-4}	3.9×10^{-4}	7.1×10^{-4}

[a]Data are the average of three independent experiments.

with 1×10^4 recipient spores of *S. mobaraensis*, 2.5×10^6 *E. coli* donor cells yielded no exconjugants, but the increase in the number of *E. coli* donor cells (≥2.5×10^7) made conjugal transfer of *S. mobaraensis* possible. The increase of the number of recipient spores without increase of *E. coli* donor cells did not affect the transconjugation frequency. Therefore, this suggests that when increased numbers of recipient spores are used and no exconjugants are present, the number of *E. coli* donor cells must be increased for transconjugation efficiency.

Conjugal transfer of actinobacteria was carried out by integration of vector (pSET152) having an *attP* site into the *attB* locus in the recipient chromosome via the integrase (*int*) function. All of the *attB* sites of actinobacteria lay within an open reading frame (ORF) coding for pirin (a newly identified nuclear protein that interacts with Bcl-3 and nuclear factor I) (Choi et al., 2004). However, some strains have another pseudo-*attB* site or no *attB* site in their genome (Combes et al., 2002). These cause potential problems such as mutagenesis, inducing phenotypic changes, or no transconjugant

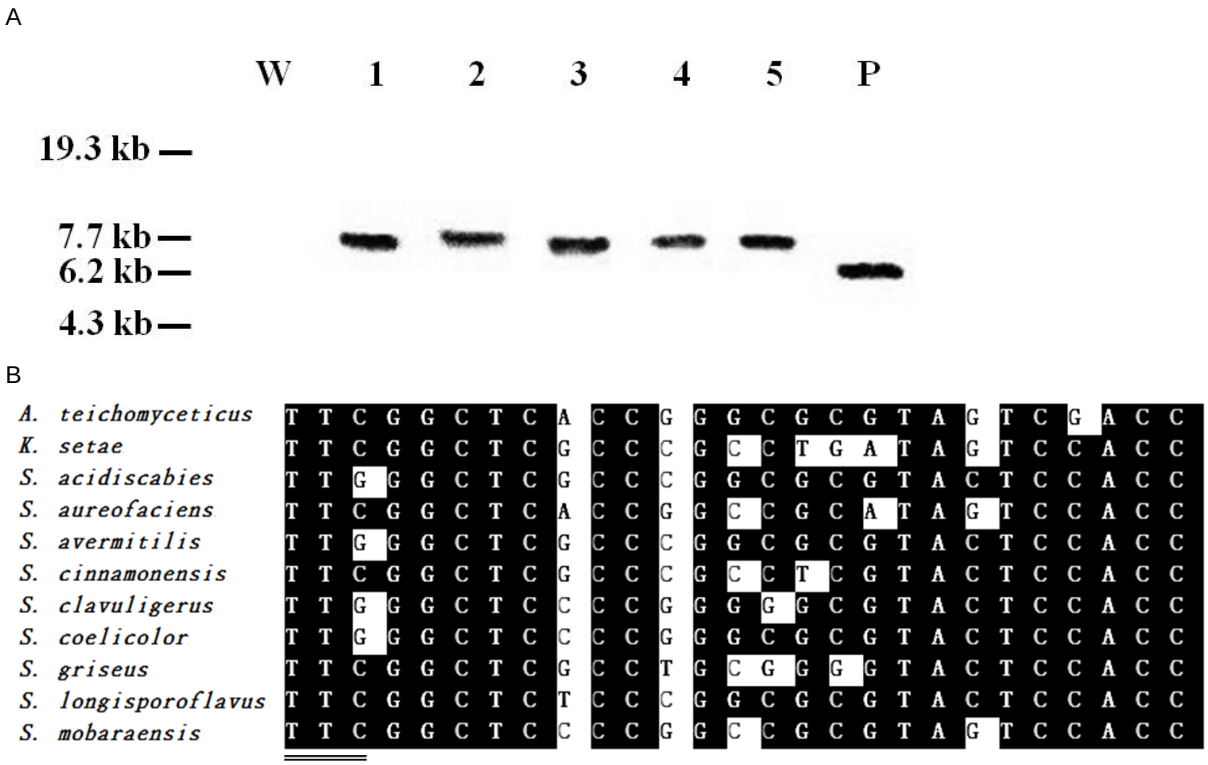

Figure 2. Southern blot analysis of *Kpn*I-digested genomic DNAs of exconjugants (a) and Alignment of the *attB* site sequences of *S. mobaraensis* and other actinomycetes (b). (a) Lanes: lane W, wild-type *S. mobaraensis*; lanes 1 ~ 5, exconjugants containing pSET152; lane P, plasmid pSET152. Genomic DNA from the wild-type strain and exconjugants were digested with *KpnI*. pSET152 was digested with *Bam*HI (lane P). The DNA was blotted onto a nylon membrane and hybridized with a DIG-labeled 0.5-kb aparamycin-resistant fragment of pSET152. (b) *Actinoplanes teichomyceticus* NBRC13999, *K. setae* NBRC14216, *S. acidiscabies* ATCC49003, *S. aureofaciens*, *S. avermitilis* MA-4680, *S. cinnamonensis*, *S. clavuligerus*, *S. coelicolor* A3(2) M145, *S. griseus* ATCC12475, *S. longisporoflavus* 83E6, *S. mobaraensis* ATCC29032 (present study). Under double line represents the core sequence of *attB* site integrated with the *attP* site.

(Gregory et al., 2003). To identify the *attB* site of *S. mobaraensis*, genomic DNA of exconjugants were prepared, digested by *KpnI* that was not included in pSET152, and then confirmed by Southern hybridization using a 0.5 kb aparamycin resistance fragment of pSET152 as a probe. As shown in Figure 2a, all of the exconjugants showed an equal single band pattern with Southern hybridization, suggesting that the *attB* site integrated with the *attP* site of pSET152 is a unique site in the *S. mobaraensis* chromosome. Genomic DNA fragments bearing the integration site were rescued as plasmids from pSET152-integrated exconjugants following *Kpn*I digestion by self-ligation and transformation into *E. coli*. Plasmid sequencing using the primers ATTPR (5'-CTGGGTGGGTTACACGACGCCCCT-3') and ATTPL(5'-CGTTGGCGCTACGCTGTGTCGCTG-3') revealed that all of the plasmids harbored a right-flanking region of the insertion site in their genomes, and the same insertion endpoints within the ORF coding for pirin (data not shown). Presently, the sequence of the right-flanking region of the *attB* site in *S. mobaraensis* was determined for the first time and registered as a core region of the *attB* site for the insertion of ØC31 *attP* derived from *S. mobaraensis* ATCC49003 in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB819044. However, although no clear data are available regarding the reason, the left-flanking region of the *attB* site in *S. mobaraensis* genomes was not detected in this study, unlike the case of other actinobacteria, despite high transconjugation efficiency. The core sequence (TTS) of *attB* site integrated with the *attP* site was found to be TTC in *S. mobaraensis* (Figure 2b). Also, the right-flanking region of the *attB* site of *S. mobaraensis* exhibited the highest levels of homology (92.6% nucleotide identity) with that of *S. aureofaciens*. The observations recorded in this investigation provide sufficient efficiency to enable conjugal transfer of genetic elements through *attB/P*-mediated site-specific integration for *S. mobaraensis*, and also should facilitate molecular genetic studies in this strain because all pSET152-integrated exconjugants revealed phenotypes identical to those of wild-type *S. scabies* (data not shown).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effects of auxins on *in vitro* reserve compounds of *Phalaenopsis amabilis* (Orchidaceae)

Sandra Sayuri Ori, Edison Paulo Chu* and Armando Reis Tavares

Institute of Botany, C. Postal 3005, 01061-970 São Paulo, Brazil.

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The effects of auxin and the endogenous levels of reserve compounds of *Phalaenopsis amabilis* (L.) Blume (Orchidaceae) were analyzed *in vitro*. Rootless plants were inoculated in modified MS media supplemented with IBA or NAA (0.0, 0.2, 1.0 and 5.0 mg L^{-1}) and with 2,4-D (0.000, 0.032, 0.160 and 0.800 mg L^{-1}). The biochemical parameters of endogenous levels of soluble carbohydrates and starch and of total soluble protein in roots, leaves and shoots were analyzed after 30 and 120 days. Carbohydrate levels in leaves showed similar patterns for all treatments. At 30 days, there was an increase in the endogenous carbohydrate level along with an increase in the concentration of auxins. At 120 days, the endogenous carbohydrate level in leaves had decreased, while the auxin concentration had continued to increase, demonstrating the mobilization of the carbohydrates. The leaf carbohydrate levels decreased from day 30 to 120; for both IBA and 2,4-D treatments, there was starch accumulation in roots as a function of the collection date. The 2,4-D concentration of 0.0032 mg L^{-1} decreased the level of total soluble protein in roots. The *in vitro* plants exhibit different growth patterns depending on the classes and concentrations of growth regulators. Biochemical analyses exhibited that metabolic activity and the degradation and accumulation of substances occurs in leaves, roots and shoots, demonstrating that roots contribute to the maintenance of plant metabolism and also act as reserve organs, even in epiphytic plants.

Key words: Storage compounds, soluble carbohydrates, starch, soluble proteins.

INTRODUCTION

The genus *Phalaenopsis* has sixty described species and thousands of hybrids derived from crosses between *Phalaenopsis amabilis* (L.) Blume and *Phalaenopsis stuartiana* (Rchb. f) (Harper, 2004). Originally from Northern Australia, Southeast Asia, the Himalayas, Indonesia and the Philippines (Arditti, 2007), these ornamental plants have high commercial value on the international market as one of the few orchids to bloom every six months with flowers of varied colors, ranging from white to bright red, and with long flower durability of up to 45 days. The species has epiphytic monopodial growth, with leaves arranged alternately from the buds, and its commercial propagation is performed by germinating seeds under aseptic conditions, as side-by-side shoots (Arditti, 2007). The *in vitro* culture techniques used for this species have a high propagation rate com-

*Corresponding author. E-mail: chu07@hotmail.com.

pared to conventional methods, with larger quantities of seedlings, better health, high ornamental value and production during a short time (Bosa et al., 2003).

The roots of epiphytic orchids have a complex structure that acts as a storage system for water and nutrients, is responsible for more than 70% of the plant's photosynthesis (Winter and Holtum, 2002) and also functions as protective tissue (Arditti, 2007). Auxins are the only growth regulators that increase consistently with root growth and are responsible for the formation of root primordia, at least in tissues that naturally have a predisposition to root (De Klerk, 2002). Auxins also influence shoot development, cell proliferation and elongation, and high concentrations are lethal with herbicidal activity (George, 1996).

The main compounds of plant reserves, including those in roots, are carbohydrates (starch and water-soluble carbohydrates), acting as an energy source for growth and as carbon skeletons for young tissue, as well as maintaining the osmotic potential of the cell (Itai and Birnbaum, 1996). Proteins correspond to approximately 30% of the total dry mass of a typical plant (Taiz and Zeiger, 2003), and they become part of the cytoskeleton (microtubules and microfilaments), protein reserves in seeds (globulins and prolamins), enzymes, and smaller amounts of peptides and amino acids. In a heterotrophic culture in which the main carbon source (sucrose), organic and inorganic salts are provided by the semi-synthetic substrate on which the plant depends throughout its growth and capacity to absorb these nutrients, which is activated by the differentiation of organs (buds and roots as large users of nutrients) and coordinated by a specific balance of auxin and cytokinin that also permits vegetative propagation.

Meristems of plants are nutrient sinks due to their high division rate, which is related to auxin induction of cell proliferation (Hartig and Beck, 2006), and multiple studies demonstrate that there are several functional types of auxin (Simon and Petrásek, 2011). The increase in soluble carbohydrate flux upon correct auxin action correlates with the C:N ratios, osmotic potentials, and formation of new organs (buds and adventitious roots), and auxins also function as signaling molecules (Gibson, 2005; Börner, 2011).

Carbohydrates and auxins function as signaling molecules and drivers of growth and developmental processes. Auxin metabolism is also regulated by the availability of free sugars, and the regulation of the biosynthesis and degradation of the main auxin, indole-3-acetic acid (IAA) by sugars, requires changes in the expression of multiple genes and metabolites linked to several IAA biosynthetic pathways (Sairanen et al., 2012).

The present study aimed to correlate the effects of different concentrations of the auxins IBA (indole-butyric acid), NAA (α-naphthalene-acetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) with the endogenous levels of soluble carbohydrates, starch and proteins from plants of *P. amabilis* hybrids cultivated *in vitro*, to obtain plants with greater levels of reserve compounds that would thus exhibit better growth.

MATERIALS AND METHODS

Culture of plants

Plantlets of *P. amabilis* were obtained from *in vitro* seed germination on MS medium (Murashige and Skoog, 1962) modified with 10% macronutrient, 30 g L^{-1} sucrose, agar (0.8%), pH 5.8 and autoclaved (15 min at 121°C, 1.2 atm). The plantlets were cultivated in 550 ml glass bottles with polypropylene caps filled with hydrophobic cotton and maintained in a culture room (temperature of 27 ± 2°C, photoperiod of 12 h and 25.0 µmol m^{-2} s^{-1} of photosynthetic active radiation (PAR). After 360 days, plants were selected based on the following criteria: 5.0 cm high, 2.0 cm wide and 2 leaves. Their roots were removed aseptically and transferred to auxin treatments (Sigma-Aldrich reagents): NAA (0.0, 0.2, 1.0 and 5.0 mg L^{-1}) or IBA (0.0, 0.2, 1.0 and 5.0 mg L^{-1}) and 2,4-D (0.0, 0.032, 0.160 and 0.800 mg L^{-1}) added to liquid of modified MS medium (same composition and process as described above). The treatments consisted of 10 glass bottles (as described above) with five plants each, in which 60 ml of culture medium was renewed every 30 days for each auxin and concentration, and the plants were maintained under the same culture conditions.

Two treatment bottles were randomly collected at 30 and 120 days of cultivation, totaling 10 plants analyzed per treatment. Plants were separated (leaves, roots and shoots), weighed for fresh mass, lyophilized and weighed for dry mass, ground in a knife micro-mill (0.2 mm mesh sieve) and divided into three samples of 100 mg for biochemical analysis.

Biochemical analysis

Extraction and determination of soluble carbohydrates

For each treatment and organ sample ground and lyophilized (100 mg), three extractions with 5 ml of 80% ethanol (homogenized for 10 min in each extraction) were made. These extracts was then centrifuged (1,000 *g* for 10 min at room temperature) resulting in the combined ethanolic supernatant extract, and its volume was measured.

The soluble carbohydrates were determined in triplicate using the phenol-sulfuric acid method (Dubois et al., 1956) and read on a spectrophotometer at 490 nm. The values were expressed as soluble carbohydrates (mg g^{-1} dry weight of soluble carbohydrate) with D-glucose as the standard.

Extraction and determination of total soluble protein

After extracting the ethanolic fraction, 5 ml 0.2 M phosphate buffer (pH 6.7) was added for each residue, homogenized for 10 min and centrifuged (1,000 *g* at room temperature), with triplicate of extraction, resulting in the soluble protein supernatant. The supernatants were collected and combined with the corresponding fraction, and the final volume was measured. Protein in the supernatant was determined by a dye-binding assay (Bradford, 1976) in triplicate, using bovine albumin as the standard. Biochemical measurements were performed on a spectrophotometer at 595 nm, and the content of total soluble protein was expressed as mg g^{-1} dry weight of soluble protein.

Extraction and determination of starch

For starch extraction, the previous residue was used with the addition of 5.0 ml perchloric acid (52% v v^{-1}), homogenized at 4°C for 15 min with periodic shaking, and centrifuged at 1,000 *g* at room temperature for 10 min, with two extractions. The supernatants were collected and their volumes were measured, yielding the starch extract, and the residue was discarded. The concentration of starch was calculated using the phenol-sulfuric acid method, with D-glucose as the standard (McCready et al., 1950) and multiplied by the correction factor 0.9 (estimated for plant starch), and the content was expressed as mg g^{-1} dry weight of starch, using the same standard curve of D-glucose as before.

Statistical analysis

Comparisons of the mean levels of soluble carbohydrates and starch and total soluble protein from leaves, roots and shoots at 30 and 120 days of growth were performed by analysis of variance (ANOVA) and the Turkey test. Measurements were conducted on three samples per experiment, and assays were performed in triplicate. Differences were considered significant at $p \leq 0.05$.

RESULTS

The level of soluble carbohydrates in roots (Figures 1B, 2B and 3B) in control samples of *P. amabilis* increased from day 30 to day 120, but it decreased in leaves (Figures 1A, 2A and 3A). The addition of the auxins IBA and 2,4-D reduced the carbohydrate levels in roots from day 30 to day 120 (Figures 1B and 2B), but treatment with NAA had no effect on root carbohydrate levels (Figure 3B).

However, with 5.0 mgL^{-1} NAA, a lethal herbicide effect was observed. Carbohydrate concentrations in leaves were reduced (Figures 1A, 2A and 3A) in all assays. Levels of soluble carbohydrates in shoots induced with 0.160 mg L^{-1} 2,4-D (Figure 2C) was high (approximately 250 mg g^{-1} dry mass) at day 30 and reduced by 80% at day 120 of the same 2,4-D treatment. Levels of soluble carbohydrates in plants treated with 5.0 mgL^{-1} NAA (Figure 3C) for day 30 was similar to those in plants treated with 2,4-D, but NAA was lethal for plants by 120 days.

Starch levels in control roots of *P. amabilis* (Figures 1E, 2E and 3E) were higher at day 120 than at day 30. Starch concentrations in roots increased at day 30 in treatments with 2,4-D (Figure 2E) and IBA (Figure 1E), except for the 5.0 mg L^{-1} IBA treatment. In NAA treatments (Figure 3E), starch levels were constant for 0.2 and 1.0 mgL^{-1} NAA at 30 and 120 days; the lowest concentration was observed for 5.0 mg L^{-1} NAA at 120 days, and there was no root induction with 5.0 mg L^{-1} NAA at 30 days. Leaf starch levels in plants treated with IBA (Figure 1D), NAA (Figure 3D) and 2,4-D (Figure 2D) were higher at day 30 than at day 120.

Shoots induced with 0.160 mgL^{-1} 2,4-D (Figure 2C, 2F and 2I) showed high levels of soluble carbohydrates, starch (both with 200 mgg^{-1} dry weight) and soluble proteins at 30 days, and a reduction was observed (75%) at 120 days, possibly due to the deleterious effects of auxin action in these tissues, not allowing their normal growth. The reserve carbohydrates (starch and soluble) were quantified at approximately 425 mgg^{-1} dry weight of leaves in the treatment with 0.160 mgL^{-1} 2,4-D at 30 days (Figure 4D). Higher amounts of reserve carbohydrate (starch and soluble) were observed in roots, at approximately 480mgg^{-1} dry weight for the 0.2 mgL^{-1} IBA treatment at 30 days (Figure 4B).

Total soluble protein levels varied between 5.0 and 8.0 mgg^{-1} dry weight in leaves of *P. amabilis* (Figures 1G, 2G and 3G), roots (Figures 2H and 3H) and shoots in treatments with 2,4-D and NAA (Figures 2I and 3I). Levels of total soluble protein over 10 mgg^{-1} dry weight were observed in roots upon treatment with 0.032 mgL^{-1} 2,4-D for 30 days, possibly related to the initial phase of organ tissues in multiplication and differentiation, and in shoots upon treatment with 0.160 mgL^{-1} 2,4-D for 120 days (Figure 2I), showing an 88% reduction in soluble protein levels. All other measurements of total soluble protein from leaves and roots showed constant levels (Figures 1G, 1H, 2G, 2H, 3G and 3H).

DISCUSSION

Research into orchids and their growth and biochemical compounds are scarce when compared with studies of *Solanum tuberosum*, a tuberous dicot rich in starch (60-75% dry weight). Both the leaves and roots of *P. amabilis* are rich in carbohydrates (soluble and starch) that may allow their re-mobilization under certain culture and propagation conditions.

Stancato et al. (2002), using pseudobulbs of *Cattleya forbessii* Lindl. × *Laelia tenebrosa* Rolf. described the translocation of reserve compounds from mature shoots to young as developing tissues, showing a direct relationship between source (mature photosynthetic tissues) and drain. Vaz et al. (2004) observed that long days also increase the concentration of starch and soluble carbohydrates in *Psygmorchis pusilla* Dodson and Dressler, an epiphytic orchid. Suzuki (2005) compared the amounts of soluble carbohydrates in the shoots and sub apical regions of *C. fimbriatum* and observed an inverse relationship between the two samples analyzed: the shoot contained approximately 25 mgg^{-1} fresh mass of soluble carbohydrates, with a lower concentration in the sub apical tissue, at approximately 5.0 mgg^{-1} fresh mass of soluble carbohydrates. Increased sucrose concentration in *Dendrobium* leads to a high level of total carbohydrates and starch, and the high level of soluble sugars allows for shoot elongation and growth (metabolic sink) (Ferreira et al., 2011).

Leaves of *P. amabilis* showed higher levels of reserve carbohydrates, mainly soluble carbohydrates, upon treatment with IBA (0.2 mgL^{-1}), NAA (1.0 mgL^{-1}) and 2,4-D (0.160 mgL^{-1}), corresponding to approximately 180 mgg^{-1}

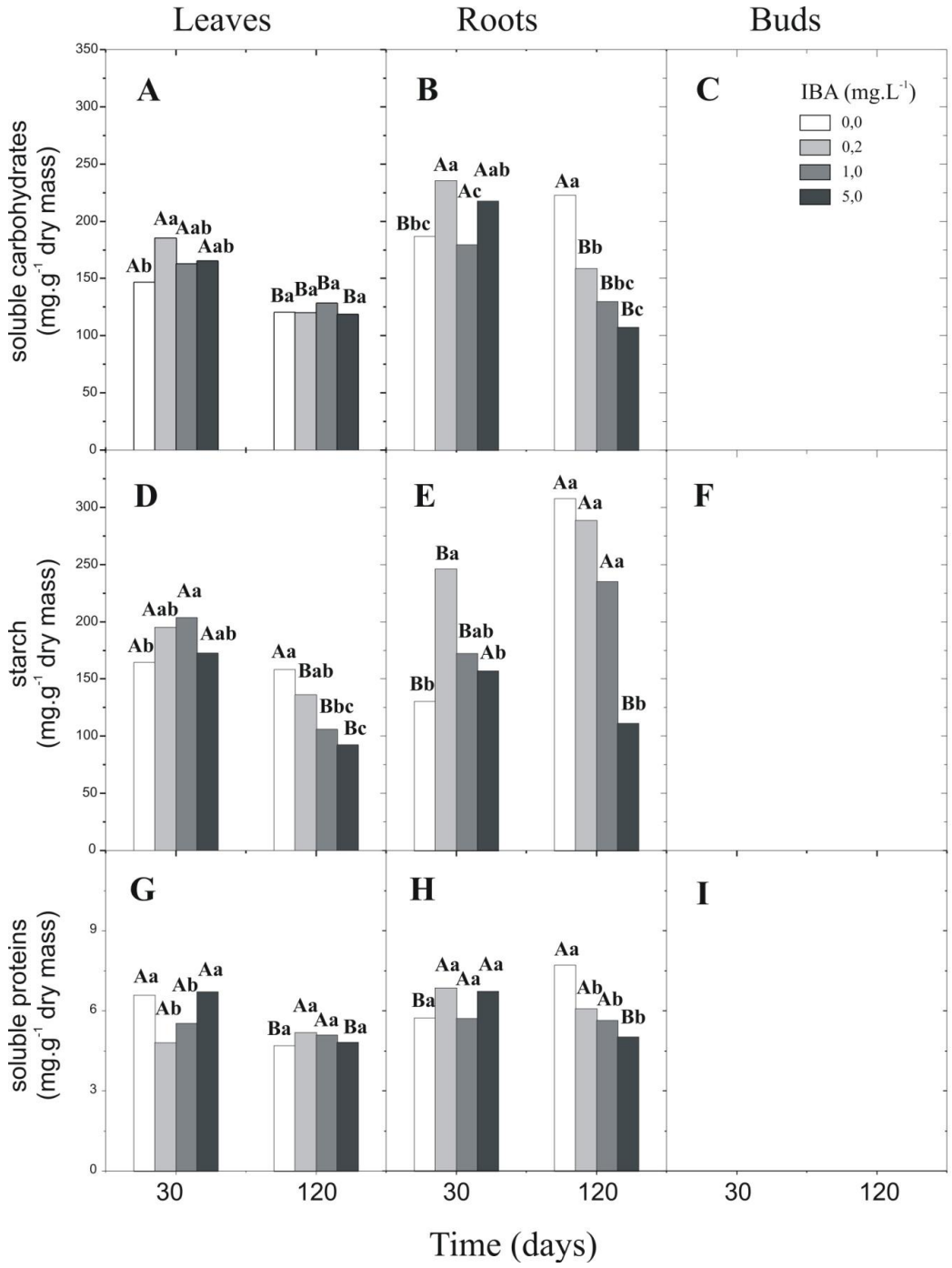

Figure 1. *In vitro* soluble carbohydrates, starch and soluble proteins in leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with IBA at concentrations of 0.0, 0.2, 1.0 and 5.0 mg L^{-1}, (*) there was no induction of shoots in this treatment; means followed by different capital letters indicate significant differences ($P \leq 0.05$) between the treatments and the lowercase letters indicate significant differences ($P \leq 0.05$) between the concentrations of growth regulators.

dry weight at 30 days. *P. amabilis* also has a higher accumulation of starch in leaves (200 and 250 mg g^{-1} dry weight) upon treatment with IBA (0.2 and 1.0 mgL^{-1}), NAA (0.2 and 1.0 mgL^{-1}) and 2,4-D (0.032 and 0.160 mgL^{-1}), resulting in five times more reserves than shown in other studies. This accumulation of carbohydrates may be related to the initial growth cycle in culture, which is followed by a decline upon growth arrest, with cell division having employed all available carbon molecules. This would preclude the existence of sink organs as

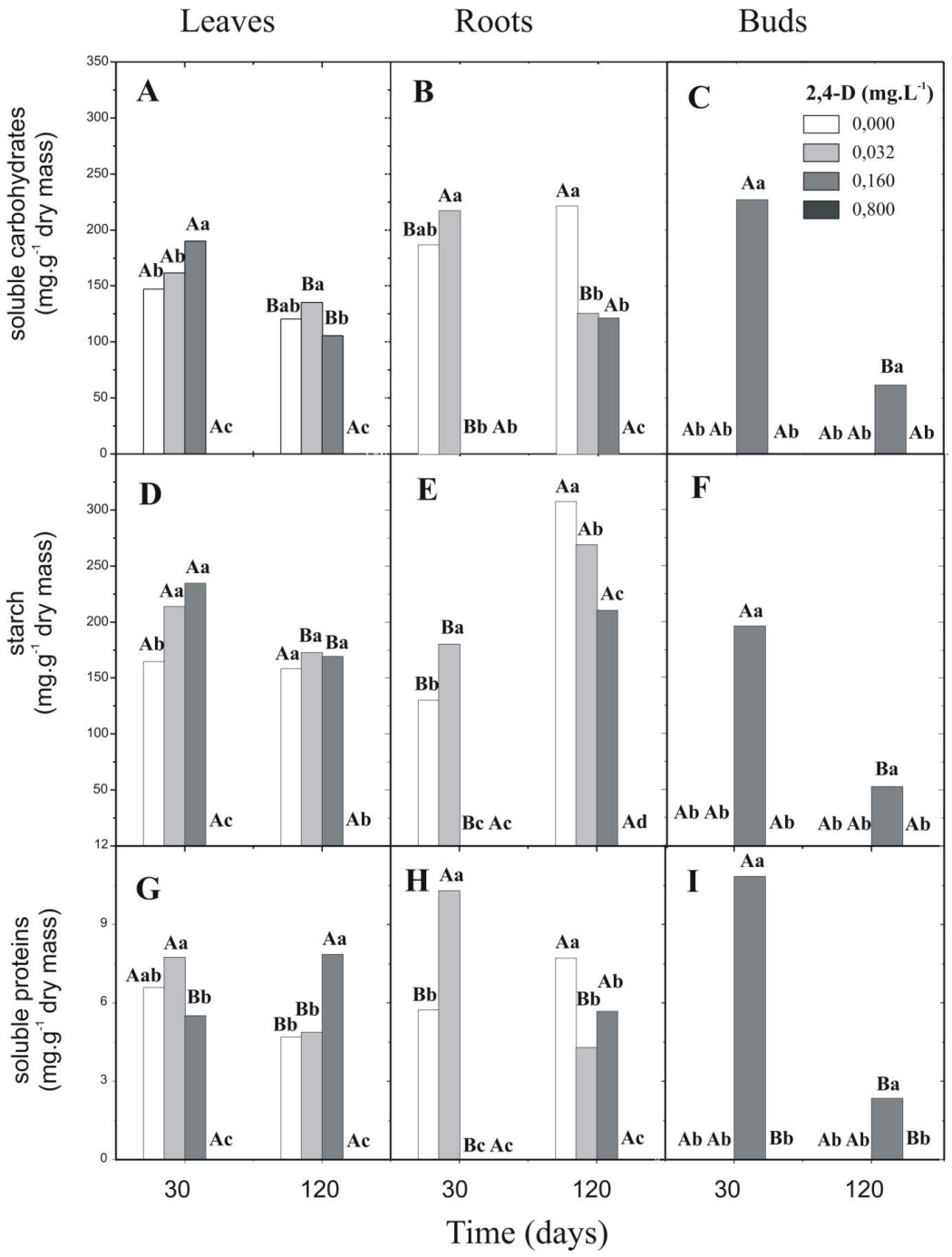

Figure 2. *In vitro* soluble carbohydrates, starch and soluble proteins in leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with 2,4-D at concentrations of 0.000, 0.032, 0.160 and 0.800 mg L^{-1}; means followed by different capital letters indicate significant differences ($P \leq 0.05$) between the treatments and the lowercase letters indicate significant differences ($P \leq 0.05$) between the concentrations of growth regulators.

reported for the life-cycle of sugarcane suspension cells (Goldner et al., 1991). In the callus cells of tomato cotyledons, starch accumulation seems to be a prerequisite for the *in vitro* development of shoots during the first days in culture, irrespective of the future development of the explants, and therefore the culture regime does not influence amylogenesis or changes in the protein pattern at a relatively late stage, when cell differentiation is visible (Thorpe et al., 1986; Stamp, 1987; Branca et al., 1994). Starch can act as a storage reserve

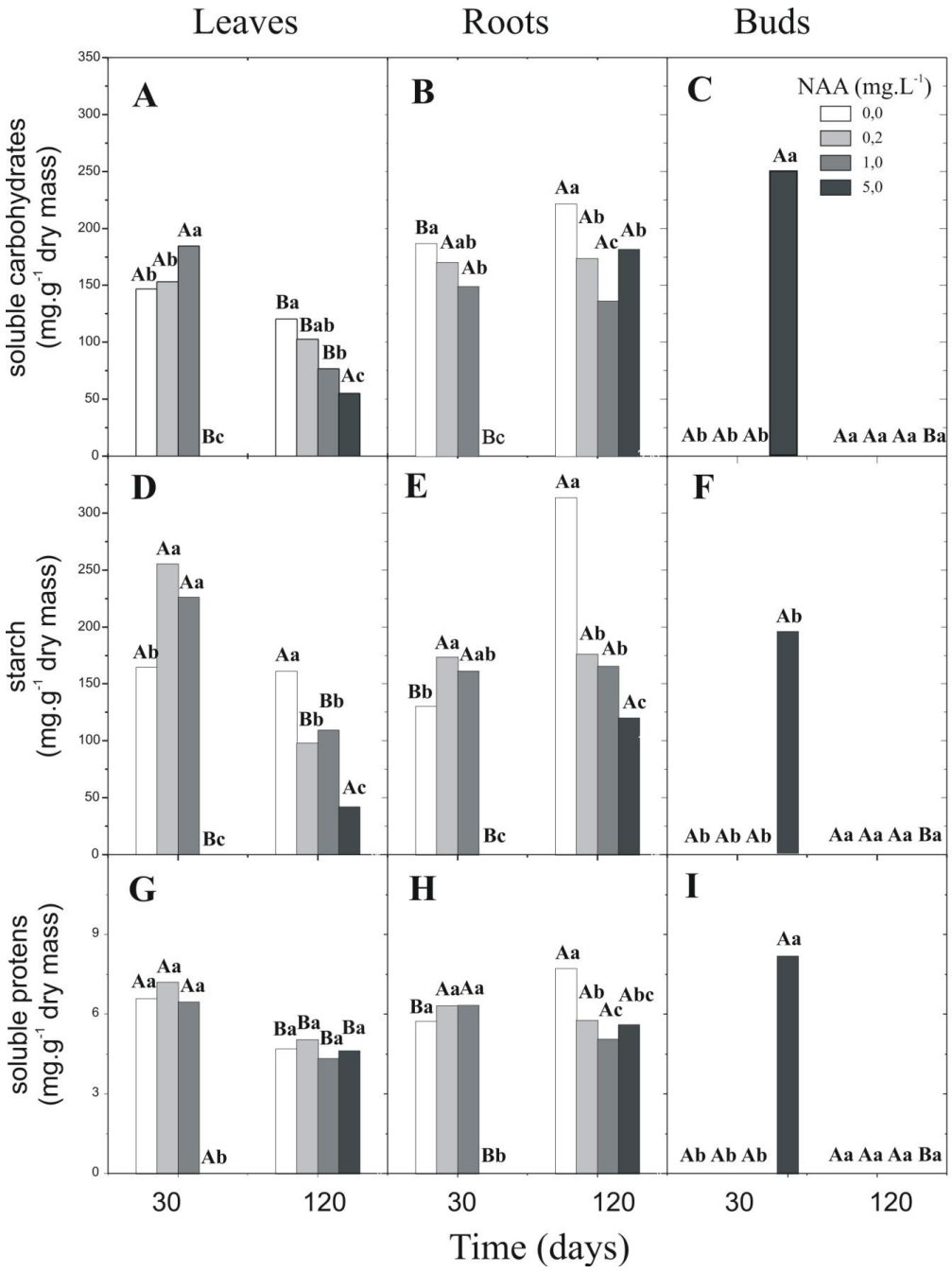

Figure 3. *In vitro* soluble carbohydrates, starch and soluble proteins in leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with NAA at concentrations of 0.0, 0.2, 1.0 and 5.0 mg L^{-1}; means followed by different capital letters indicate significant differences ($P \leq 0.05$) between the treatments and the lowercase letters indicate significant differences ($P \leq 0.05$) between the concentrations of growth regulators.

to support plant respiration and growth through the night, and several projects have focused on the synthesis and turnover of starches (Börner, 2011) and their mobilization in the acclimatization phase as important compounds during root and stem development.

Catasetum fimbriatum (Morren) Lindl. upon 90 days of *in vitro* culture, showed carbohydrate levels of 190 mgg^{-1} dry weight in mature roots, but lower levels were observed upon treatment with IBA treatment (0.061 mgL^{-1}) for 10 days, with approximately 240 to 150 mgg^{-1} dry weight at 30 days of culture. Similar effects were observed for the concentration of starch (approximately 50 mgg^{-1} dry mass for each compound in reserve), with the tips of roots and shoots showing approximately 300

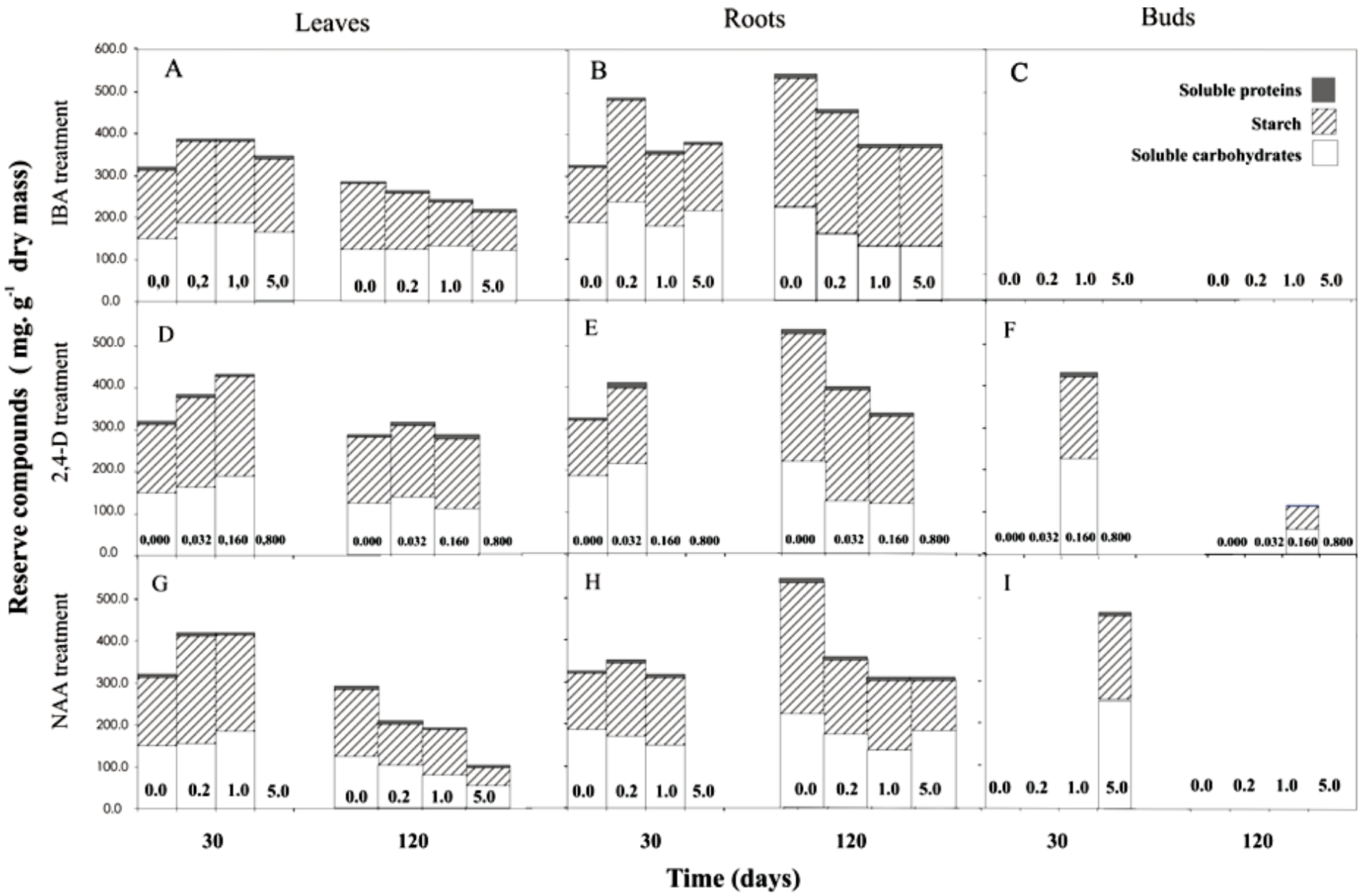

Figure 4. *In vitro* total reserve compounds (soluble carbohydrate, starch and soluble protein) of leaves, roots and buds of *Phalaenopsis amabilis* at 30 and 120 days in the treatments with auxins (IBA, 2,4-D and NAA).

mgg^{-1} and 405 mgg^{-1} dry weight of carbohydrate, respectively, and about 65 mgg^{-1} and 250 mgg^{-1} dry weight of starch, suggesting high carbon and energy costs for the development of new organs (Vaz et al., 1998).

In addition to controlling the assimilate supply to sorghum grains, the capacity to synthesize starch is also under hormonal regulation. Whereas IAA increases starch accumulation by facilitating the transport of sugars into grains and their transformation to this polysaccharide, IAA also increases the activity of the sucrose-hydrolyzing enzymes and decreases the activity of sucrose-phosphate synthase, contributing to a decrease in the proportion of sucrose in grain sugars (Bhatia and Singh, 2002).

Roots of cultivated potato showed the amount of total carbohydrates of 50 mgg^{-1} dry weight at 20 days (Schittenhelm et al., 2004), but *P. amabilis* roots showed 240 mgg^{-1} dry matter when treated with IBA (0.2 mgL^{-1}) for 30 days and approximately 100 mgg^{-1} dry matter when treated with IBA (5.0 mgL^{-1}) or 2,4-D (0.032 and 0.160 mgL^{-1}) for 120 days, both at concentrations four times higher than in potato. Starch levels in roots ranged from 100 mgg^{-1} dry weight after treatment with 5.0 mg L^{-1} IBA at 120 days to almost 280 mgg^{-1} dry matter (0.2 mg L^{-1} IBA at 120 days). Li et al. (2003) observed that *in vitro* explants of *Cymbidium sinense* (Andr.) Willd. had higher concentrations of starch grains in mature roots than in young roots. The same was observed in *C. fimbriatum* (Suzuki, 2005), in which there were an antagonism between the occurrence of sub apical shoots and the higher concentration of starch in shoots at mature stages (approximately 23 mgg^{-1} fresh weight) and lower in the sub apical development phase, with approximately 2.0 mgg^{-1} fresh weight.

Phalaenopsis has a CAM (Crassulatian acid metabolism), which requires an elevated energy level and large carbohydrate stocks. There is a competition between carbon storage for CAM to maintain a high capacity to fix CO_2 and exporting as much carbon as possible for growth (Wild et al., 2010). Four basic carbon partitioning strategies may occur in CAM species: ME (malic enzyme) starch formers, ME extra chloroplastic carbohydrate formers, PEPCK (PEP carboxylase) starch formers, and PEPCK extra chloroplastic carbohydrate formers. ME species can also combine both starch and extra chloroplastic carbohydrate storage, as *Phalaenopsis* does in our study.

The present results show that the total soluble proteins in roots and the photosynthetic aerial parts of *P. amabilis* are most likely not storage proteins, as are those found in potato tubers and soybean seeds. These proteins may be related to enzymes and their synthesis, coenzymes, nucleic acids, chlorophyll and to primary plant metabolism. According to Debergh and Maeno (1981), roots developed *in vitro* are not functional, but the biochemical analysis of roots of *P. amabilis* cultured *in vitro* shows accumulation of organic compounds that could be re-mobilized and translocated to newly formed tissue. Reduced levels of total soluble protein, from 350 to 125 mgg^{-1} dry weight, were also observed in pho-tosynthetic leaves of wild and transgenic potato grown *ex vitro* for 120 days (Schittenhelm et al., 2004), and this phenol-menon was related to the end of the growth cycle with remobilization of compounds to the storage organ. Silveira et al. (2004) observed that intracellular protein levels increased during the growth phase cells of *Pinus taeda* L. in suspension culture of embryos when 2,4-D (0.44 mg L^{-1}) was added to the medium and the starch levels were simultaneously reduced. Auxin may influence substrate distribution by determining the course and orientation of vascular strands through a mechanism by which auxin formation increases the advantage of dominant roots or shoots. The faster a root develops, the more carbohydrates and phloem-transported auxin it will receive, thus denying these to weaker roots, and the auxin in turn would enhance root branching, further increasing local carbohydrate consumption (Sachs, 2005).

The formation of new functional leaves and roots for the establishment of the *ex vitro* plant depends on the *in vitro* induction phase or on the acclimatization phase, with the mobilization of all of the existing reserves in plant tissues. Sucrose and starch (sources of carbon skeleton and energy), peptides and re-assimilated proteins (via proteases and degradation as a source of amino acids for the synthesis of new enzymes) can increase the rate of cell division and growth of the initial establishment until an autotrophic organism is produced. Therefore, increased ability to remobilize organic compounds (soluble carbohydrates, starch and total soluble protein) to perform specific tasks and coordinate developmental programs based on the availability of these crucial nutrients may increase the survival (Gibson, 2005) and growth of these explants when they are transferred from the *in vitro* stage to *ex vitro*, as observed in *Oxalis tuberosa* Mol (Conner et al., 1993) and *Rosa* (Capellades et al., 1991). Schittenhelm et al. (2004) observed that the starch from leaves of wild potato (*Solanum tuberosum* L.) and transgenic potato grown in a greenhouse were reduced from 20 to 120 days, showing nearly complete translocation of these reserves from the tubers during the growth cycle. To comprehend source-sink regulation in relation to plant development and to be able to manipulate these complex interactions for agricultural purposes (Wardlaw, 1990; Williams et al., 2000; Lemoine et al., 2013), it is vital that the underlying induction factors determining a sink organ for sugar (competition/priority system among growing tissues and storage) be correlated with growth regulators, sugar-sensing mechanisms, and the physiological process of sugar transport, and their cellular and temporal expression patterns must be defined.

Our results demonstrate the presence of high metabolic

activity with absorption of nutrients from the medium (macro- and micronutrients including sucrose, vitamins, and other organic compounds), the translocation of nutrients, and the accumulation and remobilization of carbohydrates and starch in leaves, stems and roots of *P. amabilis*. Furthermore, these results indicate the importance of the root system to epiphytic orchids that do not have specialized structural reserves, such as pseudobulbs, for use during growth, development and propagation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Identification and distribution of *Tomato yellow leaf curl virus* TYLCV and *Tomato yellow leaf curl Sardinia virus* TYLCSV infecting vegetable crops in Morocco

M. Rotbi[1], A. P. De Castro[2], M. J. Díez[2] and N. Elmtili[1*]

[1]Laboratory of Biology and Health, Department of Biology, Abdelmalek Essaadi University, PO Box 2121, 93030 Tétouan, Morocco.
[2]Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV). Universitat Politècnica de Valéncia, Camino de Vera 14, 46022, Valencia, Spain.

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Leaf samples of 177 tomato plants were collected during 2006-2007 in tomato yellow leaf curl disease (TYLCD) infected fields, as well as 100 leaf samples of sweet pepper, common bean, zucchini and the wild species *Solanum elaeagnifolium*, in order to study the population structure and genetic variation of *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato yellow leaf curl virus* (TYLCV) in Morocco. Molecular hybridization using specific probes and restriction of a genomic region corresponding to the coat protein gene were performed to differentially identify TYLCV and TYLCSV species. Both species were present in the infected plants from Southwestern and Northern Morocco analyzed in this study. This is the first report of the presence of TYLCD in Northwest area. 66% of the tomato samples and 37.8% pepper were infected, most of them with mixed infections. The rest of the species analyzed were virus free. Five of the six Moroccan haplotypes identified had previously been reported. We identified a new haplotype representing 76% of the TYLCSV infected samples collected in Agadir-Chtouka-Ait-Baha area. The genomic region corresponding to the CP gene was sequenced for 10 isolates. The TYLCV and TYLCSV sequences had 99% identity with those previously identified in Northern Morocco and of Murcia respectively.

Key words: *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Tomato yellow leaf curl virus* (TYLCV), molecular hybridization, population structure, genetic variation.

INTRODUCTION

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating diseases affecting cultivated tomato (*Solanum lycopersicum* L.) in tropical and subtropical regions. Symptoms of the disease include upward curling of leaflet margins, reduction of leaflet area, and yellowing of young leaves, as well as stunting and flower abortion. This disease is caused by several virus species belonging to the genus *Begomovirus*, family *Geminiviridae*.

*Corresponding author. E-mail: elmtili@hotmail.com. Fax: +212 539994500.

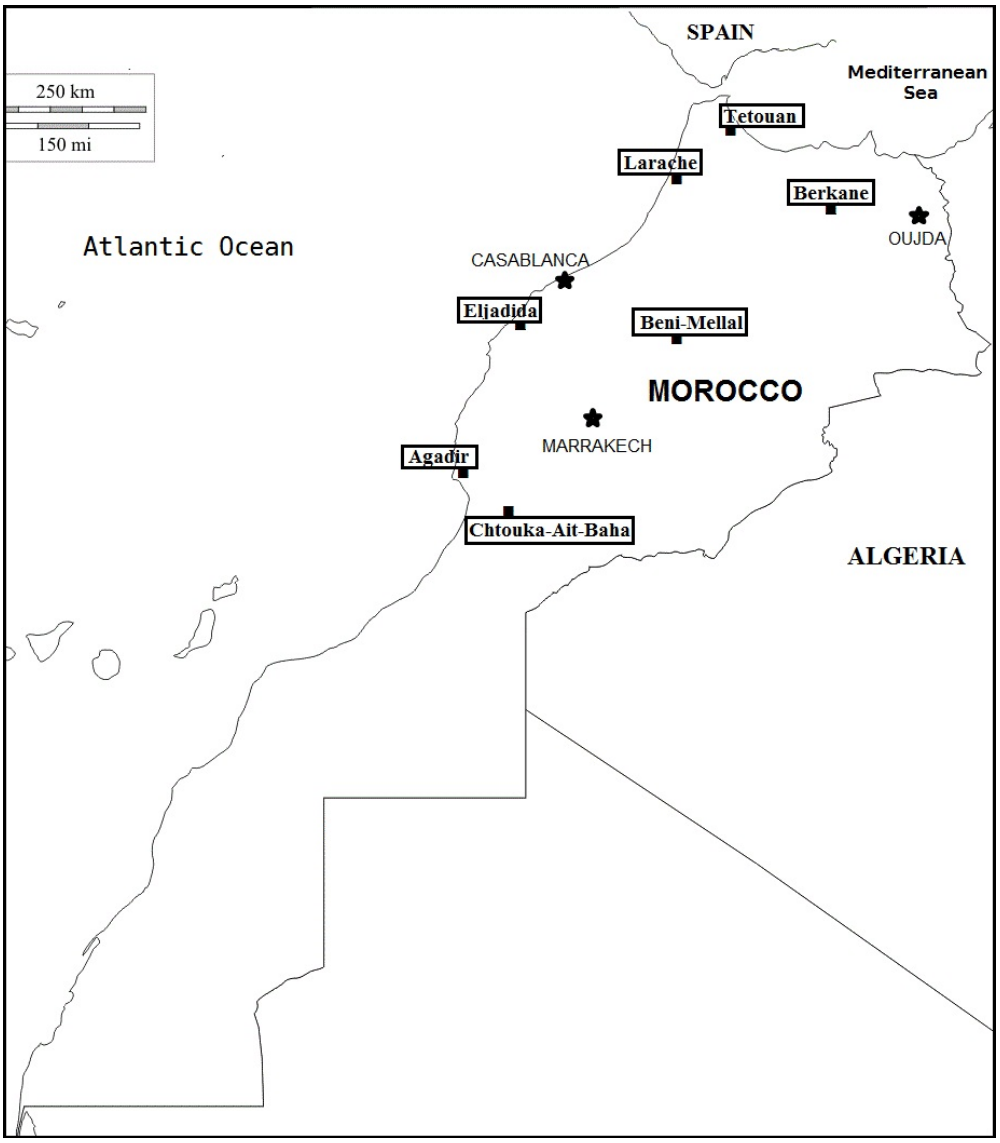

Figure 1. Geographic localization of analysed samples. Black shaded locations correspond to sampled areas.

They are transmitted by the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) in a persistent circulative manner. Presently, TYLCD is the most important tomato disease in the Mediterranean basin, causing serious economic losses in Morocco. Control of this viral disease is extremely complicated due to the high efficiency of vector transmission, as well as to the difficult eradication of the insect.

TYLCD was first identified in Morocco in 1996/1997 in surveys conducted by the Service for the protection of plants, in the region of El Jadida after importation of grafted tomato plants from the Netherlands by a farmer from this area (Peterschmitt et al., 1999a; Jebbour and Abaha, 2002) (Figure 1). Concretely, the species reported was the species form Middle East, *Tomato yellow leaf curl virus* (TYLCV); the isolate was closely related to the Dominican Republic isolate (Peterschmitt et al., 1999). The disease then spread to other tomato producer regions, particularly Berkane and Agadir (Jebbour and Abaha, 2002). Severe outbreaks of TYLCD occurred during summer and autumn 1999 in Agadir. The viral species identified were TYLCV and *Tomato yellow leaf curl Sardinia virus* (TYLCSV); the isolates corresponding to both species were in this case in closest relationship to isolates from Spain (Monci et al., 2000). TYLCD was the principal cause of the important losses registered in 1999 in the region of Agadir and reduction of 50% of the tomato cultivated area in El Jadida (Tahiri et al., 2007). The presence of both TYLCV and TYLCSV was subsequently reported in the Agadir region (Sedegui

et al., 2002). Analysis of samples harvested in 2001-2002 showed that infection of tomato crops was more common in the southwest than in the north (Tahiri et al., 2007). The sequence analysis revealed the existence of the Spanish strain of TYLCSV and of two genetically different strains of TYLCV. The Spanish origin of the TYLCSV isolate found in Morocco has been later confirmed (El Merach et al., 2007; Boukhatem et al., 2008). The degree of severity of the symptoms was evaluated by the dosage of viral DNA on the infected plants (Rotbi et al., 2010).

The exchange of plant material between countries facilitates the transfer of viral species (Hanafi, 2000). New isolates may have been introduced or the existing ones may have moved to other major growing areas, in the years since the latest studies in Morocco. The objectives of this study are identifying the geographical distribution of TYLCV and TYLCSV in tomato crops in Morocco, determining the incidence of these viral species in other vegetable crops, and studying the genetic diversity of the viral species TYLCV and TYLCSV in Morocco.

MATERIALS AND METHODS

Plant material

The sample recollection was carried out during 2006 and 2007 in 22 greenhouses and fields distributed on the most important areas of tomato production in Morocco (Figure 1). All tomato samples collected showed TYLCD symptoms, which varied from slight to severe. We also collected samples of the other cultivated and wild species with the aim of verifying the possible presence of the virus. Sweet pepper, zucchini and *Solanum elaeagnifolium* were symptomless while yellowing and curling of leaves was observed in common bean.

DNA extraction

Total DNA was extracted from dehydrated or frozen leaf samples using the CTAB method. Samples of 30 mg of dehydrated leaf tissue or 75 mg of frozen leaf tissue were ground and 450 µl of extraction buffer (2% CTAB, 20 mM EDTA, 100 mM Tris, 1.42 M NaCl) were added. Subsequently, samples were maintained at 65°C for 30 min. After adding 500 µl of chloroform:isoamyl alcohol (24:1), the mixture was centrifuged at 13400 × *g* for 10 min. The supernatant was recovered and one volume of cold isopropanol was added. After 10 min at -20°C, samples were centrifuged for 10 min at 13400 × *g*. The pellet was washed with 70% ethanol. After subsequent centrifugation, the pellet was resuspended in 50 µl of TE buffer (10 mM Tris, 1mM EDTA).

TYLCV and TYLCSV detection

Identification of the viral species was performed by dot blot hybridization and restriction of a genomic region corresponding to the coat protein gene.

Molecular hybridization

Aliquots of 5 µl of total extracted DNA from each sample and a 10-fold dilution were first denatured with 30 mM NaOH and 1 mM EDTA for 30 min at room temperature and charged on nylon positively charged membranes for hybridization. DNA was fixed on the membrane by UV crosslinking. Hybridization was carried out according to 'The DIG system user's guide for filter hybridization' (Roche Molecular Biochemicals) using digoxigenin-11-dUTP and chemiluminiscent detection. Membranes were pre-hybridized in standard hybridization buffer plus 50% deionized formamide for at least 1 h. Subsequent hybridization was done at 42°C overnight in fresh pre-hybridization solution containing 20 ng of denatured probe per ml. The probes employed represented the intergenic region of Spanish isolates belonging to TYLCV (YLCV-Mld[ES:72:97]) and TYLCSV (TYLCSVES[ES:Alm2:92]) species (kindly supplied by E.R. Bejarano, Universidad de Málaga, Spain). The probes were labelled by incorporation of digoxigenin-11-dUTP during PCR. One replicate of each membrane was hybridized with each probe, respectively. Washing steps and incubation with antibody were done according to manufacturer's instructions. Detection was carried out with CSPD and direct exposition to CCD camera (Intelligent Dark Box-II, Fujifilm, and Tokyo, Japan).

DNA typing

General primers of the *Begomovirus* genus were used to amplify the genomic region comprised of the coat protein gene (CP) of TYLC viruses. The primers were:

TY-1(+): 5'-GCCCATGTA(T/C)CG(A/G)AAGCC-30 and TY-2(-): 5'-G(A/G)TTAGA(A/G)GCATG(A/C)GTAC-3' (Accotto et al., 2000).

PCR conditions were 30 cycles of 1 min at 94°C, 1 min at 48°C and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. Final volume of PCR was 25 µl containing: 100 ng of template DNA, PCR buffer, 2 mM of $MgCl_2$, 100 µM of each dNTP, 0.4 µM of each primer, and 1 U of Taq polymerase (Roche, Spain). PCR products were digested with *Ava*II (TAKARA BIO INC.), which was predicted to cut TYLCSV differently from TYLCV. Digested DNA was separated by 2% agarose gels and stained with Ethidium Bromide.

PCR products were also digested with the restriction endonucleases used by Font et al. (2007): *Hae*III, *Hpa*II, *Rsa*I, *Taq*I and *Hin*fI (TAKARA BIO INC.), and the resulting DNA fragments were separated by electrophoresis in 3% agarose using 0.5 × TBE buffer. DNA fragments were visualized under UV light after Ethidium Bromide staining. A sample representative of each restriction pattern, obtained with all five endonucleases, was considered to define a haplotype and was sequenced and aligned using the tool nucleotide BLAST of NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

RESULTS

Identification of TYLCV and TYLCSV species

Molecular hybridization and restriction of a genomic region corresponding to the coat protein gene were successfully used to identify the species TYLCV and TYLCSV in infected samples of some crops collected in Morocco. The presence of both viral species in some areas of single and mixed infections in tomato and pepper plants was confirmed.

For dot-blot experiments, specific probes to TYLCV and TYLCSV were used. In both cases good signal intensity with the original samples and with the 10-fold dilution were obtained. In no case did healthy controls produce

Table 1. Number of infected samples with each species or with mixed infection as shown by molecular hybridization and PCR.

Crop	Origin	Number of analysed samples	Number of positive samples											
			Molecular hybridization						PCR					
			TYLCV	%	TYLCSV	%	Mixed	%	TYLCV	%	TYLCSV	%	Mixed	%
Tomato	Berkane	40	17	42.5	0	0	15	37.5	7	17,5	1	2.5	5	12.5
Tomato	Larache	53	6	11.3	0	0	10	18.9	-	-	-	-	-	-
Tomato	Tetouan	18	18	100	0	0	0	0	-	-	-	-	-	-
Tomato	Agadir	10	6	60	0	0	2	20	10	100	0	0	0	0
Tomato	Chtouka	56	11	19.6	7	12.4	28	47.4	19	33.9	22	39.2	7	12.5
Total tomato		177	58	33	7	4	55	31	36	20.3	23	13	12	8.3
Pepper	Berkane	10	0	0	0	0	0	0	0	0	0	0	0	0
Pepper	Larache	29	3	10.3	0	0	8	27.5	-	-	-	-	-	-
Pepper	Tetouan	6	0	0	0	0	6	100	-	-	-	-	-	-
Total pepper		45	3	7	0	0	14	31.1	0	0	0	0	0	0
Common bean	Berkane	10	0	0	0	0	0	0	0	0	0	0	0	0
Common bean	Agadir	11	0	0	0	0	0	0	0	0	0	0	0	0
Total bean		21	0	0	0	0	0	0	0	0	0	0	0	0
Zucchini	Tetuan	5	0	0	0	0	0	0	0	0	0	0	0	0
Solanum elaeagnifolium	Beni-Mellal	29	0	0	0	0	0	0	0	0	0	0	0	0

a signal.

Tomato showed the highest infection level (Table 1): 68% of tomato samples were infected, 33% corresponding to TYLCV, 4% to TYLCSV and 31% to mixed infections. In the case of pepper where 38% of samples are infected, most of them with mixed infections. Single infection with TYLCSV was not detected in pepper. The rest of the samples analyzed were free from these viruses. The percentage of infection varied according to the geographical origin. The highest percentage of infected plants corresponded to Tetouan, followed by Berkane, Agadir and Chtouka (Table 1).

Amplification of the coat protein gene with general primers for *Begomovirus*, followed by restriction with *Ava*II to differentially identify TYLCV and TYLCSV confirm the results of molecular hybridization. The controls (based on hybridization results) produced the expected patterns, that is, three bands of 360, 150 and 68 pb for TYLCSV, and two bands of 302 and 277 pb for TYLCV; the five expected bands were present for mixed infections. The percentages of infection obtained by PCR for the samples collected in Berkane, Agadir and Chtouka were consistently lower than the ones obtained using molecular hybridization (Table 1). In contrast, TYLCV was detected in the 100% of analyzed samples from Agadir. No amplification was obtained from the samples collected in Larache and Tetouan, in spite of the modifications performed on the PCR protocol. Samples of sweet pepper, bean, and squash were negative.

Haplotypes differentiation

PCR products obtained from 62 of the TYLCD infected tomato plant were digested with five endonucleases to characterize the virus isolates (Figure 2). The resulting DNA fragments showed

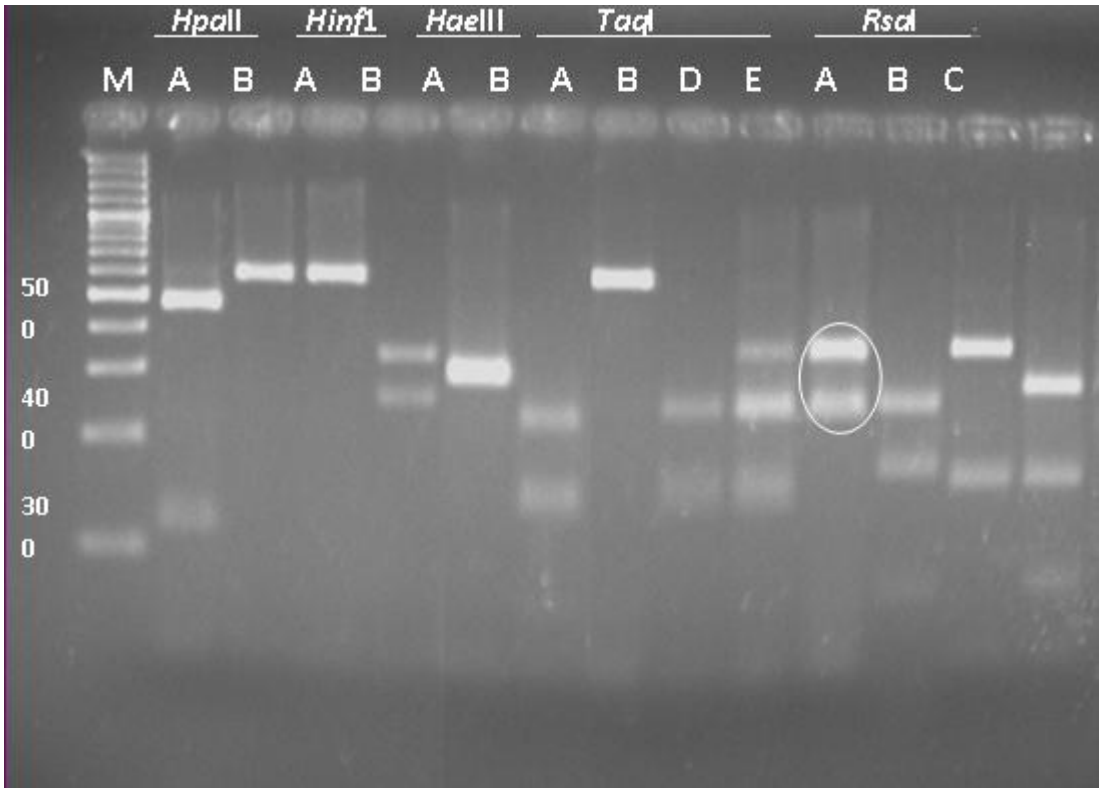

Figure 2. 3% agarose gel electrophoresis of CP PCR product digested with the restriction endonucleases *Hae*III, *Hpa*II, *Rsa*I, *Taq*I and *Hinf*I (Fermentas) corresponding to haplotypes identified from 51 Moroccan isolates of TYLC virus. Haplotypes nomenclature according to Font (2003). The new haplotype is indicated with a circle. M: 100 pb GeneRuler™ DNA Ladder (Fermentas).

Table 2. Restriction pattern of haplotypes of the coat protein gene of tomato yellow leaf curl viruses from Morocco. We respected the Font (2003) nomenclature.

Region	Species	Restriction pattern						Frequency (%)
		***Hpa*II**	***Hinf*I**	***Hae*III**	***Taq*I**	***Rsa*I**	**Haplotype**	
Berkane	TYLCV	B	A	B	B	B	VI	8 (1/ 13)
	TYLCV	B	A	B	B	C	X	23 (3/13)
	TYLCV	B	B	B	B	B	IV	30.7 (4/13)
	Mixed	-	-	-	-	-	-	38 (5/13)
Chtouka-Ait-Baha	TYLCV	B	B	B	B	B	IV	40.5 (17/42)
	TYLCV	B	C	B	B	B	XXI	2 (1/42)
	TYLCSV	A	A	A	E	A	**XXII**	38 (16/42)
	TYLCSV	A	A	A	A	A	I	9.5 (4/42)
	Mixed	-	-	-	-	-	-	9.5 (4/42)
Agadir	TYLCV	B	B	B	B	B	IV	100 (7/7)

13 polymorphic restriction sites. Eight different haplotypes were identified, 6 belonging to TYLCV species and 2 belonging to TYLCSV (Table 2). There was a high similarity between Spanish and Moroccan isolates of TYLCV and TYLCSV. Three out of the four TYLCV Moroccan haplotypes detected in this study had been previously identified in Spain (Font, 2003). The dominant TYLCV haplotype was the IV, representing 49 and 30.7% in the South West and the North East, respectively. One of the TYLCSV Moroccan haplotypes had also been previously identified in Spain (Font, 2003). However, a new restriction pattern after digesting with

Table 3. Comparison of identified haplotypes of 64 Moroccan isolates of the tomato yellow leaf curl virus with the described isolates in the database GenBank.

Origin	Species	Haplotype (Font accession, 2007)	% of homology (accession NCBI)	Number of sequenced samples
Berkane	TYLCV	VI (DQ058099)	-	0
	TYLCV	X (DQ058103)	99 (AF071228) TYLCV-Mld[ES:72:97]	1
	TYLCV	IV (DQ058097)	99 (AM409201 ; DQ503437)	2
	Mix		97 (EF625894)97 (AF071228) TYLCV-Mld[ES:72:97]	2
Chtouka-Ait-Baha	TYLCV	IV (DQ058097)	99 (EF060196) TYLCVIL[MO:Ber:05]	
	TYLCV	XXI	-	
	TYLCSV	XXII	99 (Z25751, TYLCSVES[ES:Mur1:92] AF271234) TYLCMalV- [ES:421:99]	3
	TYLCSV	I (DQ058094)	99 (DQ058094)	1
Agadir	TYLCV	IV (DQ058097)	99 (EF060196) TYLCVIL[MO:Ber:05]	1

*Taq*I was identified in TYLCSV samples collected in the province of Chtouka-Ait-Baha. All samples in which this pattern was observed corresponded to the same haplotype. The frequency of haplotype, named XXII, was remarkable, representing 38% of TYLCSV haplotypes identified in southwestern Morocco (Chtouka-Ait-Baha). In all geographic areas surveyed (South West and the North East), the structure of the virus population comprised a predominant haplotype and a few haplotypes with very low abundance.

Sequencing of the CP fragment of TYLCV haplotypes

One sample representative of each of the identified haplotypes was sequenced. All the sequences of the Moroccan haplotypes causing TYLCD belong to TYLCV and TYLCSV species. The isolates identified in the work here reported had 99% identity with those corresponding to the same haplotypes described by Font et al. (2007) (Table 3).

We sequenced a region of 580 pb of the CP of one representative of each of the identified haplotypes. We confirmed all the Moroccan haplotype sequences as TYLCV and TYLCSV species, being the percentages of identity higher that 90% in all cases. By sequences alignment we confirmed that the sequences of haplotypes I, IV, and X identified in Moroccan samples correspond perfectly to those described in Spain (Font et al., 2007) with a 99% of identity. The same grade of similarity was revealed when comparing each two of the obtained sequences corresponding to the same haplotype.

DISCUSSION

Molecular hybridization and PCR analysis performed on samples of different horticultural crops collected in Morocco allowed the identification of TYLCV and TYLCSV viruses in the main areas of tomato production. Tomato was the species with the highest infection percentage. However, a part of the samples tested were negative for TYLCV and TYLCSV with the two detection methods used despite the plants exhibited typical TYLCD symptoms. The same result was found by Tahiri in 2007 where 21 out of 54 samples resulted negative for begomovirus detection. As stated by Tahiri et al. (2007) it could be due to the presence of TYLCV like symptoms induced as a result of infection by another pathogen or by a physiological stress. Alternatively, it could also be

caused by too low concentration of the virus or an uneven distribution of TYLCV/TYLCSV in the plant preventing its detection by PCR.

The presence of TYLCV and TYLCSV in tomato in the Southwest and the Northeast of Morocco was in agreement with previous reports (Peterschmitt et al., 1999; Monci et al., 2000; Tahiri et al., 2007). Our results show higher levels of infection in Berkane and similar infection in Agadir and Chtouka. We have also noted a higher decrease in the percentage of infections by TYLCSV in all areas compared with the results obtained by Tahiri et al. (2007) except Chtouka, which stayed stable. The decrease of TYLCSV was also reported by Sánchez-Campos et al. (1999) in Spain. We note also an important increase in the level of mixed infections. This is a worrying scenario as the appearance of recombinants is very high in these conditions. In fact, recombinants from TYLCV and TYLCSV were also detected by Monci et al. (2002) and García-Andrés et al. (2006) appearing in some cases new pathogenic characteristics.

About other crops, only 17 of the 45 (38%) pepper samples are positive. This percentage was a little higher than the one found by Morilla et al. (2005), who detected TYLCV infection in 6% of the pepper plants and in about 50% of the tomato plants tested. Infection of pepper was no detected by Tahiri et al. (2007). All samples of bean, zucchini and the wild species *Solanum elaeagnifolium* were negative. Although pepper is a host for TYLCV, transmission of the virus from an infected pepper plant into a tomato plant is only possible under laboratory conditions. As stated by Morilla et al. (2005), this suggests that pepper is a dead end in the epidemiological cycle of this virus. However, it cannot be completely ruled out that pepper may serves as an inefficient reservoir when vector populations are extremely high. In contrast, bean is a host of TYLCV that from can be transmitted to tomato by the vector *Bemisia tabaci*. Though in this work we have not detected bean infected samples, it would be convenient to continue sampling in order to detect as soon as possible its appearance and to take the appropriated preventive measures.

We reported for the first time infected fields in Tétouan and Larache areas. This would be kept in mind and continuous monitoring would be conducted as they are important areas of cultivation of tomato industry. The molecular characterization of the viruses has been conducted digesting a fragment of 580 pb of the CP with five restriction enzymes. This method has proved to be useful for the characterization of the TYLCV and TYLCSV isolates present in Morocco and their comparison among them and those available in databases. All isolates identified in Morocco except the XXII had been previously detected in Spain, being the frequency of this new haplotype remarkably. It would be necessary to continue with the sampling of infected samples to monitor the spread of this new haplotype as well as to check its pathogenic characteristics and aggressiveness. A high similarity between the sequence diversity of TYLCV and TYLCSV of Moroccan and Spanish isolates has been found. Five of the six Moroccan haplotypes identified by RFLPs analysis were described in Spain (Font 2003), 4 corresponding to TYLCV species and 1 to TYLCSV species.

We identified a new haplotype (Table 1) of TYLCSV in Southwestern part of the country and its frequency was the 38% of the TYLCSV infected samples analyzed were collected from Chtouka-Ait-Baha zone. The major haplotype of TYLCV species was the IV with 49 and 31% in the Southwest and the Northeast of the country respectively. In all geographic sampled regions, the structure of virus population was composed of a predominant haplotype and a few other haplotypes with very low abundance. Then, we demonstrate the validity and efficiency of the RFLPs method used in this work for identification and characterization of TYLCD Moroccan haplotypes. The new haplotype nucleotide sequence had the highest homology with a TYLCSV isolate from Murcia "Z25751" suggesting that it was introduced from Spain.

By comparison with the results published up to 2013, accessions AF071228, Z25751 and EF060196 of TYLCV are the most similar to the ones identified in Morocco (Peterschmitt et al., 1999; Tahiri et al., 2007; Boukhatem et al., 2008). This finding supports the hypothesis that the disease was introduced in Morocco in same time that was detected in Spain. However probable that more than one introduction of TYLCV occurred in Morocco according to the observations made by Peterschmitt et al. (1999) who stated that in Morocco, TYLCV symptoms were observed during the 1996 to 1997 growing season, following importation of grafted tomato plants from the Netherlands by a farmer from the Casablanca region. Given the high rate of vegetal material exchange, it is frequent the transmission of vectors and diseases across countries and continents as has been evidenced in this work.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Gibberellic acid, amino acids (glycine and L-leucine), vitamin B_2 and zinc as factors affecting the production pigments by *Monascus purpureus* in a liquid culture using response surface methodology

Fateme Baneshi[1], Majid Azizi[2], Mohsen Saberi[3] and Mohammad Farsi[4]

[1]Horticulture Science, Ferdowsi University of Mashhad, Iran.
[2]Agriculture College, Ferdowsi University of Mashhad, Iran.
[3]Industrial Microorganism Department-ACECR, Mashhad bunch, Iran.
[4]Agriculture College, Ferdowsi University Of Mashhad, Iran.

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The combined effects of zinc, gibberellic acid, vitamin B_2, amino acids (glycine and L-leucine) on pigment production were evaluated in a liquid culture of *Monascus purpureus*. In this study, response surface design was used to optimize each parameter. The data were analyzed using Minitab 14 software. Five parameters were applied by using response surface methodology for pigment production in liquid cultures. Four zinc, 11 gibberellic acids, three vitamins B_2, eleven and fifteen amino acids (glycine and L-leucine) respectively; levels were evaluated. The highest production of pigment was reached with a 10 mg/l vitamin B_2, 50 mg/l gibberellic acid and 50 mg/l glycine amino acid.

Key words: *Monascus purpureus*, response surface methodology, amino acid, gibberellic acid, zinc, vitamin B_2.

INTRODUCTION

Since many kinds of synthetic dyestuffs have been found to be hazardous to human health, only limited kinds of such dyestuffs are permitted to be used in food in many countries, and therefore, there is a need to develop alternative sources of natural food colorants (Lee et al., 2000). *Monascus* is an ascomycetous fungus discovered by Van Tieghem (1884) traditionally used for the production of food colouring, fermented foods and beverages (Alsarrani and Elnaggar, 2005). Pigments synthesized by the fungi *Monascus* spp. have been traditionally used in Asia for colouring and securing a number of fermented foods (Lee et al., 2000). The angkak has long been recognized as a folk medicine for improving food digestion and blood circulation and for treatment of muscle bruising and dysentery. The manufacturing process for angkak and its therapeutic applications are well documented in the ancient Chinese pharmacopoeia (Ben- Taso-Gum-Mu) (Miyake

*Corresponding author. E-mail: baneshi_fa@yahoo.com. Tel: (+98) 9171080023. Fax: (+98) 7112271458.

Abbreviation: ANOVA, analysis of variance; **PDA,** potato dextrose agar.

et al., 2005; Panda et al., 2010). Metals are an integral part of all ecosystems (Raza et al., 2010). Some of them are vital components of living systems and known as essential metal ions (Raza et al., 2010). Secondary metabolisms are affected by the presence or absence of these essential metal ions, as they may be responsible for activation of some of the biosynthetic pathways (Raza et al., 2010). The zinc ions and a particular combination of amino acids (glycine, L- leucine, L-tryptophan) were identified as important components in the richer medium responsible for the increased growth (Johnson and Mchan, 1975). *Monascus purpureus* can synthesize many secondary metabolites including red and yellow pigments, monacolins and gamaaminobutyric acid, which are industrially and medicinally important compounds (Jia et al., 2010). Monascus fungi produce at least six major related pigments which can be categorized into three groups based on color as follows: yellow pigments: monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$); orange pigments: monascorubrin ($C_{23}H_{26}O_5$) and rubropunctatin ($C_{21}H_2O_5$); and red pigments: monascorubramine (C23H27NO$_4$) and rubropuntamine ($C_{21}H_{23}NO_4$) (Subhasree et al., 2011). Several papers concerning the influence of cultivation media composition on the lovastatin biosynthesis have already been published (Bizukojc et al., 2006).

Considering the biochemical mechanisms of the process, a hypothesis was proposed that the supplementation with chosen B-group vitamins might exert a positive effect on the biosynthesis of mevinolinic acid (Bizukojc et al., 2006). Plant hormones are involved in several stages of plant growth and development (Mukhopadhyay et al., 2004). It might be of interest to investigate if they influence the growth of microorganisms also (Mukhopadhyay et al., 2004). It has been shown that gibberellic acid at an optimum concentration of 10 mg/l increased the cell division rate of different strains of *Hansenula wingei* (Mukhopadhyay et al., 2004).

In the previous study, we have found that the highest production of pigment was reached with a pH value of 3, maltose 250 g/l and a temperature of 25°C (Baneshi et al., 2011). In this work, determination of best conditions for pigment production was investigated on the effect of various zinc, gibberellic acid, vitamin B_2, amino acids (glycine and L-leucine) in a liquid cultured with the best conditions obtained from previous study. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes (Subhagar et al., 2009). RSM, an experimental strategy was used for finding the optimum combination of mixed substrate for pigment production using *M. purpureus*.

MATERIALS AND METHODS

Microorganism

M. purpureus ATCC 1603 was purchased from the Leibniz Institute DSMZ German Collection of Microorganisms and cell cultures (DS MZ, Germany). *M. purpures* was maintained on the PDA (Merck, Germany) slants at 4°C, and cultured at 37°C for 10 days were used for inoculum preparation (Figure 1).

Culture conditions and inoculum preparation

Ten day-old PDA pure cultures of *M. purpureus* (1 pool standard) were used for inoculation of conical flask containing the fermentation medium: 4% of glucose, 1% of yeast extract and 0.1% of KH_2PO_4 in 30 mL of distilled water, adjusted to pH 6. These cultures were incubated at 25°C for 48 h in a shaking incubator at 100 rpm. The basal medium consisted of: 0.15% of NH_4Cl, 0.1% of KH_2PO_4, 0.05% of $MgSO_4{\cdot}7H_2O$, 0.05% of NaCl, 0.01% of $FeSO_4{\cdot}7H_2O$, 250 g/l of maltose and 150 ml of deionized water, adjusted to pH 3. The content of the flasks were mixed and autoclaved at 121°C at 15 psi for 20 min. After inoculation (7.5%, v/v), the fermentation was carried out at 25°C for 14 days in a shaking incubator at 150 rpm.

Pigment estimation

After fermentation, estimating extracellular pigment using ethanol 96% (20 ml) in each medium of pigment taken. The sample were kept on a rotary shaker at 120 rpm for 2 h, allowed to stand for 15 min and filtered through Whatman GF/C filter paper (47 mm). Ethanol extract of control medium was kept as the blank for pigment and analysis was done using a spectrophotometer (Cecil 2010UV-visible) set at 400 nm for yellow pigment, 460 for orange pigment and 500 nm for red pigment. The results were expressed as optical density units per gram of dried medium multiplied by dilution factor (Lin and Iizuka, 1982; Lee et al., 2007).

Experimental design

The optimum conditions, a response surface design were selected. The involved crucial factors were zinc (X_1), gibberellic acid (X_2), vitamin B_2 (X_3), glycine amino acid (X_4) and L- leucine amino acid (X_5). These factors and the level at which the experiments were carried out are given in Table 1. The low and high levels were coded as -1 and +1; the middle level was coded as 0. A total of 33 runs with 7 central points were generated. The central point of the design arrangement decided on was: Concentrations of each zinc, gibberellic acid, vitamin B_2 and amino acids (L- luecine and glycine) were 10, 25, 5, 37.5 and 25 mg/l, respectively.

Response surface methodology

The data were analyzed using Minitab 14 software (Minitab Inc., USA). The quadratic model for predicting the optimal point was expressed as follows:

$$Y = b_{\circ} + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{44} x_4^2 + b_{55} x_5^2 + b_{12} x_1 . x_2 + b_{13} x_1 . x_3 + b_{14} x_1 . x_4 + b_{15} x_1 . x_5 + b_{23} x_2 . x_3 + b_{24} x_2 . x_4 + b_{25} x_2 . x_5 + b_{34} x_3 . x_4 + b_{35} x_3 . x_5 + b_{45} x_4 . x_5$$

Where, Y is the amount of pigment produced (mg/g dry substrate), and x_1, x_2, x_3, x_4 and x_5 are input variables. X_1, x_2, x_3, x_4, and x_5 indicate zinc, gibberellic acid, vitamin B_2 and amino acids (glycine and L- luecine) concentrations, respectively. B_0 is a constant and B_1, B_2, B_3, B_4 and B_5 are linear coefficients. B_1, B_2, B_3, B_4, and B_5 indicate zinc, gibberellic acid, vitamin B_2 and amino acids (glycine and L- luecine) concentrations, respectively. B_{11}, B_{22}, B_{33}, B_{44} and B_{55} are quadratic coefficients and B_{12}, B_{13}, B_{14}, B_{15}, B_{23}, B_{24},

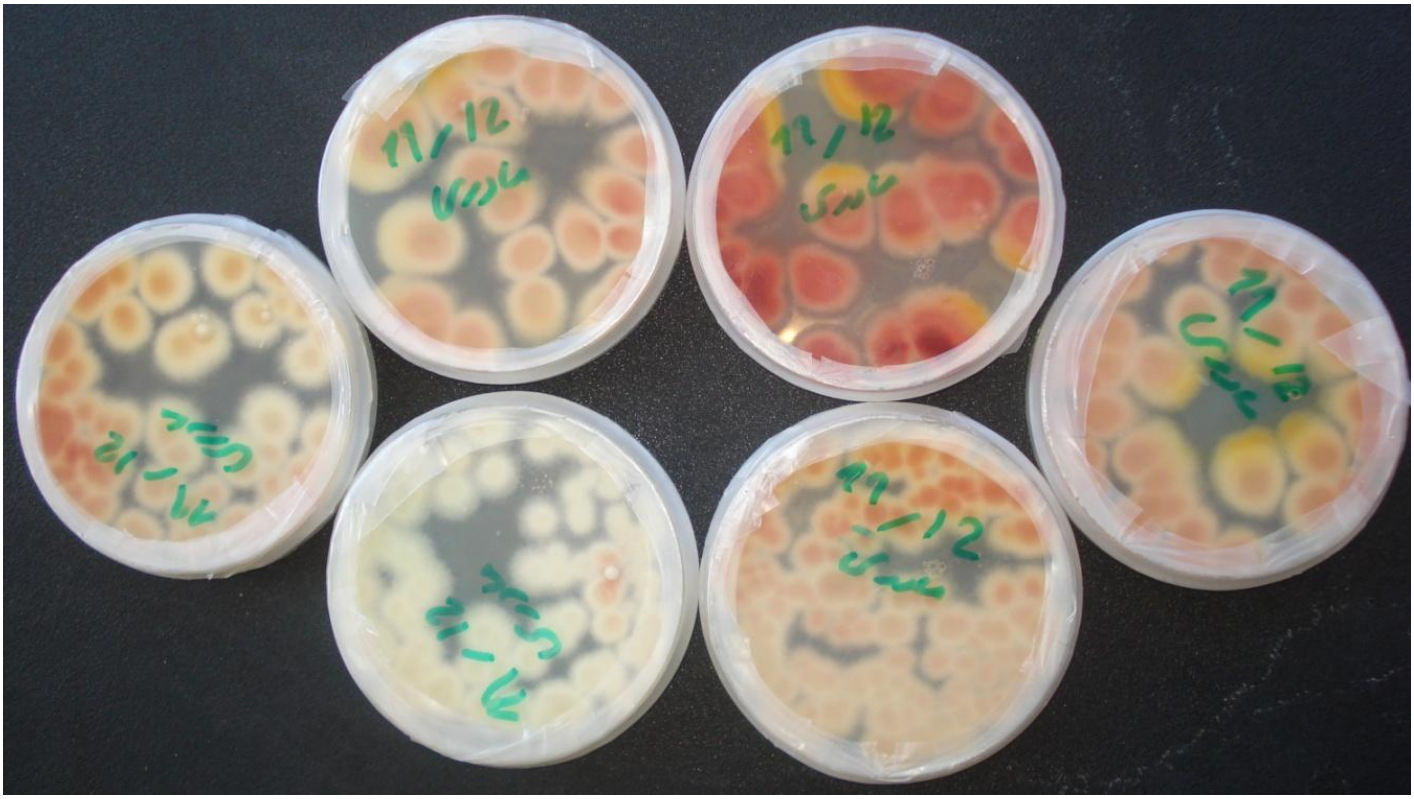

Figure 1. *Monacus purpureus* ATTC 1603 cultivated on potato- dextrose agar for 10 days at 37˚C.

Table 1. Levels of variable used in response surface design.

Variable		Coded-variable level		
	Symbol	-1	0	1
Zinc concentration	X_1	0	10	20
Gibberellic acid concentration	X_2	0	25	50
Vitamin B2 concentration	X_3	0	5	10
Glycine amino acid concentration	X_4	0	25	50
L-luecine amino acid concentration	X_5	0	37.5	75

B_{25}, B_{34}, B_{35} and B_{45} are cross-product coefficients. B_{12}, B_{13}, B_{14}, B_{15}, B_{23}, B_{24}, B_{25}, B_{34}, B_{35}, and B_{45} indicate zinc.gibberellic acid, zinc.vitamin B_2, zinc.amino acid glycin, zinc.amino acid leucine, gibberellic acid.vitamin B_2, gibberellic acid.amino acid glycine, gibberellic acid.amino acid leucine, vitamin B_2.amino acid glycine, vitamin B_2.amino acid leucine and amino acid glycine.amino acid leucine, respectively.

RESULTS AND DISCUSSION

Among the five factors used in the central composite design zinc, gibberellic acid, vitamin B_2 and amino acids (L- leucine and glycine) were used for pigment production. Table 2 shows the results of experimental data and simulated values. Multiple regression analysis of the response surface design for the pigment production:

Yellow Pigment production (A400/g): $0.557+ 0.854\ x_1 + 2.005\ x_2+ 2.433\ x_3 + 0.279\ x_4 +238x_5 – 0.027\ x_1^2 – 0.236x_2^2 – 2.080x_3^2 – 0.012\ x_4^2 + 0.477\ x_5^2 – 1.275\ x_1.\ x_2 – 1.207x_1.\ x_3 – 0.291x_1.\ x_4 – 1.341x_1.\ x_5 – 0.466x_2.\ x_3 – 0.070\ x_2.\ x_4 – 2.546\ x_2.\ x_5 – 0.323\ x_3.\ x_4 – 0.842x_3.\ x_5 – 0.704\ x_4.\ x_5$

Orange pigment production (A460/g): $2.113 – 0.086x_1 + 2.974\ x_2+3.014x_3 – 0.642\ x_4 + 1.357\ x_5+ 0.693\ x_1^2 – 1.585\ x_2^2 – 1.343\ x_3^2 + 0.444\ x_4^2 – 0.998x_5^2 – 0.479\ x_1.\ x_2 - 2.395x_1.\ x_3 + 0.798\ x_1.\ x_4 – 1.214\ x_1.\ x_5 – 1.329\ x_2.\ x_3 + 0.563\ x_2.\ x_4 – 1.891x_2.\ x_5 – 1.562\ x_3.\ x_4 – 2.451x_3.\ x_5 + 0.060\ x_4.\ x_5.$

Table 2. Response surface design with actual and predicted pigment production.

Run	Block	Code level					Pigment production (UA400)		Pigment production (UA460)		Pigment production (UA500)	
		X_1	X_2	X_3	X_4	X_5	Actual	predicted	Actual	predicted	Actual	predicted
1	1	-1	-1	-1	-1	1	2.225	2.165	2.530	2.528	2.120	2.121
2	1	1	-1	-1	-1	-1	2.740	2.558	2.900	2.753	2.550	2.475
3	1	-1	1	-1	-1	-1	5.154	5.021	4.710	4.736	2.920	2.994
4	1	1	1	-1	-1	1	4.098	4.130	4.146	4.131	3.174	3.100
5	1	-1	-1	1	-1	1	1.800	1.774	5.304	5.275	1.390	1.471
6	1	1	-1	1	-1	1	2.375	2.514	2.920	2.850	2.570	2.503
7	1	-1	1	1	-1	1	4.362	4.550	5.016	5.119	4.056	4.138
8	1	1	1	1	-1	-1	5.682	5.779	6.465	6.423	4.620	4.625
9	1	-1	-1	-1	1	-1	1.374	1.119	1.192	1.146	0.923	0.931
10	1	1	-1	-1	1	1	2.920	2.830	3.000	2.912	2.630	2.490
11	1	-1	1	-1	1	1	4.428	4.386	4.140	4.226	2.720	2.729
12	1	1	1	-1	1	-1	6.465	6.332	6.090	6.030	4.020	3.952
13	1	-1	-1	1	1	1	2.410	2.475	2.760	2.790	2.240	2.256
14	1	1	-1	1	1	-1	2.940	2.914	3.714	3.599	2.700	2.639
15	1	-1	1	1	1	-1	6.240	6.263	6.135	6.193	4.302	4.390
16	1	1	1	1	1	1	2.380	2.563	2.640	2.657	2.070	2.010
17	1	0	0	0	0	0	4.710	4.692	5.592	5.272	4.074	3.705
18	1	0	0	0	0	0	2.650	4.692	3.690	5.272	2.610	3.705
19	1	0	0	0	0	0	4.962	4.692	5.526	5.272	3.948	3.705
20	1	0	0	0	0	0	5.700	4.692	5.568	5.272	3.762	3.705
21	1	0	0	0	0	0	4.986	4.692	5.646	5.272	3.726	3.705
22	1	0	0	0	0	0	4.902	4.692	5.316	5.272	3.930	3.705
23	2	-1	0	0	0	0	4.494	4.763	5.724	5.498	4.104	3.746
24	2	1	0	0	0	0	5.022	4.997	4.896	5.416	3.552	4.091
25	2	0	-1	0	0	0	3.000	3.465	2.940	3.407	2.250	2.487
26	2	0	1	0	0	0	6.270	6.050	5.538	5.365	3.924	3.868
27	2	0	0	-1	0	0	2.760	3.653	3.852	4.097	2.840	3.105
28	2	0	0	1	0	0	4.338	3.690	4.854	4.903	3.594	3.510
29	2	0	0	0	-1	0	4.890	4.865	5.430	5.606	4.080	4.054
30	2	0	0	0	1	0	4.644	4.914	4.956	5.074	3.594	3.801
31	2	0	0	0	0	-1	4.926	5.561	4.866	5.221	3.492	3.440
32	2	0	0	0	0	1	5.184	4.794	4.164	4.103	2.940	3.173
33	2	0	0	0	0	0	6.120	4.896	6.600	5.131	4.614	3.708

Red pigment production (A500/g): $0.763 + 0.849\, x_1 + 3.242\, x_2 + 1.839\, x_3 - 0.236\, x_4 + 2.288\, x_5 + 0.630\, x_1^2 - 1.589\, x_2^2 - 1.200\, x_3^2 + 0.657\, x_4^2 - 1.203\, x_5^2 - 1.863\, x_1.\, x_2 - 1.781\, x_1.\, x_3 - 0.571\, x_1.\, x_4 - 2.416\, x_1.\, x_5 + 0.736\, x_2.\, x_3 - 0.728\, x_2.\, x_4 - 2.796\, x_2.\, x_5 - 0.408\, x_3.\, x_4 - 1.105\, x_3.\, x_5 - 1.305\, x_4.\, x_5$

The analysis of variance of regression for pigment production was summarized in Table 3. The r^2 values of red pigment level, orange pigment level, and yellow pigment level were 0.88, 0.89, and 0.85, respectively. Also, the test statistics p-value for the overall regression is significant at the 5% level, which means the model is adequate in approximating the response surface of the experimental design. The linear effects of the factors were found to be more significant than the interaction and quadratic effects of the factors. The suitable factors for pigment production, was applied at 10 mg/l vitamin B_2, 50 mg/l gibberellic acid and 50 mg/l glycine amino acid (Figure 2). Figure 3 (A to E) shows response surface plots of the effect of cultivation vitamin B_2, gibberellic acid and glycine amino acid addition on the production of pigment. As shown in Figure 3 (A, B and C) production is optimum at a vitamin B_2 of around 5 mg/l, and increased gibberellic acid addition increased yellow, orange and red pigments content. As shown in Figure 3 (D, E and F), when the vitamin B_2 was around 5 mg/l, the production of pigment gradually increased along with glycine amino acid addition. It was reported that RSM is a reliable and useful statistics methodology for the investigation of the optimal condition (Lee et al., 2007). RSM has some

Table 3. ANOVA for response surface design during pigment production by *M. purpureus.*

Source	Df[a]	Sum of square		
		Red pigment (A500/ g)	Orange pigment (A460/ g)	Yellow pigment (A400/ g)
Regression	20	21.887	48.606	52.088
Linear	5	10.472	27.096	32.985
Square	5	4.659	9.527	8.094
Interaction	10	6.757	11.982	11.008
Residual	11	2.999	5.942	9.330
Lack of fit	6	1.556	3.057	3.950
Pure error	5	1.443	2.885	5.380
Variability explain (r^2)		0.888	0.896	0.859

[a]df: Degree of freedom.

Figure 2. Extracellular pigment production with various zinc, gibberellic acid, vitamin B_2, amino acids (glycine and L-leucine) concentration in liquid media.

advantages that include fewer experiment numbers, suitability for multiple factor experiments, search for relativity between factors, and finding of the most suitable condition and forecast response (Popa et al., 2007; Panda et al., 2009). This facilitates the determination of optimum values of the factors under investigation and prediction of response under optimized conditions (Panda et al., 2009; Chakravarti and Sahai, 2002).

This study aimed at optimizing the medium composition for higher pigment production in a liquid culture of *M. purpureus* with the best conditions obtained from previous study (Baneshi et al., 2011). The only trace element which was reported to support growth and pigment production by *Monascus* species was zinc and has been reported from different laboratories (Juzlova et al., 1996). Ng et al. (2004) found that the higher certain

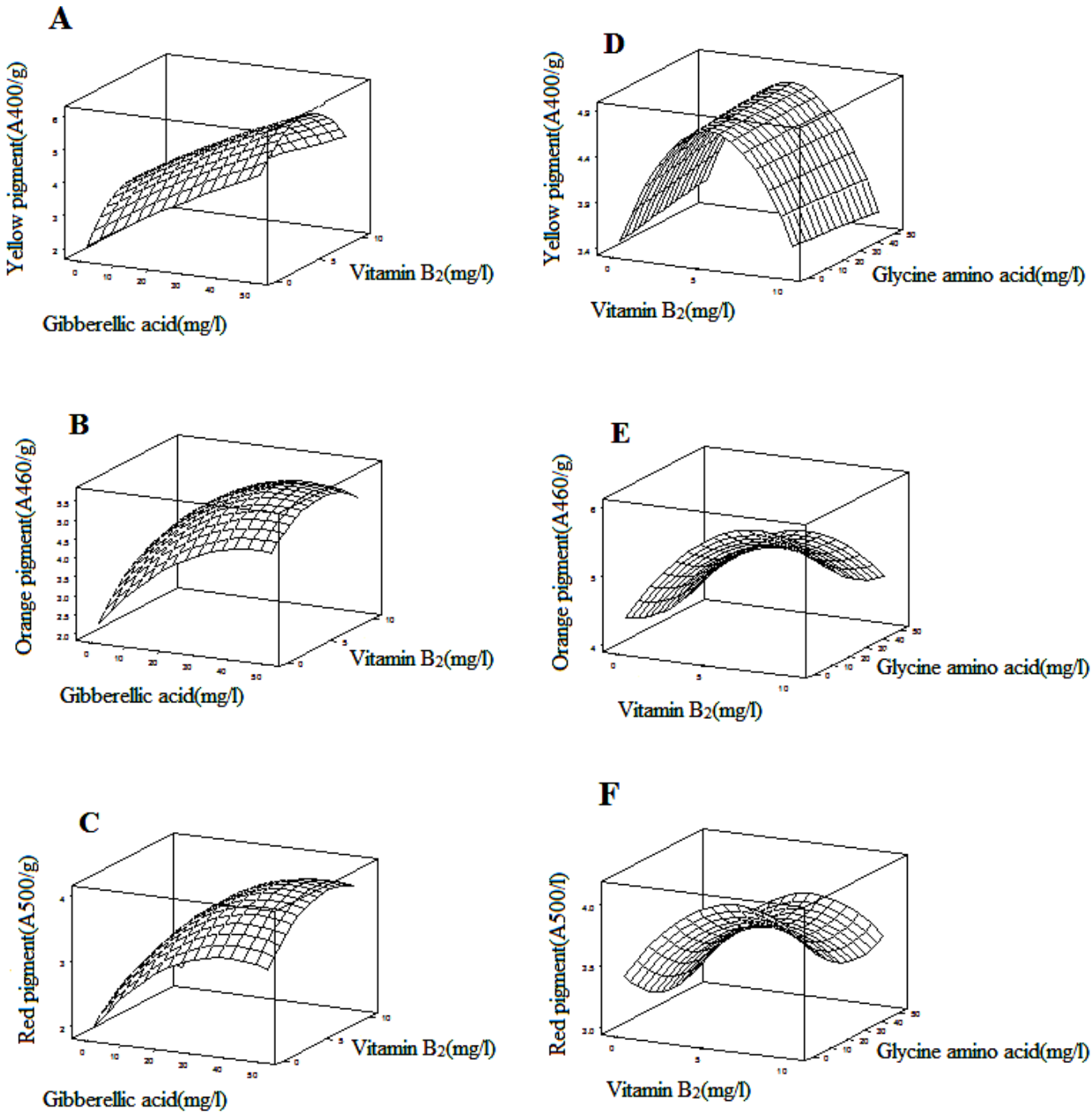

Figure 3. (A-C) Response surface plots showing the effect of gibberellic acid and vitamin B_2 on yellow, orange and red pigments production, respectively, (D-F) Response surface plots showing the effect of glycine amino acid and vitamin B_2 on yellow, orange and red pigments production, respectively while keeping others at constant levels.

zinc concentration, the lower biomass produced but the higher pigment produced. Lee et al. (2001) found that the zinc inhibited red pigment production. Bau and Wong (1979) showed that the growth, pigmentation and antibacterial activity of *M. purpureus* (starch fungus) were affected by zinc. Zinc at concentrations of 2 to 3 mM nearly stopped the growth, pigmentation and antibiotic production of both wild type and strain NI IS in liquid medium. Also, their investigation revealed that the zinc may act as a growth inhibitor and concomitantly as a

stimulant for glucose uptake and for the synthesis of metabolites such as pigments and antibiotics. Timotius and Lestari (1998) proposed that two types of response were observed, first, the higher certain amino acid concentrations, the higher the biomass produced but the production of pigment decreased (Ile, Ala, Leu, Met, Arg, Cys), secondly, the higher certain amino acid concentration, the lower the biomass produced but the higher pigment produced (Tyr, Trp, Thr, Glu, Cys, Gly) of *M. purpureus* UKSW 40. Lin and Demain found that Leucine, valine, lysine and methionine had strong negative effects on the formation of hydrophilic red pigments (Juzlova et al., 1996). Researchers proved that in a nitrogen-deficient medium, the B-group vitamins, both single, especially nicotinamide, pyridoxine and calcium d-pantothenate, and a mixture of thiamine, riboflavin, pyridoxine, calcium d-pantothenate and nicotinamide increased the efficiency of lovastatin biosynthesis (Bizukojc et al., 2006). Mukhopadhyay et al. (2004) suggested that the hormones (indole-3-acetic acid (IAA), gibberellic acid (GA_3) and kinetin (KIN)), at different concentrations, increased the biomass production of *Pleurotus sajor-caju* by 15 to 26%.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

In this study, response surface design showed clearly to be a best tool for optimizing pigment production by *M. purpureus* ATCC 1603. RSM results indicated that increasing gibberellic acid and glycine amino acid along with optimum at a vitamin B_2 addition were able to increase the pigment. In conclusion, the optimum conditions of different parameters (10 mg/l vitamin B_2, 50 mg/l gibberellic acid and 50 mg/l glycine amino acid) had an effect on pigment production.

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Full Length Research Paper

Antibacterial activity of some selected plants traditionally used as medicine in Manipur

Ishwori, L.[1]*, Anupam Das Talukdar[2], Singh P. K.[3], Dutta Choudhury, M.[2] and Deepa Nath[2]

[1]S. K. Womens'College, Kongkhampat,Nambol-795134, Manipur, India.
[2]Department of Life Science and Bioinformatics, Assam University, Silchar, Assam, India.
[3]Centre of Advanced Studies in Life Sciences, Manipur University, Imphal, Manipur, India.

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Methanolic leaf extracts of the plant species *Elsholtzia blanda* Benth., *Elsholtzia communis* (Collett & Hemsl) Diels., *Polygonum posumbu* Buchanam-Hamilton ex D. Don and *Zanthoxylum acanthopodium* DC. using methanol as a solvent were tested against 10 human pathogenic bacteria for potential antibacterial activity. The study revealed that all extracts show varied degree of antibacterial activity against the tested bacterial pathogens. The antibacterial activity was determined using agar well diffusion method. Methanolic extract of the leaf *Zanthoxylum acanthopodium* DC. showed antibacterial activity against five bacterial strains from among the ten bacteria tested followed by *Polygonum posumbu*, *Elsholtzia communis* and *Elsholtzia blanda*. *Clostridium sporogenes* was found to be susceptible to all the plants tested. Minimum inhibitory concentration of the plants against the tested organism ranged between 3.125-12.5 mg/ml. Hence these plants can be used to discover bioactive natural products that may serve as leads in the development of the new pharmaceuticals.

Key words: Antibacterial, human pathogens, methanolic extract, traditional medicine.

INTRODUCTION

The use of plants as therapeutic agents in addition to being used as food is an age long practice (Motley, 1994). Over the ages humans have relied on nature for their basic needs for the production of food stuffs, shelters, clothings, means of transport, fertilizers, flavours and fragrances and not least medicines (Chhetri et al., 2008). Medicinal plants are considerably useful and economically essential. They contain active constituents that are used in the treatment of many human diseases (Menghani et al., 2011). Medicinal plants are cheap and handy to most of the population on the globe (Oluma et al., 2004). Of the 250,000 higher plant species on the earth, more than 80,000 are medicinal (Egwaikhide et al., 2007). Over three quarters of the world population relies mainly on plants and plant extract for health care. More than 30% of the entire plants species at one time or the other was used for medicinal purposes. However, the therapeutic uses of plants by the primitive people lack scientific explanation (Dutta, 1994).

Infectious diseases account for high proportion of health problems in the developing countries including India. Antibacterial resistance among bacterial pathogens in recent time is a critical area of public health concern (Hart and Kariuki, 1998). There has been an increased

*Corresponding author. E-mail: ishlaitonjam@gmail.com. Tel: 0385-2453242 or 09856149972. Fax: 0385-2453319.

bacterial resistance to antimicrobial agents over the past decades and the outlook for the use of antimicrobial drugs in the future is still uncertain. Moreover, antibiotics are sometimes associated with adverse effects on the host, including hypersensitivity, immune-suppression and allergic reaction (McDonnell and Russell, 1999). This situation has forced scientist to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents, but the cost of production of synthetic drugs is high and they produce adverse side effects compared to plant derived drugs (Abiramasundari et al., 2011). Biomolecules of plant origin appear to be one of the alternatives for the control of these antibiotic resistant human pathogens (Raghavendra et al., 2006). Plants have provided a good source of anti-infective agents; emetine, quinine, berberine, tannins, terpenoids, alkaloids and flavonoids continue to be highly efficient instruments in the fight against microbial infections (Cowan, 1999).

Therefore, the increasing prevalence of multidrug resistant strains of microorganisms and the recent appearance of strains with reduce susceptibility to antibiotics put forward an urgent need to search for new sources of antimicrobial agent (Sieradzki et al., 1999). Hence, in the present study an attempt has been made to evaluate antibacterial potential of four medicinal plants namey: *Elsholtzia blanda* Benth., *Elsholtzia communis* (Collett & Hemsl) Diels., *Polygonum posumbu* Buchanam-Hamilton ex D. Don and *Zanthoxylum acanthopodium* DC. which are used as traditional remedies of cough, throat infection, gastric problems and bronchitis respectively in Manipur, India.

MATERIALS AND METHODS

Plant material

Four medicinal plants viz *Elsholtzia blanda* Benth. (leaf), *Elsholtzia communis* (Collett & Hemsl) Diels. (leaf), *Polygonum posumbu* Buchanam-Hamilton ex D. Don (leaf) and *Zanthoxylum acanthopodium* DC. (leaf) were screened.

Preparation of the plant extract

Plant parts were cleaned, air dried in the shade and powdered into fine powder. 10 g of the powdered plant material was soaked in 100 ml of 80% methanol and extracted for 24 h at room temperature with shaking at 150 rpm and further centrifuged at 15000 × *g* for 10 min to pellet solids. The mixture was then filtered and evaporated using the Buchi rotavapor. The dried extracts were resuspended in 5% dimethyl sulphoxide to a final concentration of 100 mg/ml.

Microorganisms used

All bacteria selected are ATCC cultures which include *Bacillus cereus* (10876), *Clostridium perfringens* (13124) *Clostridium sporogenes* (11437), *Klebsiella pneumoniae* (10031), *Niserria gonorrhoea* (19424), *Staphylococcus aureus* (11632), *Pseudomonas aeruginosa* (15442), *Shigella boydii* (9207), *Shigella flexineri* (9199), *Shigella sonnie* (25931).

Preparation of inoculums

Stock cultures were maintained at 4°C on nutrient agar. Fresh cultures are prepared by transferring a loopful of cells from the stock cultures to Mueller Hinton Broth.

Antimicrobial susceptibility testing

The antimicrobial activity of the methanolic plant extracts were screened against the pathogens by using the agar well diffusion assay (Bauer et al., 1966). An inoculum suspension was swabbed uniformly to solidified 20 ml Mueller Hinton agar and the inoculums were allowed to dry for 5 min. Holes of 6 mm in diameter were made in the seeded agar using sterile cork borer. Aliquot of 50 µl from each plant crude extract (100 mg/ml) was added into each hole on the seeded medium and allowed to stand for 1 h for proper diffusion and further incubated at 37°C for 24 h. The antimicrobial activity was evaluated by measuring the inhibition zone diameter in millimetres (mm) around the wells. Aliquots of phosphate buffer saline were used as negative control. These studies were accomplished in triplicates.

Determination of minimum inhibitory concentration (MIC)

The MIC is determined for the highly active plant that showed significant antibacterial activity against the test bacteria according to the methods of Nakamura et al. (1999) and Dulgar and Aki (2009) with some modifications. Serial dilution of each of the extracts were prepared using the 5% DMSO (dimethyl sulphoxide) to produce the final concentrations of 100, 50, 25,12.5, 6.25 and 3.125%. This dilution represents concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml, respectively. 100 µl from each dilution is transferred to 96 well microplate. The bacterial suspension is adjusted to 1×10^8 CFU/ml in Mueller Hinton broth and 100 µl of it is transferred to the respective wells. The microplates were incubated for 24 h at 37°C. MIC values were determined by plating 50 µl from clear wells onto Mueller Hinton Agar. The MIC was considered the lowest concentration of the sample that prevented visible growth. The samples were examined in triplicates.

RESULTS AND DISCUSSION

The antibacterial activity of plant parts used in folk medicines in Manipur from four medicinal plant species has been evaluated *in-vitro* against ten human pathogens (Figure 1). All the four methanolic extracts resulted in consistent inhibition zones against one or more of the bacteria tested in well diffusion assays. The tested plants showed negative as well as positive activities against tested bacteria. *Z. acanthopodium* DC. shows moderate to good (11-15 mm diameter zone of inhibition). The largest zone of inhibition 18 mm was recorded against *Shigella sonnie* with the leaf of *Elsholtzia communis* (Collett & Hemsl) Diels. All the four plant extract namely: *Elsholtzia blanda* Benth., *Elsholtzia communis* (Collett & Hemsl) Diels, *Polygonum posumbu* Buchanam-Hamilton ex D. Don and *Z. acanthopodium* DC. were found active against *Clostridium sporogenes*, 3 extracts active against

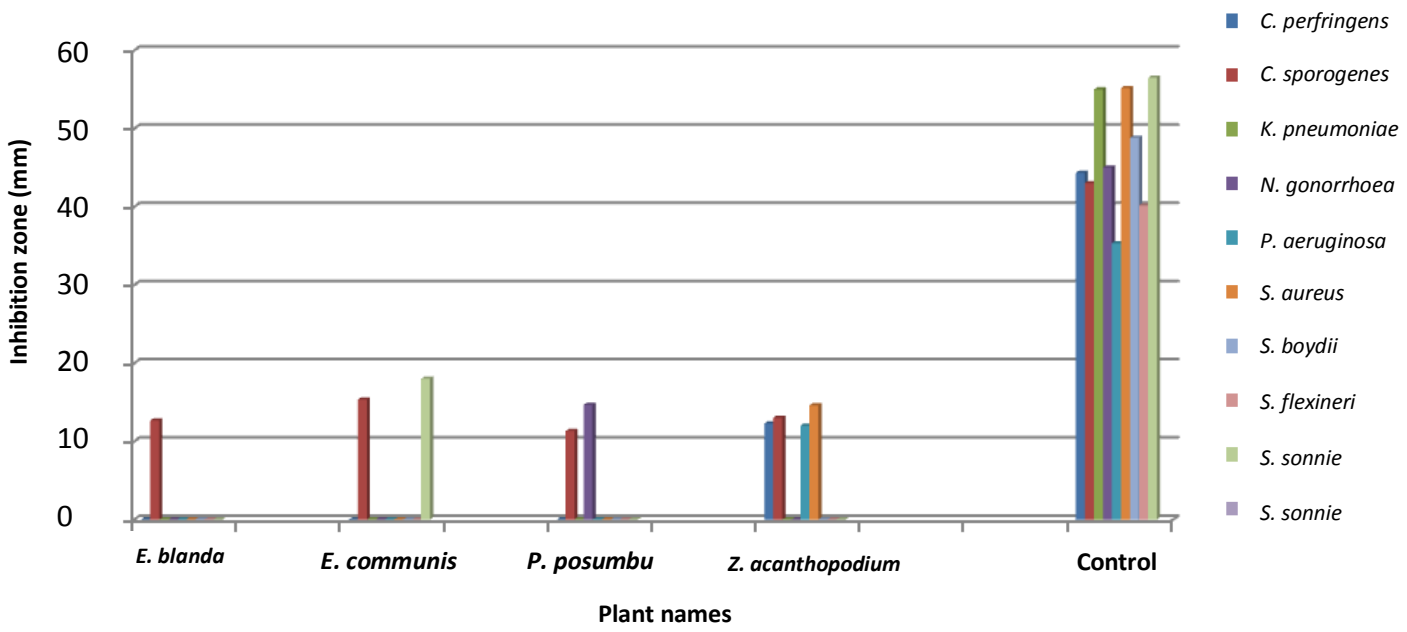

Figure 1. Antibacterial activity of four plant extract.

Table 1. Ethnobotanical information on the plant used as medicine in Manipur (Singh et al., 2003).

Scientific name (family)	Local name in Manipuri	Plant part used	Traditional used
Elsholtzia blanda Benth.	Kanghuman	Leaf	Cough, dyspepsia. Leaf paste applied on forehead in dizziness
Elsholtzia communis (Collett & Hemsl) Diels. (Lamiaceae)	Lomba	Leaf	Fever, cough, high blood pressure, nose bleeding and menstrual disorder
Polygonum posumbu Buchanam-Hamilton ex D Don.	Phakpai	Leaf	Fever and dyspepsia
Zanthoxylum acanthopodium DC. (Rutaceae)	Mukthrubi	Leaf	Fever, dyspepsia, cough, bronchitis. Seed oil applied on rheumatism

Bacillus cereus. The extract of *Elsholtzia communis* (Collett & Hemsl) Diels and *Polygonum posumbu* Buchanam-Hamilton ex D. Don showed active against only for *Shigella sonnie*, *Niserria gonorrhoea,* respectively (Table 1). On the basis of the result obtained in this present investigation it can be concluded that methanolic extract of *Z. acanthopodium* DC. leaves have significant antibacterial activity (Table 2). The highest inhibition was shown by *Elsholtzia communis* (Collett & Hemsl) Diels followed by *Polygonum posumbu* Buchanam-Hamilton ex D. Don, *Z. acanthopodium* DC. and *E. blanda* Benth. respectively. *Z. acanthopodium* DC. shows inhibition to five different bacterial strains from among the ten bacteria tested (Table 3). *C. sporogenes* is susceptible to all the plant extract tested whereas *Klebsiella pneumoniae* does not show susceptibility to all the four plants tested.

Minimum inhibitory concentrations (MIC) were established for all the active plant extracts. The MIC values range from 3.125-12.5 mg/ml. *E. blanda* Benth. and *Polygonum posumbu* Buchanam-Hamilton ex D Don. shows the highest antibacterial efficacy against *B. cereus* at 3.125 mg/ml and *Elsholtzia communis* (Collett & Hemsl) Diels against *C. sporogenes* and *S. sonnie* at 3.125 mg/ml. Also *Z. acanthopodium* DC also proved to possess the highest antibacterial activity against *P. aeruginosa* and *S. aureus* at an MIC value of 3.125 mg/ml. The least inhibition was shown by *Polygonum posumbu* Buchanam-Hamilton ex D. Don against *C. sporogenes* (MIC value of 12.5 mg/ml). In classifying the

Table 2. Antibacterial activity of methanolic extract of selected plant leaves against ten human pathogenic bacteria.

Human pathogenic bacteria	Zone of inhibition (mm)				
	Elsholtzia blanda	***E. communis***	***Polygonum posumbu***	***Z. acanthopodium***	***Control**
Bacillus cereus	12 ± 0.00	0.00 ± 0.00	12.33 ± 0.58	10.33 ± 0.57	30 ± 0.50
Clostridium perfringens	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.30 ± 0.49	44.33 ± 0.58
Clostridium sporogenes	12.67 ± 0.57	15.33 ± 0.57	11.33 ± 0.58	13.00 ± 1.00	43 ± 00.00
Klebsiella pneumonia	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	00.00 ± 00.0	55 ± 00.00
Niserria gonorrhea	0.00 ± 0.00	0.00 ± 0.00	14.67 ± 0.70	00.00 ± 00.00	45 ± 0.50
Pseudomonas aeruginosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12 ± 1.00	35.33 ± 0.29
Staphylococus aureus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	14.60 ± 0.58	55.16 ± 0.28
Shigella boydii	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	00.00 ± 00.00	48.83 ± 0.29
Shigella flexineri	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	00.00 ± 00.00	40.17 ± 0.29
Shigella sonnie	0.00 ± 0.00	18.00 ± 0.00	0.00 ± 0.00	00.00 ± 00.00	56.5 ± 0.5

*Imepenem. Each value represented by Mean±Standard Deviation.

Table 3. Minimum inhibitory concentration.

Plant	Bacteria	MIC (mg/ml)
E.blanda Benth.	*B. cereus*	3.125
E.blanda Benth.	*C. perfringens*	6.25
Elsholtzia communis (Collett & Hemsl) Diels	*C. sporogenes*	3.125
Elsholtzia communis (Collett & Hemsl) Diels	*S.sonnie*	3.125
Polygonum posumbu Buchanam-Hamilton ex D Don.	*B. cereus*	3.125
Polygonum posumbu Buchanam-Hamilton ex D Don.	*C. sporogenes*	12.5
Polygonum posumbu Buchanam-Hamilton ex D Don.	*N. gonorrhoea*	3.125
Z. acanthopodium DC.	*B. cereus*	6.25
Z. acanthopodium DC.	*C. sporogenes*	6.25
Z. acanthopodium DC.	*C. perfringens*	6.25
Z. acanthopodium DC.	*P. aeruginosa*	3.125
Z. acanthopodium DC.	*S. aureus*	3.125

antibacterial activity as Gram positive or Gram negative, it would generally be expected that a much greater number would be active against Gram positive than Gram negative bacteria (McCutcheon et al., 1992). The present finding also revealed that from among the ten human pathogens investigated, seven Gram positive bacteria showed sensitivity against the plant extracts supporting the above views. Hence from the above observation it can be concluded that Gram positive bacteria are more susceptible than the Gram negative bacteria.

The present study justify the use of *Z. acanthopodium* DC., *Elsholtzia communis* (Collett & Hemsl) Diels, *E. blanda* Benth. *and Polygonum posumbu* Buchanam-Hamilton ex D Don. in the traditional system of medicine to treat various infectious diseases. The investigation also supports the used of tested plants in traditional medicine. The used of these plants in folk medicine suggest that they represent an economic safe alternative to treat infectious diseases. Based on this, further pharmacological investigations need to be undergone in order to isolate and identify active compounds.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Comparison of polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) for diagnosis of *Fusarium solani* in human immunodeficiency virus (HIV) positive patients

Ferdousi A.[1]*, Shahhossein M. H.[2,3], Bayat M.[1], Hashemi S. J.[4] and Ghahri M.[5]

[1]Department of Veterinary Mycology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Microbiology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran.
[3]Iranian Gene Fanavar Institute (IGF), Tehran, Iran.
[4]Department of Parasitology and Mycology, School of Public Health and Institute of Public Health Researches, Tehran University of Medical Science, Tehran, Iran.
[5]Department of Biology, Faculty of Science, Imam Hossein University, Tehran, Iran.

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***Fusarium solani* is the most virulent *Fusarium* sp., frequently reported in the fatal disseminated fusariosis in immunocompromised patients. However, it has been always considered, as a very rare or case report infection among HIV positive patients. Anyhow, because of its irreparable consequences, early diagnosis is very important. In this study, loop-mediated isothermal amplification (LAMP) method was developed for rapid and specific detection of *F. solani* in serum samples of human immunodeficiency virus (HIV) positive patients. Transcription elongation factor (TEF-1α) region was considered as the target gene. The test was carried out in 1 h reaction at 65°C in a heater block. The specificity of the test was 100% and its sensitivity was a copy of genome. Using this method among 45 DNAs samples extracted from HIV positive patients' serums, 9 (20%) cases were positive for *F. solani.* All of the samples were rechecked by polymerase chain reaction (PCR) and the results were the same. Considering these results, it was concluded that due to advantages of the LAMP technique, it can be a better alternative for PCR, even in low technology laboratories. In addition, these findings revealed that the possibility of fatal fusariosis due to *F. solani* is not so rare in HIV positive patients.**

Key words: *Fusarium solani,* loop-mediated isothermal amplification (LAMP), HIV, polymerase chain reaction (PCR).

INTRODUCTION

Fusarium species are common saprophytic fungi that cause a broad spectrum of superficial and systemic infections in human. Systemic or disseminated infection, occurred exclusively in severely immunocompromised

*Corresponding author. E-mail:atousaferdousiir@yahoo.com.

Table 1. The features of primers.

Name of primer	Sequence of primer
F3fso	5'-GCTTCTCCCGAGTCCCAA-3'
B3fso	5'-AGGAACCCTTACCGAGCT-3'
FIPfso	5'-CTTTGTCCAACGTCGCCCGAGTTTTGCGGTTCGACCGTAAT-3'
BIPfso	5'-AACACCAAACCCTCTTGGCGCAGCGGCTTCCTATTGTTGAA-3'
Lbfso	5'-GCATCACGTGGTTCATAACAGACA-3'
Lffso	5'-GGGGTAAATGCCCCACCAAAAA-3'

patients (Jain et al., 2011; Nucci and Anaissie, 2002). *Fusarium solani* being one of the most virulent species is associated with disseminated disease more frequently than other *Fusarium* sp. The mortality rate of this fusariosis is almost 100%. It can penetrate into the body following a simple onychomycoses or even from the hospital environment (Jain et al., 2011; Nucci and Anaissie, 2007; Lodato et al., 2006; Guarro et al., 2000; Nucci et al., 2003; Ashwini et al., 2013; Galimberti et al., 2012). Invasive infections occur in immunocompromised patients, mainly in association with prolonged and profound neutropenia or severe T-cell immunodeficiency. However, for unknown reasons, this infection has been always considered to be rare among HIV positive patients (Ashwini et al., 2013; Galimberti et al., 2012). It must be mentioned that because of the limitation of diagnostic method the real incidence of these infections is not clear (Galimberti et al., 2012; Azor et al., 2008).

Early diagnosis of fusariosis is important because of its high level of resistance to several antifungal drugs, and its response often requires combination therapy (Ashwini et al., 2013). The utility of classical or conventional methods, however, is limited, as most of the invasive fungal infections are proven only at autopsy. Furthermore, culture or histopathological analysis are usually time consuming and serological techniques are not specific enough to identify the species (Ashwini et al., 2013; Lewis et al., 2006).

Molecular methods based on nucleic acid sequencing, especially gold standard PCR method, are powerful tools for diagnosis of fungal infections and specific identification of etiological agent. However, because of the expensive equipments, like thermal cycler, needed in gold standard method, it cannot be used in a low-technology laboratory. Loop-mediated isothermal amplification (LAMP) is one of the molecular techniques, which was first designed by Notomi and his colleagues in 2000. This method employs a set of six primers that can recognize a total of eight distinct sequences on the target DNA. They are named FIP and BIP as inner primers, B3, F3 as external primers, BLP and FLP as the loop primers. In this method, strand displacement DNA polymerase in isothermal conditions (approximately 65°C) eliminates the need for a thermal cycler. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour (Mori et al., 2006; Nagamine et al., 2002). Currently, LAMP is mainly applied in the fields of medicine, virus detection, food safety testing and so forth, with less application in detection of fungi, bacteria, nematode and insects (Fukuta et al., 2004; Guan et al., 2010; Jing et al. 2013; Nemoto et al., 2009; Tsujimoto et al., 2007; Nie et al., 2005).

In the present study, LAMP method is established for rapid and specific diagnosis of *F. solani* in serum of HIV positive patients. The results were compared with the results obtained from PCR reference method through chi-square test.

MATERIALS AND METHODS

Extraction of DNA from standard strain

The standard lyophilized *F. solani* strain belonging to bacterial and fungal collection of Iranian Research Organization for Science and Technology (IROST), *PTCC* No. 5284 (UMAH 7419), was cultured in Sabouraud dextrose broth (SDB). After one week incubation, 500 µl of the medium was taken and centrifuged at 10,000 rpm for 2 min.

The supernatant was discarded and the sediment was suspended in 100 µl double distilled water (DDW). Afterwards, DNA was extracted by DNG-plus method. Besides reference strain PTCC NO. 5284 (UMAH 7419), 10 other references strains belong to the collection of TEHRAN university had been used, to be more sure about the accuracy of the obtained results.

Designation of specific primers for LAMP technique

Primers were designed by Primer explorer V4 based on transcription elongation factor (TEF-1α) region of *F. solani* genome (Table 1). The sequences between 29736 to 30046 from mitochondrial genome of the organism were selected and 62978 bps were amplified. The ACC number is JNO 41209.

Reaction mixture for LAMP

LAMP reaction mixture was prepared as following: DDW 5.2 µl, Betaine 5 Mol 4 µl, dNTP (10 mM) 3.5 µl, 10× buffer 2.5 µl, $MgSO_4$ (100 mM) 1.8 µl, primer Mix (A) 1 µl, primer Mix (B) 1 µl, Bst DNA polymerase enzyme (New England BioLabs; Lot:33/110806) 1 µl, target DNA (extracted DNA from standard strain) 5 µl, and total

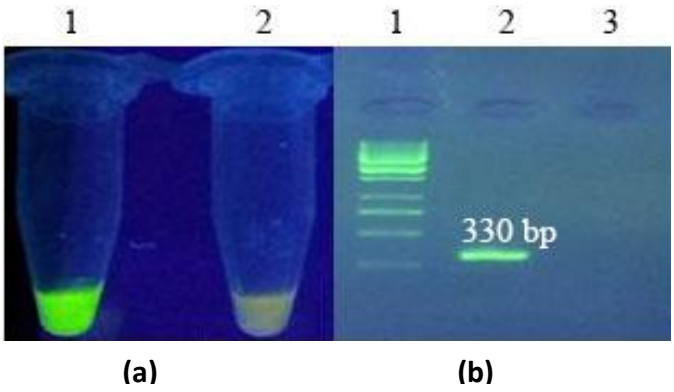

Figure 1. (a) LAMP optimization, **(b)** PCR optimization. 1: Positive reaction 1: Size marker 1 kb termoscientific 2: Negative reaction 2: Positive control (330 bp). 3: Negative control.

volume is 25 µl. Primer Mix(A) containing FIP, BIP primers concentration are 40 and 10 µl DDW in 100 µl total volume, respectively, and primer Mix (B) containing LF, LB concentration are 20 and 60 µl DDW in 100 µl total volume, respectively. The reaction was followed by incubation at 65°C for 1 h.

Analysis of LAMP product

At the end of the reaction, 1 µl of 0.1% SYBER Green (*In vitro* gen) was added to each tube. Afterwards the tubes were visualized under UV light.

Identification of LAMP sensitivity and specificity

To determine the sensitivity of the test, different dilution of suspension agent with specified numbers was obtained to study paired primers sensitivity. DNA extraction was done by these dilutions using Boiling with DNG-plus. To provide serial dilution, after measuring the optical density (OD) of extracted DNA from *F. solani*, Genome Copy Number (GCN) was calculated for it. Then a serial dilution of fungal DNA from 1,000,000 copies of DNA (10^{-1} dilution) to 1 copy of DNA (10^{-6} dilution) was prepared. This serial dilution was used for both PCR and LAMP methods, and their sensitivity was compared. DNA extraction was done by these dilutions using Boiling with DNG-plus.

To provide serial dilution, after measuring OD of extracted DNA from *F. solani*, genome copy number (GCN) was calculated for it. Then a serial dilution of fungal DNA from 1,000,000 copies of DNA (10^{-1} dilution) to 1 copy of DNA (10^{-6} dilution) was prepared. This serial dilution was used for both PCR and LAMP methods, and their sensitivity was compared.

DNA of *HSV, Streptococcus pneumoniae, Staphylococcus aureus* and some other species of *Fusarium* except for *F. solani* including F. *oxysporum, F. verticillioides, F. poea, F. graminearum* and *F. prolifratum* were used for specificity test.

Reaction mixture for PCR

The *mtcytb* gene was considered as a target gene for PCR, and ffuso1 (5'-CTC TGT TAA TAA TGC AAC TC-3') and rfuso2 (5'-TGG TAC TAT AGC TGG AGG A-3') were used as specific PCR primers (Dan et al., 2011). PCR was carried out in a total 25 µl reaction mixture containing 5 µl DNA sample, 2.5 µl PCR 10x buffer, 1 µl of forward and reverse specific primer 10 mM, 0.75 µl $MgCl_2$ (50 mM), 0.5 µl dNTP10 mM, 0.4 µl Taq DNA polymerase 5 u/µl followed by primary denaturation at 94°C for 2 min, cycles denaturation at 94°C for 1 min, polymerization at 72°C for 2 min, and final polymerization at 72°C for 10 min. The total number of cycles was 35. The sensitivity and specificity of the test were evaluated by the same method described for LAMP technique.

Analysis of PCR product

The electrophoresis of the reaction product was carried on the agarose gel containing 1.5% SYBER Green (Sina Colon Cat. No.MR7730C) in TBE 0.5 x buffer.

Clinical sample collection and extraction of samples DNAs

A total of 45 serum samples from blood of HIV positive patients (35 samples belonging to Pasture Institute in Tehran and 10 samples belonging to Iranian Research Center for HIV/AIDS (IRCHA), Tehran University of Medical Sciences, and Tehran, Iran) were collected. For safety reasons, firstly, they were inactivated by heating and then their DNA was extracted through DNG-plus method.

Application of optimized LAMP and PCR test for the clinical samples

Both the optimized LAMP and PCR tests were conducted using the entire 45 DNAs, and the results were compared by chi-square test.

RESULTS

At the end of the LAMP reaction, after adding 5 µl SYBR Green to each tube, the positive reaction tube was demonstrated by a bright green fluorescence under UV 366 nm light, while in negative control and negative reaction tubes no fluorescence was observed under UV light (Figure 1a). In parallel, the PCR products were confirmed by electrophoresis on 1.5% agarose jel, the size of PCR product was 330 bp and the target gene was *mtcytb* (Figure 1b). In addition, the results approved by all other 10 mentioned references too.

The results of specificity test indicated that there was no cross reactivity in LAMP technique and its specificity for *F. solani* detection was 100% (Figure 2a). The same results were obtained by PCR method (Figure 2b). In online research with nblast, this data were approved. The detection DNA limit of LAMP technique is one copy of genome. The used PCR technique had the same level of sensitivity and specificity. Using the LAMP method for 45 clinical samples, 9 cases (20%) of them were found to be positive for *F. solani* and 36 cases of them were found to be negative (Figure 3a). The same results were obtained for the same samples by PCR test (Figure 3b). Comparison of the results obtained from two methods by

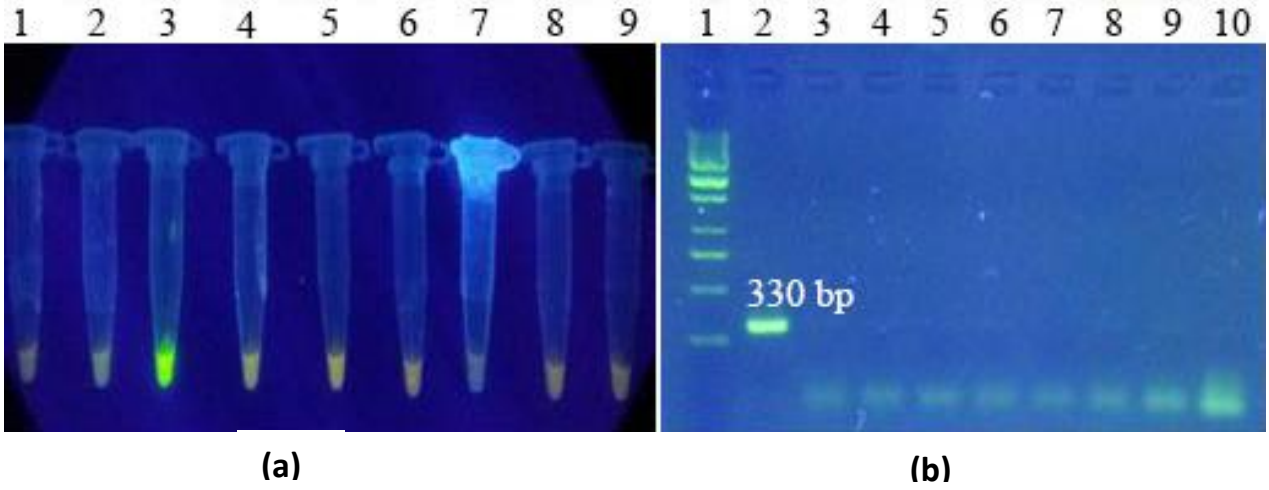

Figure 2. (a): 1: *Streptococcus pneumoniae* (**b):** 1: Middle Range DNA Ladder 1113 Termoscientific. 2: *HSV* 2: *F. solani*; 3: *F. solani* 3: *HSV*; 4: *Staphylococcus aureus* 4: *Streptococcus pneumoniae*; 5: *F. oxysporum* 5: *Staphylococcus aureus*; 6: *F. verticillioides* 6: *F. oxysporum.* 7: *F. poea* 7: *F. verticillioides*; 8: *F. graminearum* 8: *F. poea*; 9: *F. prolifratum* 9: *F. graminearum* ; 10: *F. prolifratum.*

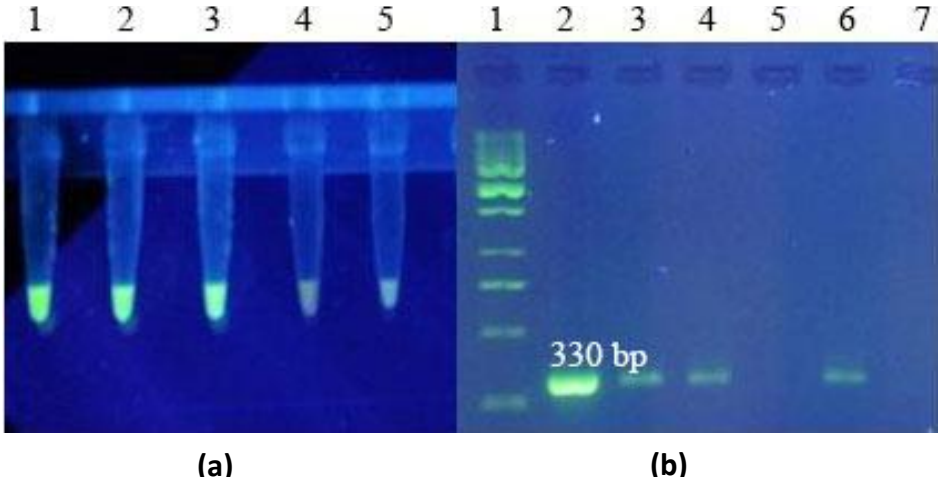

Figure 3. (a): 1: Positive control, (**b):** 1: Size marker DNA Ladder 1 Kb Termoscientific. 2 and 3: Positive sample; 3, 4 and 6: positive sample; 4: Negative sample; 5: Negative sample; 5: Negative control; 7: Negative control.

Chi-squared test showed that both techniques are equally for diagnosis of *F. solani* in serum of HIV positive patients.

DISCUSSION

Fusariosis as the second most frequent systemic mycosis is associated with high mortality (Jain et al., 2011; Lodato et al., 2006) and *F. solani* as the most virulent species, is also associated with disseminated disease more frequently than other *Fusarium* sp. (Jain et al., 2011). Successful treatment is linked to early diagnosis of this infection (Guarro et al., 2000; Galimberti et al., 2012). Identification of species level is highly important because different species have different antifungal sensitivity (Alcazar et al., 2008; Tortorano et al., 2008; Lo¨effler et al., 2002; Lo¨effler et al., 1997). This is particularly more vital when two different species cause infection at the same time. Guarro et al. (2000) reported a case of mixed infection caused by two species of *Fusarium* in a human immunodeficiency virus-positive patient with lymphoma who was neutropenic due to chemotherapy. The patient showed the typical signs of a disseminated fusarial

infection, with *F. solani* isolated from skin lesions and *F. verticillioides* isolated from blood. The report discusses how difficult it is to make an accurate diagnosis when an immunosuppressed patient is infected with more than one fungal species, especially when the species are morphologically very similar (Guarro et al., 2000).

LAMP method is a very powerful tool for identifying the species, particularly, in a mix infection. Due to the use of six primers for target gene, this technique is highly specific in identifying the species. Some important factors including efficacy of DNA extraction and quality of designed primers will affect the sensitivity and specificity of the assay (Lo¨effler et al., 1997; Einsele et al., 1997; Francois et al., 1999). In order to use molecular methods like RAPD, RFLP and PCR for isolation of *Fusarium*, the DNAs were previously developed (Godoy et al., 2004; Thomas et al., 2003; Oriel et al., 2005). The major objective of the current study was to develop the LAMP method and further optimize it for specific, sensitive and rapid detection of organism directly in sample, even in low technology laboratory. In this study, LAMP test was carried out in 1 h; however, for the same analysis by PCR method, the passed time was 3 h. Apart from rapidity, we observed an equal level of sensitivity and specificity using the LAMP method compared to gold standard PCR test. However, in some other studies, a higher level of sensitivity was reported for the LAMP method as compared to the PCR method (Kuboki et al., 2003; Bakheit et al., 2008; O'Donnell et al., 2007; Helm et al., 1990). In general, LAMP offers a better alternative, and the major advantages of this method are possibility of visual judgment by color, being time saving, and not requiring the costly PCR apparatus and gel scanner.

As it is mentioned above, quality of designed primers and the sequences of elected target genes can affect the sensitivity and specificity of the molecular assays (Jain et al., 2011; Lewis et al., 2006; Kuboki et al., 2003). Different molecular studies employ richly varied sequences of multiple loci such as elongation factor 1α (EF-1α)), β-tubulin (β-TUB), calmodulin (CAM), RNA polymerase II second largest subunit (RPB2), the nuclear ribosomal Internal Transcribed Spacer (ITS) region, domains D1 and D2 of the 28S ribosomal DNA (rDNA) large subunit for *Fusarium* sp. identification. However, the available data clearly demonstrate that sequences from the (ITS) region and domains D1 and D2 of the 28S ribosomal DNA (rDNA) large subunit are too conserved to resolve most of the clinically important fusaria at the species level. Moreover, use of ITS and β-tubulin within *F. solani* species complex should be avoided due to paralogous or duplicated divergent alleles (Jain et al., 2011; Guarro et al., 2003; Arif et al., 2012; Eljaschewitsch et al., 1996). In this study, TEF-1α was considered as the target gene. This sequence, according to the reported results (Arif et al., 2012) was highly specific for *F. solani* and did not show cross reaction with any other similar species of *Fusarium* (Eljaschewitsch et al., 1996). This agreed with the obtained results in the current study. As it has been shown in Figure 2a, the specificity of the test is 100%. In online research with nblast, this data was approved. Therefore, the established test can precisely detect *F. solani* in the sample and differentiate the organism even in a mixed infection. Moreover, as it can detect even one copy of genome in the sample, this technique can be very effective for rapid and accurate diagnosis of *F. solani* in serum of HIV positive patients at the early stages of infections.

In the present research, the total number of samples was 45, and nine cases (20%) of them were detected to be positive for *F. solani* by both PCR and LAMP methods. As previously described, for unknown reasons this infection has been always considered to be rare in the population of HIV positive patients (Ashwini et al., 2013; Muhammed et al., 2011). A review of 294 patients published in 2005, identified only two cases of disseminated *Fusarium* infection in HIV positive patients (Perfect, 2012) and no cases of disseminated *Fusarium* infection have been reported since 2005 (Galimberti et al., 2012).

In April 2013, a case report of fatal disseminated fusariosis due to *F. solani* in a HIV positive patient was published. This case represented the first report of fatal disseminated *Fusarium* infection in a non-neutropenic HIV positive patient (Ashwini et al., 2013). As it has been mentioned, although fusariosis is the second responsible organism for disseminated fusariosis, but this infection has been always considered as a rare infection in HIV positive patients. Ashwini et al. (2013) claimed that the two most significant predisposing risk factors for disseminated filamentous fungal infections are severing prolonged neutopenia and T cell deficiency. The first one is less common in HIV patients and the second one has also become less common because of highly active antiretroviral therapy (HAART), at the asymptomatic stage of disease. The mentioned report concluded that because of these reasons this infection is rare in this population. This can be true; however, it seems that due to limitation of diagnostic methods, the real incidence of these infections is unknown. Since they are poorly detected, laboratories and clinical microbiologists are not generally aware of their possible presence in human infections (Azor et al., 2008). Sometimes just after appearing disseminated lesions on the skin of patient, or after positive blood culture this can be identified as Fusariosis, however; in some cases like patient in the article, there is no lesion on the skin of patient. Therefore it causes unawareness of the presence of organism in the body of the host. It seems that, the number of hidden infections must be more than what has been reported. On the other hand because of using corticostoid to prevent, (IRIS) syndrome in these patients, the risk of IFI is increased. Maybe in the first stages of the disease,

because of HAART, it cannot be so obvious, but gradually after decreasing the number of T cell, progressively, it will appeared.

However, proof of the presence of organism in the serum sample does not mean that there is infection at the moment, but it shows that the potentiality of infection is not so rare but maybe it has been ignored or has not been detected clearly by now, but by this developed method it could be distinguished very soon and this can be useful for prevention of disseminated infection.

The obtained results are very highly reliable, because the same samples have been rechecked by gold standard PCR, and the positive and negative controls have been always used in each group of samples.

Generally, early diagnosis is important to manage the consequences of infections. For instance, the results of different researches have shown increased survival of patients by combination therapy (Perfect, 2012; Ho et al., 2007; Vagace et al., 2007). LAMP, as a more recent molecular technique for diagnosis, has some advantages like rapidity, sensitivity, cost effectiveness and easy visual result judgment. To our knowledge, this is the first attempt to establish this technique for detection of *F. solani* in serum of HIV positive patients. The results were compared with the results of gold standard PCR method by chi-squared test. As the results show, both methods to the last dilution of DNA (10^{-6} = 1 copy of DNA) were positive. In addition, using both of these techniques, for clinical samples, the same specimens had the same results. Considering these findings, it can be concluded that both tests had the same level of sensitivity and were equal for diagnosis of *F. solani* in serum samples. Besides the general advantages which were mentioned previously for LAMP techniques, the high level of specificity and sensitivity of this optimized test makes it proper for precise identification of pathogen at early stage of disease and it can be used in all clinical laboratories instead of PCR. It suggests that this method can be applied for other high risk groups for disseminated infection like patients with hematologic malignancies in cytotoxic therapy and transplant recipient.

Conclusion

According to the results obtained in this research, it can be concluded that because of accuracy, rapidity and its cost effectiveness, the LAMP technique can be a better alternative for gold standard PCR, even in low technology laboratories. In addition, the results imply that this fatal infection should no longer be considered so rare in HIV positive patients.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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