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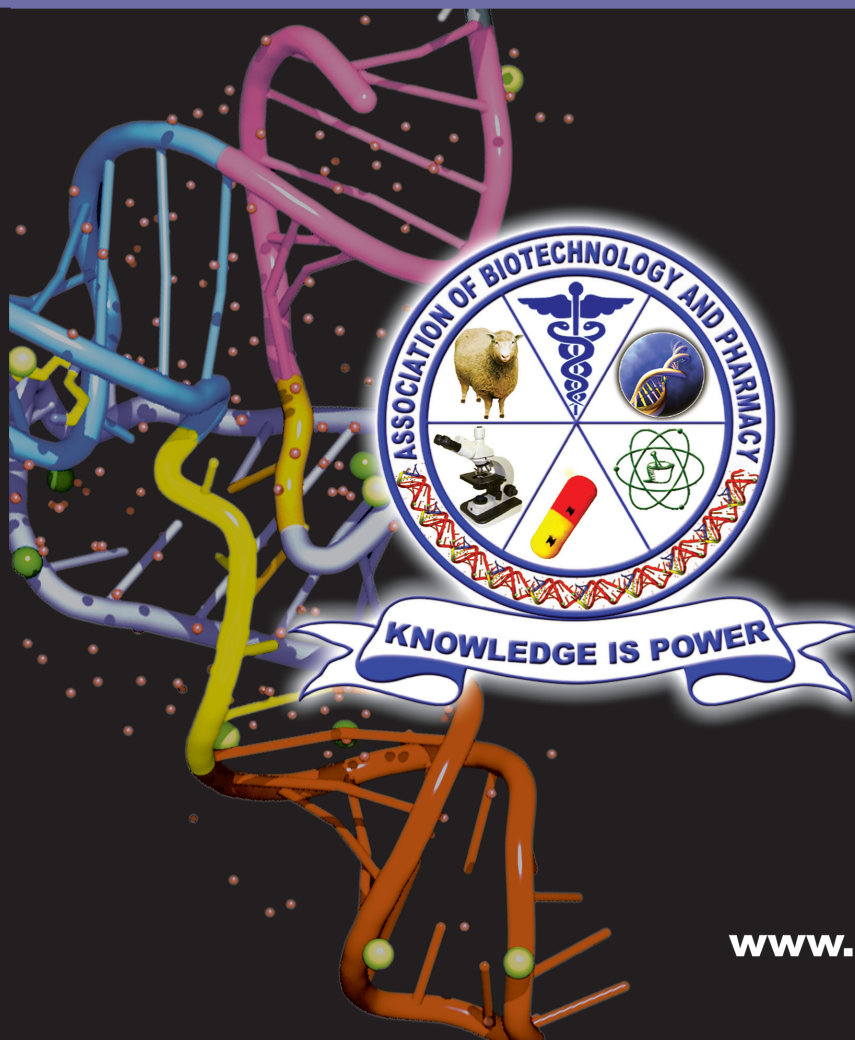

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Seroprevalence of Antibodies to HPV L1 in a Limited Population study determined by the GST-capture ELISA

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Abstract

Human papillomavirus (HPV) infection elicits an antibody response in most individuals, mainly to L1- the major capsid protein of the virus. The Glutathione S-Transferase (GST) capture ELISA was evaluated in a limited population study (n=187 women donors) for its suitability in determining the seroprevalence of antibodies specific to L1 of five high-risk HPV genotypes (16, 18, 31, 45 and 52). Serum samples were obtained from some of the hospital-visiting women aged between 18-60 years with an unknown history and/or status of infection. Most samples (42%) showed sero-positivity to HPV 45 (80/187) followed by sero-positivity to HPV 16 (32%), 18 (16.5%), 52 (10%) and a single (0.5%) positive sample for HPV 31. There was significant cross-reactivity in the samples across the assayed genotypes. The results suggest that the GST-capture ELISA might serve as an efficient alternative to VLP-ELISA in sero-epidemiological studies especially in the large-scale, preliminary, screening of individuals hitherto untested for HPV infection.

Keywords: HPV genotype 16, 18, 31, 45 and 52; GST-L1; GST-capture ELISA; Sero-positivity; VLP-ELISA

Introduction

Cancer of the uterine cervix or cervical cancer is the second most common cancer in women worldwide. Every year, 450,000 new cases are diagnosed and 220,000 patients succumb to the disease (1). It is now firmly established that infection with HPV of the high-risk genotypes, predominantly by the type 16 and 18 (2, 3) precludes cancer. HPV genotypes 16, 18, 31, 45 and 52 together are associated with more than 75% of cancers (4). The infection with the virus is common in majority of women who are sexually active. Although, the first encounter with the virus may occur at an early age coinciding with sexual maturity, the manifestation of the symptomatic disease is observed much later, usually after 35 years of age. In most individuals the virus is cleared spontaneously within months or a couple of years following infection. However, a small number of infected individuals fail to clear the virus and carry persistent infection; a significant proportion of these individuals acquire the disease (5, 6).

Considering the long latency from infection to cancer, early detection is the key to the management of the disease (7). Tests based on detection of the HPV DNA in the cervical smear samples provide a definitive answer to the

current status of the infection (presence or absence of the virus) but offer no clue to the history of infection (previous infections) (8). Besides, the current DNA detection tests are based on PCR and/or hybridization methods, which require considerable skill, training, instrumentation and complexities in both sampling procedures and testing.

ELISA technique, it may be argued, is relatively simple, better understood and has been in wide usage in clinical diagnoses. In view of the persisting serum antibodies to the major capsid protein L1 of HPV, in majority of infected subjects (9), serological diagnosis by ELISA is a promising tool for early detection of present as well as past infections. The major capsid proteins, L1, expressed in heterologous hosts, self-assemble into virus-like particles (VLPs) (10). Since VLPs are structurally near-identical to the native virus current vaccines for prophylaxis of HPV infection are based on VLPs produced from recombinant L1 proteins. Logically hence, the evaluation of vaccine efficacy is based on ELISA with VLPs as coating antigens (11, 12). The VLP based ELISA is also the current gold standard for HPV L1 serological studies (13). Recently, an alternate format namely the rapid capture ELISA based on the glutathione-S-transferase L1 fusion protein (GST-L1) expressed in *E. coli* was reported for the serological evaluation of HPV infection (13). The ELISA involved coating of microtitre well plates with glutathione coupled casein for capturing the GST-HPV16 L1 antigens present in the crude lysate of recombinant *E. coli*. The results of the capture assay, in their study, showed good concordance with the VLP ELISA in the determination of HPV 16 L1 specific antibodies in women. Since the production of GST-L1 antigens involve little downstream processing relative to the production of VLPs it offers a convenient alternative in sero-epidemiological and vaccine efficacy studies (14).

This paper reports the development of a standard ELISA protocol using the purified HPV16 GST-L1, rather than the earlier reported (13) use of crude lysates of bacteria expressing the protein, for the detection of L1 specific antibodies of the five high-risk HPV genotypes (16, 18, 31, 45 and 52).

Materials and methods:

Cloning, expression and purification of HPV major capsid protein : HPV16, 18, 31, 45 and 52 L1 genes, borne on a plasmid flanking with *EcoR*I and *Not*I restriction sites were chemically synthesized (GeneArt, Germany). The genes were sub-cloned into pGEX-4T-1 vector (GE Healthcare, USA) using the Rapid DNA Ligation Kit (Roche, USA) and subsequently transformed into competent *E. coli* BL21 cells. Recombinant *E. coli* BL21 cells were cultured in LB broth and were induced with 1mM IPTG at 0.6 OD_{600} for 4h. Post induction the cells were harvested and later re-suspended in 50mM potassium phosphate buffer containing 200mM NaCl, 1mM EDTA, 1mM DTT, 2mM ATP and 5mM $MgCl_2$. The cell mass was then lysed by sonication (Sonics Vibra cell, USA) and the respective recombinant L1 proteins of the HPV genotypes (16, 18, 31, 45, 52) were purified by affinity chromatography using the Glutathione Sepharose® 4B affinity media as per the manufacturer's manual (GE Healthcare, USA). Analysis of purification was performed with 10% SDS-PAGE and by Western blotting with anti-HPV16 L1 monoclonal antibody (Camvir™; Novus Biologicals, USA) and anti-GST polyclonal antibody (GE Healthcare, USA).The concentrations of the proteins were determined using the Bicinchoninic acid (BCA) protein assay kit (Sigma Aldrich, USA).

Serum samples : Serum samples analyzed in this study were obtained from 187 donor women on consent who visited the Sir Sundarlal Hospital, Banaras Hindu University, Banaras. The age of the volunteers ranged between 20-60 years. The volunteers had not undergone any prior screening for HPV infection or cervical abnormalities.

Positive sera : Sera from cancer patients (n=10) who also tested positive for HPV DNA of either 16, 18 and 45 genotypes in the DNA hybridization

assay (Linear array HPV Genotyping test Kit, Roche Molecular System, USA) were screened for L1 specific antibodies in an indirect ELISA coated with respective VLPs (VLP-based ELISA) as described elsewhere (12). The samples which showed high titres were further screened in the neutralization assay with the pseudovirions of the respective HPV genotypes (courtesy Dr. J.T. Schiller, NIH, USA) as described elsewhere (15). The samples showing significant neutralization titres in the assay were classified as positive sera that contained L1 specific antibodies of the respective genotypes. The HPV16 L1 human reference anti-serum (NIBSC, The UK) was also used as one of the positive sera in the 16 L1 specific GST capture ELISA.

Negative sera : Negative sera (n=20) were constituted by sera obtained from young adolescent girls that showed no reactivity either in the VLP-based ELISAs or the pseudovirion neutralization assays for HPV genotypes 16, 18 or 45.

Standardization of ELISA : Microtitre plates with matrices of different binding strengths for proteins, (low=Polysorp™, medium=-Medisorp™ and high=Maxisorp™) from Nunc, Denmark were evaluated for coating efficiency of casein–glutathione at a concentration of 200ng/well. Three different blocking agents- *viz.*, casein hydrolysate (2%), fish gelatine (2%) and a commercial blocking reagent (Qiagen, USA) in phosphate buffered saline with 0.05% of Tween 20 (PBST) were studied for their blocking efficiency. Optimum concentration of capture antigen was determined by two fold serial dilution with the starting concentration of 400ng/well. Further, optimum concentration of binding antigen (GST-HPV16 L1) was determined by the addition of antigen in two-fold serial dilutions with a starting concentration of 2400ng/well. The bound antigen was probed with commercial HPV16 L1 specific monoclonal antibody (Camvir™; Novus Biologicals, USA). The optimal time of exposure to the chromogenic substrate-TMB/H_2O_2, was determined by incubating the assay plates for various time periods (10, 15, 20 and 30 min) prior to stopping the reaction with 1.25N H_2SO_4. The absorbance was measured at 450nm in an ELISA reader (Beckman Coulter DTX 880 Multimode detector USA).

GST capture ELISA : Ninety six well Maxisorp plates (Nunc Denmark) were coated with Glutathione casein (200ng/well) in 0.5M carbonate-bicarbonate buffer, pH 9.6. The wells were blocked with 2% (w/v) casein in phosphate buffer saline (PBS-casein) containing 0.05% Tween 20 (Sigma-Aldrich, USA) for 1 hour at 37°C. Post washing, binding antigen- GST-HPVL1 (16, 18, 31, 45 or 52), was added at concentration of 600ng/well to respective wells and plates were incubated 37°C for 1 h. Subsequently sera for analysis was added in two-fold serial dilutions (1:25 to 1:3200) along the rows of wells and incubated at 37°C for 1h. Bound antibodies were detected using anti-human IgG HRPO (Sigma-Aldrich, USA; at the dilution of 1:10K in 2% casein-PBST. Appropriate control wells *viz.,* non-specific antigenic control (GST-Bovine Papilloma virus L1 protein) and the assay control.

Method qualification: *Assay Precision:* The assays were repeated thrice on five different days. The inter-assay and intra-assay precision was determined as a function of the coefficient of variation (%CV) across assays.

Statistical analysis : *Cutoff values:* Mean absorbance (A_{450}) of negative sera plus 3 standard deviations was taken as the cut-off values for the determination of sero-positivity in the GST capture ELISA for HPV 16, 18 and 45 L1 (16). The initial cut-off values for HPV serotypes (31 and 52) were determined by frequency distributions (17). The final ELISA cut-off values were calculated using the Receiver Operator Curve (ROC) analysis (MedCal® V 11.5.1.1).

Determination of Sensitivity and Specificity: Sera characterized as positive and negative (20 samples each) as detailed above for the L1 antigens of genotypes HPV 16, HPV18 and HPV45 were assayed for the reactivity in VLP

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based indirect ELISAs and GST capture ELISAs. The sensitivity and specificity were calculated using the 2 x 2 diagnostic test statistics (MedCal® V 11.5.1.1.) against the VLP ELISA readings as standard

Results

Purification of recombinant GST-L1 proteins : The affinity purified GST-L1s conformed to a size of ~82.0 kDa as visualized on SDS-PAGE profile (Fig.1a) and Western blot that was probed with anti-HPV L1 antibody (Fig. 1b). The additional product in the SDS-PAGE and western blot corresponds to the GST protein (Fig. 1c), most likely a cleavage artefact normally observed in recombinant expression of proteins with N-terminal fusion with GST.

ELISA Standardization : Maxisorp™ microtitre plates seemed to be the the most efficient binding matrix as indicated by the relatively higher A_{450} values even at higher dilutions of HPV 16 L1 specific antibody (Camvir™). The most efficient blocking was achieved with 2% casein in PBST. The optimum concentration of the casein-glutathione and GST-HPV L1 was 200ng/well and 600ng/well, respectively, as indicated by the A_{450} absorbance profile in the standardization trials (Fig. 2a and 2b). The optimum period of incubation with the chromogenic substrate (TMB/ H_2O_2) was 10 min.

Statistical Analysis : The variability tests for the GST-capture assay of each of the HPV genotypes showed percent coefficient of variation CVs well below the limit set for precision and accuracy (Table 1). The results therefore indicate that the capture ELISAs meet the acceptance criterion prescribed for a validated immunoassay (17, 18, 19).

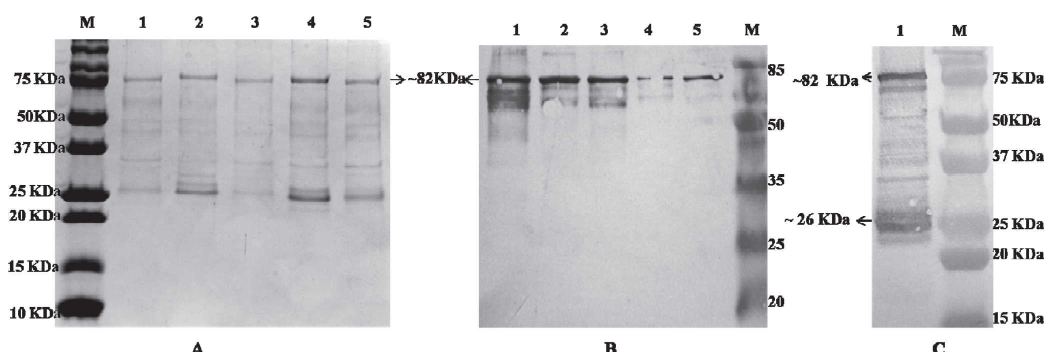

Fig. 1. Analysis of the affinity-purified recombinant GST-L1 protein
A: SDS PAGE and B: Western blot analysis of GST- L1 protein purified using Glutathione Sepharose® 4B affinity matrix. Lane 1- GST-16L1; Lane 2- GST-18L1; Lane 3- GST-31L1; Lane 4- GST-45L1; Lane 5- GST-52L1; Lane M- Pre-stained marker. C: Western blot analysis of HPV16 GST L1 probed using anti-GST anti-body.

Table 1. Coefficient of variation (CV) of genotype specific GST-capture ELISAs.

Assay	%CV				
	GST-16 L1	GST-18 L1	GST-31L1	GST-45L1	GST-52L1
Inter-plate	6.05	3.02	8.44	9.10	6.53
Day-to-day	5.15	6.53	7.34	8.01	9.02
Inter-laboratory	8.61	9.63	11.02	9.03	8.04
Intra-laboratory	7.18	4.18	6.02	9.20	8.02

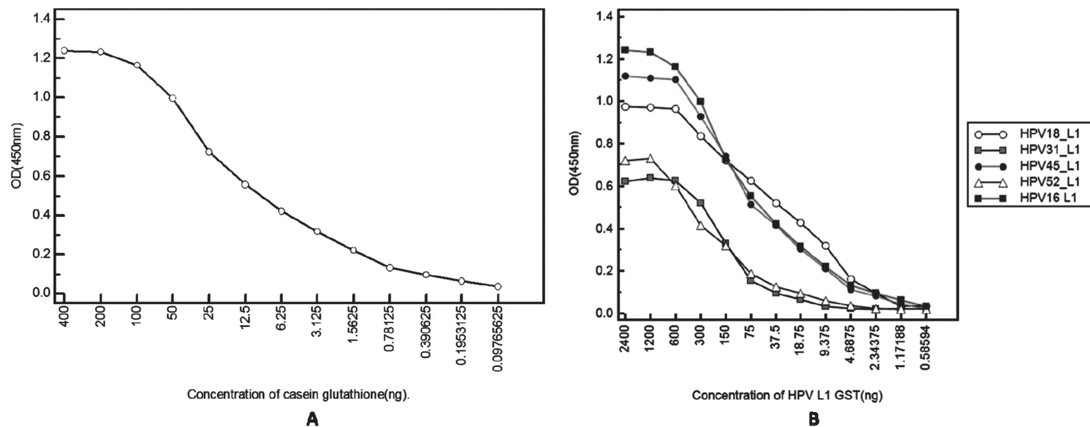

Fig. 2. Standardization of GST-capture ELISA.
A: Curve plotted as absorbance (A450) vs. concentration of coating agent (casein coupled with glutathione) performed with a two-fold serial dilution of coating agent (starting concentration; 400ng/well). The binding antigen (GST-16L1) was used at a constant concentration (600ng/well).
B: Curve plotted as absorbance (A450) vs. concentration of binding antigen (GST-16, 18, 31, 45, and 52L1) performed with a two-fold serial dilution of GST-L1 (starting concentration; 2400ng/well). The capturing antigen (casein-glutathione) was used at a constant concentration of 200ng/well. The commercial monoclonal antibody specific to HPV16 L1 (Camvir™) was used for detection of the bound antigen.

Seroprevalence : The cut-off values for the GST-capture ELISAs of L1 genotypes 16, 18, 31, 45 and 52 at 1:200 dilutions of sera were 0.473, 0.638, 0.329, 0.368 and 0.225 respectively. Out of 187 tested 80, 61, 31, 19 and 1 samples showed absorbance (A_{450}) above the cut-off values with GST-L1 capture ELISAs with HPV genotypes 45, 16, 18, 52 and 31 respectively (Fig. 3). There was significant cross-reactivity of the serum samples across genotypes (Fig. 4). All the sero-positive sera for HPV18L1 and the 52L1 reacted with one or the other genotype assayed. Only 4 sero-positive samples of 16L1 and 10 of the total 80 sero-positive samples of 45 L1 were specific to the respective genotypes.

The GST-capture ELISAs for the HPV genotypes 16, 18 and 45 showed sensitivity values of 94.7 %, 88.9% and 89.5% respectively. The specificity values for the ELISAs were 95.2%, 90.9% and 95.2 % respectively.

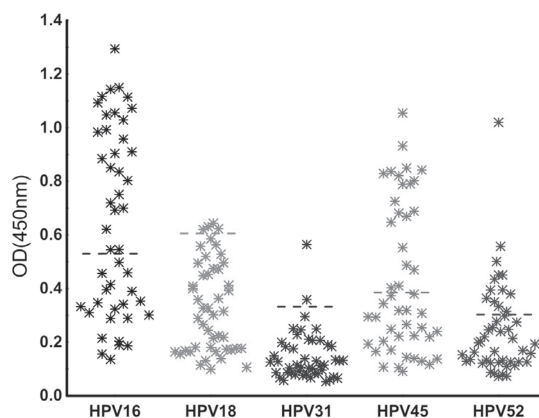

Fig. 3. Seroprevalence of antibodies to HPV L1 type 16, 18, 31, 45 and 52 in Indian women. (n=187). Dotted lines denotes the cut-off OD in the ELISA for the respective HPV types.

Discussion:

The majority of the estimated total deaths from cervical cancer, especially in the past few decades, have been reported to occur in the developing countries (20). The absence of systematic screening of women for cervical abnormalities is the primary cause for this skewed mortality rate in the less developed regions (20). Periodic and mandatory screening

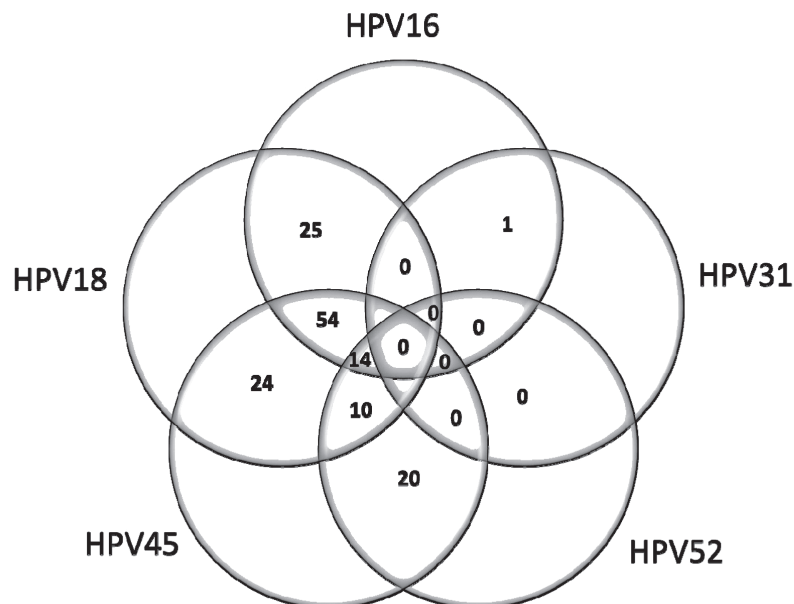

Fig. 4. Venn diagram depicting cross-reactivity of sero-positive samples across HPV genotypes. The number of samples showing sero-positivity to L1 of a specific HPV genotype is denoted in the corresponding set. The cross-reacting serum samples between HPV genotypes are denoted in the intersections of the respective sets.

of the large population of sexually active women in these regions with the current method of tissue-sampling (Papanicolaou test, Colposcopy, Biopsy etc.) is an expensive proposition. Besides, the clinical procedures involved, also require considerable skill and logistical resources that are seriously limiting in the nation states of these regions (21). Hence, a simple assay system that also holds promise in cost effectiveness such as the GST-capture ELISA, might just be the answer for large-scale screening of women in the less developed parts of the world.

The GST-capture ELISA first proposed by Sehr *et al.* 2001 (13) has been modified in this study only to the extent of using a purified form of the antigen than the crude recombinant *E. coli* lysates. We reckoned that a well characterized antigen is essential for the development of test kits and/or protocols that meet the precision and accuracy desired of a diagnostic assay (22). Further, the capture ELISA format was also evaluated for the determination of the seroprevalence of antibodies to the L1 of HPV genotypes 31, 45 and 52 besides the commonly studied 16 and 18 HPV types.

The yield of purified GST-L1 fusion proteins of the HPV genotypes ranged between 6.0mg-9.0mg/L of recombinant *E. coli* BL21 culture. Although modest, we reckon that the production costs would still be relatively less expensive than involved in the production of VLPs that need considerable downstream processing (23).

The GST-capture ELISA format was further standardized to establish a robust, reproducible assay protocol. The precision values (%CV) suggest that the ELISA meets the diagnostic assay criteria laid down by the International *Conference on Harmonization (*http://www.ich.org/; Quality Guidelines).

Seroprevalence studies in a population would invariably require established reactive and non-reactive sera (positive and negative control

sera) to determine for actual positive and negative serum samples in the ELISA. However, reference standards (except the HPV 16L1 human sera, NIBSC, the UK.) for the L1s of most HPV genotypes are not readily available. A characterized negative serum from a known, uninfected, individual is also difficult to obtain. We therefore designed a logical approach for the identification of non-reacting serum, and reacting serum from the available pool of human sera for use as the necessary negative and positive controls in the HPV 16, 18 and 45 specific GST-capture ELISAs. A combination of two established serological assays namely the pseudovirion neutralization assay (15) and the VLP-ELISA was used for this purpose. VLPs, which are identical in structure to the native virus, are used as antigens in the indirect HPV VLP-ELISA. Hence, VLP-ELISA is generally accepted as the "gold-standard" for the determination of virus specific antibodies (16). Further, the pseudovirion neutralization assay is a well-established *in vitro* assay for the determination of neutralizing antibody titres in a sample. The results of these two assays taken together, prospectively identifies genuinely positive sera; that which consists of L1 capsid specific antibodies. Sera that originally showed no reactivity in either the GST capture ELISA or the VLP ELISA, as was expected, did not show neutralizing titres in the pseudovirion assay and hence formed the panel of negative sera for the HPV 16, 18 and 45 specific GST capture ELISAs.

Lack of either VLPs or pseudovirions of HPV31 and 52 genotypes did not allow a similar approach to be followed for identification and classification of known control sera for assays specific to the two genotypes. Therefore the standard statistical methodology of determination of cut-off values by ROC was followed for the HPV 31 and 52 specific ELISAs in this study

The capture ELISAs were performed individually for each HPV genotypes (16, 18, 31, 45 and 52) to determine the serological status of the samples to the respective L1s. Surprisingly, most samples (42%) showed sero-positivity to HPV 45 (80 out of the 187 samples tested) followed by HPV 16 (32%), 18 (16.5%), and 52 (10%) respectively. There was only a single positive reactor for HPV 31. The results, except for the large number of positive reactors to HPV 45L1, generally followed the world-wide genotype specific prevalence pattern (24). There was significant cross-reactivity of sero-positive samples to L1 of one genotype to the L1s of other genotypes (Fig.4). Nearly, all samples sero-positive to the L1 of either 16 (61/187), 18 (31/187), or 52 (19/187) genotypes, also cross-reacted with the L1 of HPV 45. Only 12.5% (10 out of 80 sero-positive samples) were specific reactors to L1 of HPV 45. Similarly a minuscule 6% (4 out of 61 sero-positive samples) was specific to HPV 16 L1. All the sero-positive samples for 18L1 and 52L1 also reacted with the other genotypes, mainly either HPV45 L1 or 16L1. This prompted us to evaluate the sensitivity and specificity of the captures ELISA vis-à-vis the gold-standard assay, the VLP-ELISA. However, this could not be performed for the GST-capture ELISAs of HPV31 or 52 owing to the non-availability of genotype specific VLPs.

The determination of sensitivity and specificity was performed with the confirmed, genotype specific positive and negative sera for HPV16, 18 and 45 in the VLP-ELISA. However, the cross-reacting nature of the sera that were determined as positive reactors in the VLP-ELISA could not be ascertained due to the scarcity of VLPs. It is evident from the results that the GST-capture ELISA compares well with sensitivity and specificity *vis-à-vis* the VLP ELISA in determining sero-positivity and/or negativity of a sample to HPV L1. But the determination of genotype specificity of the antibodies in a sample by the GST-capture assay *vis-à-vis* the VLP-ELISA cannot be commented on with the limited data for the latter assay in this study.

A direct correlation between the cross-reactivity of sera to the homology of protein sequence of L1s among HPV genotypes could not be established. But the L1 capsid protein of the high-risk HPV genotypes share significant

homology in amino acid sequence (25). Hence, cross-reactivity is most likely, due to the presence of non-conformational antibodies to the linear epitopes of L1 (26).

The L1 proteins expressed in fusion with the GST are reported to be compromised in the ability to form VLPs but are shown to assemble in a pentameric structure where the GSTs occupy the periphery (27). The antigen in the GST-L1 pentameric form has the internal regions involved in the capsid assembly exposed. These regions are also fairly conserved across HPV genotypes and hence might contribute to the antibody cross-reactivity of the serum samples across genotypes. Another aspect worth consideration is the evaluation of the infection status of the volunteers involved in the study. Multiple infections with different HPV genotypes would in all probability influence the antibody repertoire of the host. At least the current infection status can be ascertained with the established HPV DNA testing methodologies (28).

Conclusion:

The study strengthens the case for application of GST-capture ELISA in preliminary screening of women for exposure to HPV. The comparable sensitivity and specificity of the capture ELISA to the current gold-standard (VLP-ELISA), the ease of antigen production, and the format of the assay (capture versus indirect), makes it a promising assay for large scale screening of individuals in low–resource settings. However, significant cross-reactivity of the positive reactors across genotypes was observed in this study. Therefore the application of the assay format for delineating the genotype specific humoral response, especially in studies to determine vaccine efficacy, would be limited.

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Phylogenetic Reconstruction of five Solanaceous species by Genome-wide Analysis of Simple Sequence Repeats in Organellar Genomes and their Utility in Establishing Species Relationships of genus *Nicotiana*

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Abstract

Simple sequence repeats (SSRs) in organellar genomes offers better opportunity in understanding the phylogenetic relationships among important Solanaceae crop species. Even though organellar genomes of some crop species are available in the public domain, a comparative analysis of SSRs has been reported only for major cereals. Since Solanaceae has been proved to be an excellent model for comparative genomics, there is immense need to understand the phylogenetic relationships by comparative analysis of organellar SSRs. The purpose of this study was to understand the organization and distribution of SSRs in the genic and intergenic regions of organellar genomes of Solanaceous species *viz.,* tomato, potato, tobacco and progenitors of tobacco and demonstrate the utility of organellar SSR markers in phylogenetic analysis.

Keywords Microsatellites. Phylogenetic relations. Chloroplast DNA. Mutations. Flanking sequences. Organellar genomes.

Introduction

Many crop species belonging to Solanaceae such as potato, tomato, pepper, tobacco etc have agronomical and medicinal importance. Among the crop species, tomato and pepper are used as excellent model system to study fruit development and plant defense (1,2,3), potato for tuber development (4) tobacco and *Nicotiana benthamiana* for plant transformation and virus-induced gene silencing (VIGS) respectively (5). The family is extremely diverse in terms of habit, habitat, flower and fruit morphology (6), which makes it very interesting for evolutionary studies. Furthermore, significant macro- and micro-synteny conservation among the genomes of tomato, potato, pepper and eggplant make Solanaceae an excellent model for comparative genomics (6). Prior to molecular systematics, morphological characters such as floral and fruit / berry characters were used for determining the phylogenetic relationships. With the shift towards molecular systematics, chloroplast DNA (cpDNA) has emerged as an excellent source of molecular variation available for higher order (i.e., generic level and above) phylogenetic studies in plants (7) and this has helped to resolve some long-standing issues in Solanaceae systematics. With the current trend of whole genome phylogeny, it will be worthwhile to determine the phylogenetic relationships based on genome-wide microsatellites as they play a major role in the evolution of genomes due to their high rate of mutation. Despite the availability of

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complete organellar genomes of many crop species, a comprehensive analysis on the abundance and distribution of microsatellites has been recently reported only in rice and major cereals (8, 9). The main objective of this study is to determine the comparative abundance and distribution of microsatellites in the genic and intergenic regions of organellar genomes of few Solanaceous species and their utilization in understanding their phylogeny.

Materials and methods

Identification of SSRs: Complete chloroplast genome sequences of five crop species of Solanaceae available in GenBank (http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html) were used for identification of SSRs. The genome sequences were *Nicotiana tabaccum* (Genbank: NC_001879), *N. sylvestris* (Genbank: NC_007500), *N. tomentosiformis* (Genbank: NC_007602), *Lycopersicum esculentum* (Genbank: NC_007898) and *Solanum tuberosum* (Genbank: NC_008096). The complete mitochondrial genome sequence of *N. tabaccum* (Genbank: NC_006581) was also used for analysis. Identification of perfect di-, tri-tetra-, penta- and hexanucleotide repeats possessing the repeat motifs repeated e"3 times was performed using the online resource Simple Sequence Repeat Identification Tool (SSRIT) (10). The sequence annotation in the Genbank database was used to identify repeats in genic and intergenic regions.

Whole genome analysis for detection of indels and SNPs: Gene content comparisons were performed with MultiPipMaker (11) (http://pipmaker.bx.psu.edu/pipmaker/tools.htm). MultiPipMaker has a suite of software tools to perform comparisons between more than two sequences. Comparisons included five chloroplast genomes listed above and N. tabaccum was used as the reference genome.

Phylogenetic analysis: SSRs with a repeat length e" 12 nt (class II) along with 200 nt flanking sequences were used for analysis. Alleles corresponding to each motif were identified based on the presence of same flanking sequences in other genomes. Absence of a particular repeat was considered as a null allele. Duplicate loci were identified based on the occurrence of same repeat motif as well as flanking sequences. Allelic information was converted in to binary data and a phylogenetic tree was constructed based on Unweighted Pair Group Method with Arithmetic Averages (UPGMA) algorithm using the TREECONW software (12).

To assess the utility of organellar SSRs as potential genetic markers, about 12 cpSSRs with a maximum repeat length of e"12 nt were selected from N. tabaccum. Primers were designed using the software FastPCR (13) with standard parameters (14) and were used for amplification of DNA isolated from 25 species of the genus Nicotiana (Table 1 and 4). Total DNA was isolated from leaves of 30 day-old tobacco seedlings following the standard protocol. PCR was performed in 10 ml reaction volume containing 25 ng of template DNA, 0.2 mM of each primer, 200 mM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 1 U of Taq polymerase in a 96-well thermal cycler (Eppendorf, Germany). The thermal profile was: 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min and a final extension at 72ºC for 7 min. Amplified products were resolved in 6% denaturing polyacrylamide gel electrophoresis (PAGE) followed by silver staining (15). SSR alleles were precisely sized using the software utility Alphaease™ (Alphainnotech, USA) with 50 bp ladder (MBI Fermentas, Lithuania) as the size standard. The allelic data was converted to binary data and the phylogenetic tree was constructed as described above.

Results and Discussion

Comparative analysis of organellar SSRs among Nicotiana group: Comparative analysis of tobacco chloroplast SSRs (cpSSRs) was performed with two of its progenitors (*N. sylvestris* and *N. tomentosiformis*) and with its mitochondrial SSRs (mtSSRs). Approximately 2% of the mitochondrial and chloroplast genomes of

tobacco possessed SSRs. Total number of cpSSRs ranged from 444 (*N. tabaccum*) to 507 (*N. tomentosiformis*) while the mitochondrial genome of tobacco had 1400 SSRs (Table 2).

About 1.44 % of cpSSRs and 1.47 % of mtSSRs were present in the genic region. The frequency of cpSSRs in the genic region of *N. tabaccum* was 2.7 SSRs / Kb, which was close to the *N. tometosiformis* (2.3 SSRs / Kb) than *N. sylvestris* (2.2 SSRs/Kb) and it, was similar to mtSSRs (2.8 SSRs/Kb). With respect to the intergenic region, *N. tabaccum* had fairly low frequency (3.4 SSRs / Kb) of SSRs than its progenitors (6.2; 6.9 SSRs / Kb). This may be due to the fact that the progenitors have more number of SSRs and most of them reside in intergenic region. But the frequency of genic mtSSRs (3.28 SSRs/Kb) was almost same as cpSSRs. The density of cpSSRs in the genic region of *N. tabaccum* (17.8 bp/Kb) was slightly higher than *N. tomentosiformis* (15.3 bp/Kb) and *N. sylvestris* (14.7 bp /Kb) (Supplementary Figure 1).

In contrast, the density of mtSSRs was higher (22.46 bp /Kb). Among the different classes of repeats, dinucleotide repeats were higher in proportion in both the chloroplast (76-81%) and mitochondrial genomes (81.35%) of *Nicotiana* group. The motif AT/TA was predominant in the genic and intergenic regions of *Nicotiana* chloroplast genomes, which was similar to that observed in liverworts, pea (16) and rice chloroplasts (8). In contrast to chloroplast genome, the motif AG/TC occurred frequently in genic and intergenic regions of the mitochondrial genome. This observation is in contrast to the pattern in major cereals where both the organellar genomes had higher frequency of AT/TA motifs (17). The next predominant repeat was tri nucleotides which contribute 17-22% of total repeats in the chloroplast genome and 15.5% in the mitochondrial genome. Among tri nucleotide repeats, mitochondria possessed 47 different types whereas chloroplast had 27. The motif AAG/TTC was more abundant in genic region of both the organelles and intergenic region of mitochondrial genome while the intergenic region of chloroplast genome had predominantly AGA/TCT motif. Similar to chloroplast, majority of tri nucleotides repeats were present in the genic region of mitochondrial genome. Recent stable studies have shown that certain tri nucleotides and hexa nucleotides are more abundant in coding regions than in non-coding regions of higher eukaryotic genomes (18). Tobacco mitochondrial genome possessed 33 different types of tetra-, and 4 types of penta nucleotide repeats while the chloroplast genomes of *Nicotiana* group had only 8-13 types of tetra nucleotide and a single penta repeat. Most of the tetra nucleotide repeats in chloroplast genomes reside in the intergenic region except *N. sylvestris*. All penta nucleotide repeats were found in the intergenic region of chloroplast genome with the motifs $(TTTAA)_3$ and $(TTAAT)_3$ unique to *N. tomentosiformis* while the motif $(TAATT)_3$ was common among *Nicotiana*. Interestingly, these genomes were devoid of hexa repeat, which is in contrast to rice chloroplast genomes (8). Generally, repeat length of di nucleotide and tri nucleotides motifs tend to be longer than other repeats. But, in the present study, the penta-and hexa nucleotides were longer than other classes of repeats. The lack of longer di and tri nucleotides repeats could possibly be explained by the downward mutation bias and short existence time (19).

Comparison of cpSSRs in *Nicotiana* with tomato and potato.

Comparative analysis was also performed among *Nicotiana*, tomato and potato cpSSRs. The frequency of SSRs in the genic region of tomato and potato was similar (2.6 SSRs/ Kb) to *Nicotiana* group. But the frequency of SSRs in the intergenic region of tomato is almost double (3.9 SSRs/ Kb) than that of potato (2.1 SSRs/ Kb), close to that of cultivated tobacco (3.4 SSRs/ Kb) and higher than tobacco progenitors (~7 SSRs/ Kb). The density of genic SSRs in tomato and potato was almost same (17 bp/Kb), which was similar to cultivated tobacco (17.8 bp/ Kb). Significant variation was observed in the

proportion of genic SSRs in potato (81%), cultivated tobacco (75%), tomato (69%) and wild tobacco (57%) in the decreasing order (Supplementary Figure 1). Among the dinucleotide repeats, AT/TA motif was predominant in the genic and intergenic region of potato and tomato, which was similar to *Nicotiana* group (Supplementary Figure 3). Among the trinucleotide repeats, AAG/TTC motif was predominant in the genic region in tomato and *Nicotiana* group while, AGA/TCT motif was predominant in potato. Predominance of ATT/TAA was observed in potato and tomato, which was similar to wild tobacco and different from cultivated tobacco. Tomato and potato had 7 & 8 tetra nucleotide repeats respectively, which is less compared to the members of *Nicotiana* group (9-13). The penta nucleotide repeat motif (AATTG)$_3$ was present both in tomato and potato, while *Nicotiana* group had (TAATT)$_3$ (TTTAA)$_3$ and (TTAAT)$_3$ motifs. This non-random distribution of repeats may be the result of differences in mutability and the bias in repair efficiency of the mismatch repair system, which could lead to overrepresentation of microsatellites in certain genomes (20). Our results suggest that the relative abundance of microsatellites increases with the genome size (Supplementary Figure 4). A positive correlation between SSR content and genome size was reported in some eukaryotic genomes (21). Similarly, the relative abundance of each class of microsatellites (di nucleotides to hexa nucleotides) also differs among the species.

Phylogenetic relationships among members of Solanaceae as revealed by cpSSRs: SSRs identified in chloroplast genomes in the present study were classified into hyper-variable markers (class I, repeat length e” 20 nucleotides), potentially variable markers (class II, repeat length e” 12 and < 20 nucleotides) and stochastic markers (class III, repeat length e” 6 and, < 12nucleotides) based on the length of repeat motif as suggested earlier (23). Tobacco mitochondrial genome possessed 51 Class II repeats with no Class I repeats. The mtSSRs can be used for the development of molecular markers for distinguishing CMS lines from their cognate maintainer lines and also for phylogenetic analysis. Recently, a mitochondrial SSR based marker has been reported in rice for distinguishing cytoplasmic male sterile lines from their isonuclear maintainer lines (24). With respect to chloroplast genomes, about 11 (tomato) to 38 (tobacco) SSRs belonged to class II type while two Class I SSRs were identified in tobacco. Lack of very long microsatellites has been considered as evidence that selection is also involved in maintaining microsatellites within a certain range (25). In a preliminary study to assess the phylogenetic relationship among members of Solanaceae, all the Class II SSRs with their flanking sequence were selected for analysis as detailed in material and methods. Cross-genome comparisons indicated that some SSR loci are highly conserved and some were highly unique to a particular species Table 3. Conservation of SSR loci across species over long evolutionary time periods with the number of repeats never reaching long values was also reported earlier (26). The phylogenetic tree constructed in this study using the SSR data indicated that cultivated tobacco (*N. tabaccum*) is closer to one of its progenitor (*N. sylvestris),* clearly confirming the maternal origin of cultivated tobacco (Fig. 1b). Flower morphology, chromosome segregation patterns, GISH, chloroplast and mitochondrial sequence data also suggest that a close relative of *N. sylvestris* (2n = 2x = 24) is the maternal S-genome donor (27,28,29,30). The present study also showed that *N. tomentosiformis* formed the out-group, while cultivated tobacco is closer to tomato than potato (Fig. 1a). Potato and tomato were grouped together and this observation was also noticed through cytogenetic studies (31). Careful survey of whole genome alignment for the identification of SNPs and indels indicated that N. *sylvestris* is the maternal parent of *N. tabaccum* as the patterns were almost similar in both the species. The similarity in the SNP-indel patterns also revealed the closeness of tomato and potato genomes. This result is in confirmation with that obtained in this study based on genome-wide SSR analysis. The phylogenetic relationship

among members of the Solanaceae determined in this study matched with the earlier reports, which were based on nuclear genome colinearity (31, 32) and analysis of chloroplast genes (32,33).

Phylogenetic relationship among the species of Nicotiana using cpSSR markers: In order to assess the potential of cpSSRs for their utility in determining the phylogenetic relationships, 12 markers were analyzed in a set of *Nicotiana spp* as described in Materials and methods. All SSR markers showed clear, robust amplification and were polymorphic (Supplementary Fig. 5). A dendrogram constructed based on chloroplast SSR polymorphism grouped all the 25 species of genus *Nicotiana* into four major clusters (Figure 1b). First major cluster consisted of three sub-clusters. Three species each of subgenus

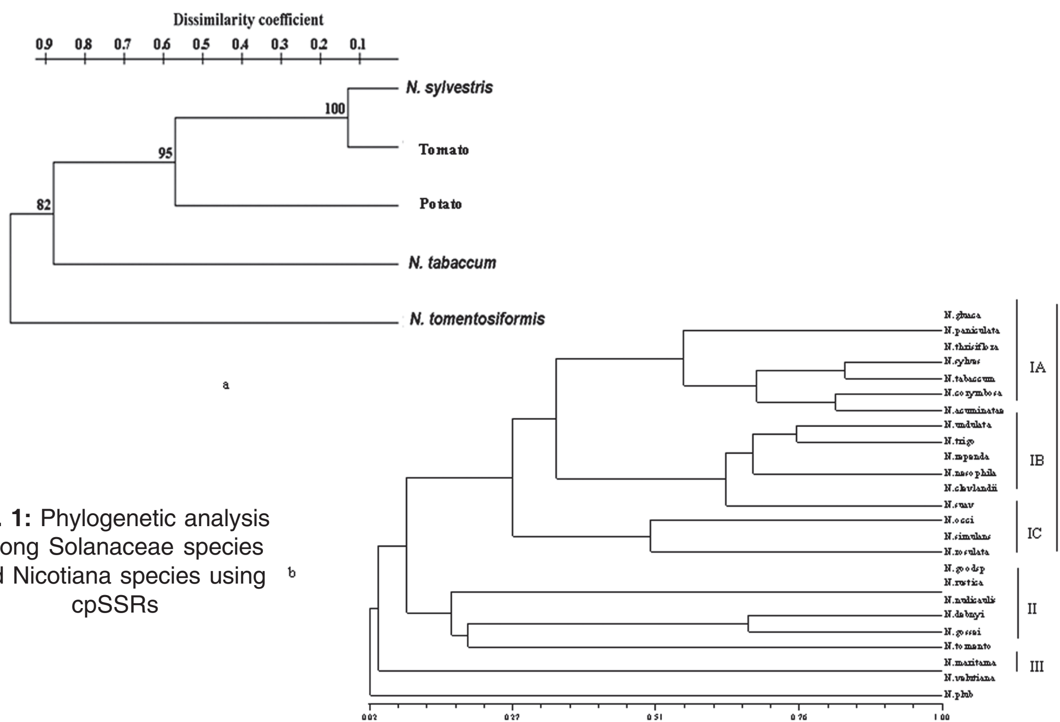
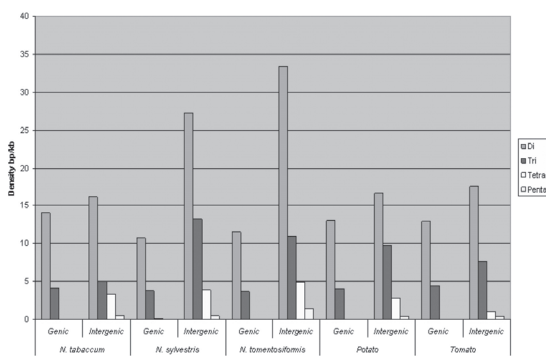
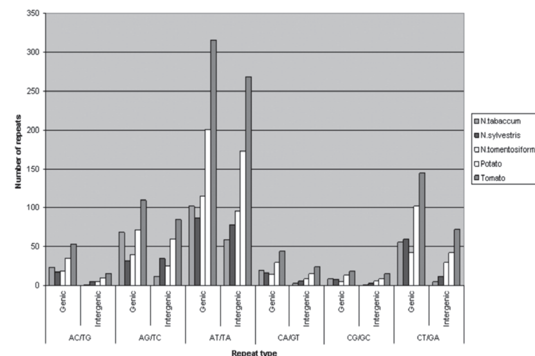


Fig. 1: Phylogenetic analysis among Solanaceae species and Nicotiana species using cpSSRs



Supplementary Fig. 1 Density of SSRs in genic and intergenic regions of chloroplast genomes



Supplementary Fig. 3 Distribution of Di-repeats in chloroplast genomes

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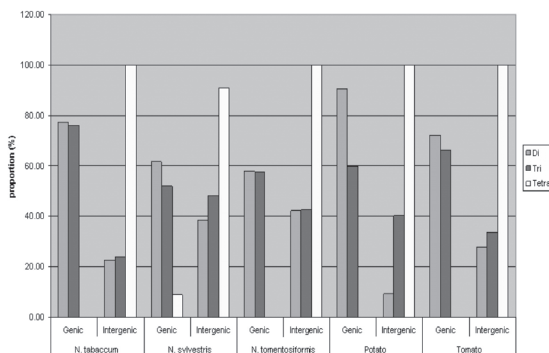
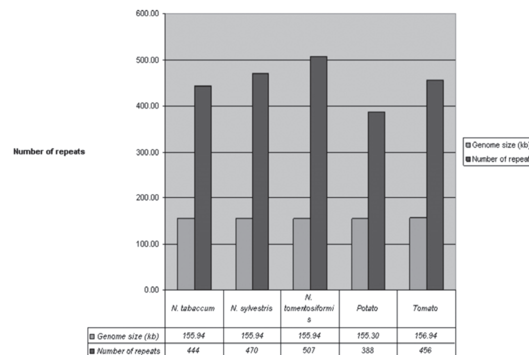
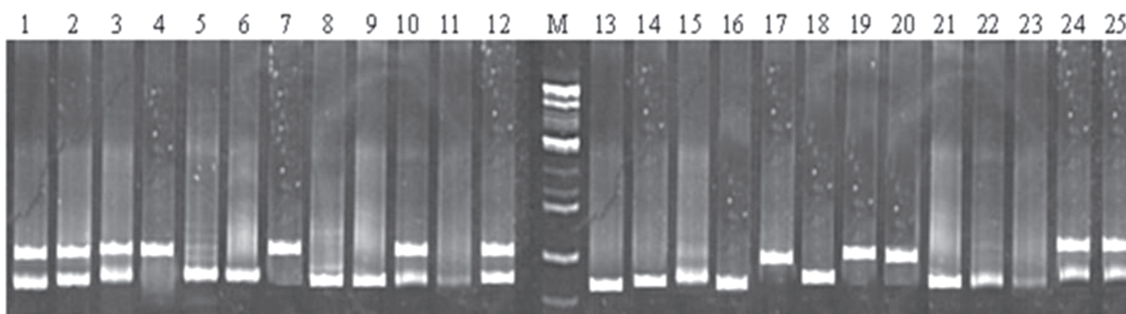


Supplementary Fig. 2. Proportion of different classes of repeats in chloroplast genomes



Supplementary Fig. 4. Genome size and abundance of repeats



Supplementary Fig. 5. Amplification profile of tobacco cpSSR-6 with 25 wild Species of Nicotiana. Details of the names are indicated in supplementary table 1.

Rustica and *Petunioides* and *N. tabaccum* belonging to subgenus *Tabaccum* were clustered together in Cluster 1A. All these species have identical chromosome number (2n=24), except *N. tabaccum* (2n=48). The grouping of *N. tabaccum* with *N. sylvestris* clearly indicated that the latter could be the maternal progenitor. Sub-clusters 1B and 1C has six and four species, respectively, belonging to the subgenus *Petunioides*. The clustering pattern indicated that these species were grouped according to their ploidy status. Cluster 2 had five species, of which, *N. rustica* belongs to Subgenus *Rustica*, *N. nudicaulis, N. debnyi* and *N. gossei* belongs to *Petunioides* while *N. tomentosiformis* belongs to *Tabaccum*. Among these, except *N. gossei* and *N. tomentosiformis*, other three species are having genomic constitution 2n=48. Cluster 3 possessed two species viz., *N. maritama* and *N. velutina* of the section *Suaveolentes* in subgenus *Petunioides*, which shares identical chromosome number (2n=32). The species *N. plumbagnifolia* formed the out-group with all the clusters, having 2n=20. *N. tabaccum*, being a tetraploid, grouped with the diploid species *N. sylvestris* rather than *N. tomentosiformis*, clearly approving the genetic relationships based on *in silico* SSR analysis discussed above. The grouping pattern is in accordance with the traditional classification (33), which was done mainly based on chromosome number.

Conclusions

With the availability of complete sequence of organellar genomes, the present study has helped in a better understanding of the

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Table 1 List of *Nicotiana* species used in the present study

Sub-genus	Section	Species	Somatic Chromosome no.
Rustica	*Petunioides*	*Glauca*	24
	Thrisiflora	*Paniculata*	24
	Rusticae	*Thyrisiflora*	24
	Undulatae	*rustica*	*48*
		undulata	24
Paniculatae	Trigonophyllae	*trigonophylla*	24
	Alatae	*plumbagenifolia*	*20*
	Repandae	*sylvestris*	24
	Acuminatae	*repanda*	*48*
	Bigelovinae	*nesophila*	*48*
	Nudicaules	*corymbosa*	*24*
	Suaveolentes	*acuminata*	*24*
		clevilandii	*48*
		nudicaulis	*48*
		suaveolenes	*32*
		debneyi	*48*
		gossei	*36*
		maritima	*32*
		velutina	*32*
		occidentales	*42*
		simulans	*40*
		goodspeedii	*40*
		rosulata	*40*
Tabacum	Genuinae	*tabacum*	*48*
		tomentosiformis	24

Table 2. Distribution of SSRs in organellar genomes

Repeat type	***Mitochondria***		***Chloroplast***									
	N. N. tabaccum		**N. tabaccum**		**N. tomentol sylvestris**		**siformis**		***Tomato***		***Potato***	
	G	I	G	I	G	I	G	I	G	I	G	I
Di	790	218	278	81	220	138	232	170	259	100	263	27
Tri	185	50	57	18	52	48	51	38	59	30	53	36
Tetra	47	6	-	9	1	10	-	13	-	7	-	8
Penta	-	4	-	1	-	1	-	3	-	1	-	1
Hexa	-	-	-	-	-	-	-	-	-	-	-	-
Total	1022	278	335	109	273	197	283	224	318	138	316	72
Frequency per kb	2.8	1.4	2.6	3.7	2.2	6.2	2.3	6.9	2.6	3.9	2.6	2.1
Density (bp/kb)	25.6	13.0	17.8	27.5	14.7	44.7	15.3	50.5			17.0	29.6

G – Genic; I - Intergenic

Table 3: Unique repeat motifs present in chloroplast genomes of Solanaceae species

Species	Repeat motif	Start position
N. sylvestris	(aag)4	3013
N. tomentosiformis	(aata)3	6721
N. tomentosiformis	(at)6	10455
N. tomentosiformis	(att)4	53140
N. tomentosiformis	(attt)3	33941
N. sylvestris	(ta)6	47423
N. tomentosiformis	(tat)4	53139
S. tuberosum	(tta)4	52747
N. tomentosiformis	(ttaat)3	14842
N. tomentosiformis	(ttc)4	36720
N. tomentosiformis	(tttaa)3	14841
N. tabaccum	(tttc)3	4106

Table 4 List of designed and validated tobacco chloroplast primers

Primer Name	Forward sequence	Reverse sequence	No.of alleles	PIC values
NtcpSSR-1	gtagaaagacgaaagtggattcg	Aataccctaccctgttcatctgg	1	0.3648
NtcpSSR-2	tagctaccgagatcaatgcagtc	Cattggatctcctgtctcatctc	1	0.2688
NtcpSSR-3	ctttccgtaccttcgcttaattc	Cccccatttttgtatcatagacc	1	0.4032
NtcpSSR-4	agaccttctcggtaaaacaggtc	Gctcccaaataatgaatcagagc	1	0.32
NtcpSSR-5	tgcaagaaaataacctctccttc	Tggcctagtctataggaggttttg	2	0.80
NtcpSSR-6	aattaagaacaaaagctcgttgc	Taaagtttggaagaccacgactg	2	0.7552
NtcpSSR-7	tatatatgttctgggacggaagg	Catcgcaaaatcctagtaccaatc	2	0.8832
NtcpSSR-8	ccatatcaaatgcagcctctatc	Ctgagttcttagccaaaattgacg	1	0.4608
NtcpSSR-9	ttcgtcgtcgagaattgaatag	Actggaagtggaagagctatttg	1	0.4608
NtcpSSR-10	ctttgccaaggagaagatgc	Atcactacactatcacggccaac	1	0.4032
NtcpSSR-11	aaaagaagaggtggtccgaatag	Agtcgtcaacatgaaagcgtaag	1	0.48
NtcpSSR-12	cctattttgggtggatttaaactg	Tcaaggtcaatctattcactcgtc	1	0.2688

nature, abundance and distribution of SSRs in these genomes in five different species of Solanaceae family. The study also helped in the identification of organellar SSRs that can be converted in to PCR-based markers. Phylogenetic relationship of few Solanaceous crop species established in this study based on *in silico* analysis of organellar SSRs as well whole genome alignments corroborated with those determined earlier by nuclear genome co linearity and chloroplast gene based phylogenetic analysis. More importantly, *N. sylvestris* was confirmed to be the maternal progenitor of the cultivated tobacco both by *in silico* SSR analysis as well as cpSSR markers analysis. The phylogenetic relationships among the genus *Nicotiana* deduced from this study using cpSSR markers was similar to that reported earlier.

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Neonatal Hypothyroidism alters Expression of Antioxidant Enzymes and Redox status in adult Rat Seminiferous Tubule Cells

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Abstract

The present study reports the effect of 6-n-propyl-2-thiouracil (PTU) induced hypothyroidism from day one of birth on the expression of antioxidant enzymes in seminiferous tubule cells of 90 days old rat. The findings indicate that, persistent neonatal hypothyroidism has induced permanent changes in the expression of genes in seminiferous tubule cells that are involved in the metabolism of reactive oxygen species, such as superoxide dismutase, catalase and glutathione peroxidase. Such alterations in their mRNA, protein and activity levels are associated with increased oxidative stress as evident by elevated levels of oxidative stress index. Withdrawal of PTU treatment on 30 days of age (transient hypothyroidism) restored the expression of catalase in seminiferous tubule cells of adult rats as that of control rats. Results suggest that PTU-induced neonatal hypothyroidism may affect testicular physiology in adulthood by causing a permanent impairment of antioxidant defences in rat seminiferous tubule cells.

Keywords : Hypothyroidism, rat testis, seminiferous tubule cell, antioxidant enzyme expression, glutathione redox status

Introduction

Testis performs two important physiological functions, such as spermatogenesis and steroidogenesis. In the first process, sperms are produced from germ cells present in seminiferous tubules for reproductive purpose. The second important function of testis is to synthesize androgens from Leydig cells that are necessary for general maintenance of organ systems and reproduction in male. Several studies have established the crucial role of reactive oxygen species (ROS) in spermatogenesis (1) as well as in steroidogenesis (2). Reactive oxygen species are generally produced as by-products of cellular metabolic activities due to incomplete reduction of oxygen molecules and are considered harmful to cells due to their higher oxidizing potential (3). Therefore, cells are equipped with both enzymatic as well as non-enzymatic antioxidant defences (AODs) to neutralize ROS and thus protect themselves from the oxidative stress. Enzymatic antioxidant defence is comprised of three principal enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (GPx) while non-enzymatic antioxidants constitute of small molecules such as reduced GSH, ascorbic acid, etc. (3). In testis, seminiferous tubule compartment consists of somatic (such as Sertoli cells and peritubular cells) as well as different types of germ cells (spermatogonia, spermatocytes and spermatids) and germ cells constitute the major population (4). Further, the levels of antioxidant enzymes in different types of germ cells are more or less similar in magnitude (5).

Thyroid hormones (THs) have been shown to have considerable modulatory effects on

hypothalamo-hypophyseal testicular axis and thereby influence testicular spermatogenesis and steroidogenesis (6). Sexual dysfunction and/or testicular degeneration noticed in adult mammals are commonly endorsed to impairment of thyroid functions (7). Thyroid hormones have important contribution in testicular development and spermatogenesis (8, 9). Many studies in past have demonstrated that hypo- or hyperthyroidism- induced impairment of testicular physiology in adult rats is attributed to elevated oxidative stress as a consequence of disruption of testicular antioxidant defences (10-12).

Our laboratory data demonstrated that, deficiency of thyroid hormone(s) during early part of the life of rats led to endocrine alterations that not only affected post-natal growth of testis, but also influenced its antioxidant defences (13), indicating that first month of post-natal life of rats deserves special attention. Important changes such as gradual and stepwise differentiation of germ cells to spermatocytes, spermatids and spermatozoa (14) and formation of mature hypothalamus-pituitary-thyroid axis (15) take place during second and third week of post-natal development of rats and completed approximately by one month of the age. It is reported that thyroid hormones play an important role in the development, differentiation and growth of organs in vertebrates (16) including testes (17). Therefore, exposure to anti-thyroid drugs such as 6-n-propyl-2-thiouracil (PTU) during early part of the post-natal life can act as an alluring model to identify thyroid hormone dependent testicular genes whose disturbances at early phase of life will have physiological impact in adulthood. PTU-induced neonatal hypothyroidism in rats is known to influence activities of testicular antioxidant enzymes in adulthood (13). This indicates that, antioxidant genes are such candidate genes, whose expressions are also regulated under altered thyroid state. Since the above study was performed in total testicular tissue homogenate, it was difficult to infer the specific impact of thyroid hormones on the expression of antioxidant enzymes in seminiferous tubule cells (STCs). Therefore, in the present study we reported the impact of persistent neonatal hypothyroidism induced by PTU on the expression of genes of antioxidant defence enzymes in seminiferous tubule cells of adult rats and their consequences in terms of oxidative status. Besides, we also investigated whether such changes are of permanent in nature or not in adulthood by withdrawing PTU treatment to rats after 30 days of birth.

Materials and Methods

Animals and treatment: Animal care, maintenance, breeding and experiments were performed under the supervision of the Institutional Animal Ethics Committee (IAEC) in accordance with the ethical standards provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India as reported earlier (13). Briefly male pups of Wistar rats obtained from breeding were made hypothyroid from day 1 of neonatal age until day 30 or day 90 of postnatal age. Hypothyroidism was induced in neonates by feeding the lactating mothers with 0.05% (w/v) PTU (Sigma, USA) through the drinking water (13). From the day of parturition until weaning (day 25 postpartum), the pups received PTU through mother's milk or drinking water and then directly through drinking water for the remaining period of experimentation. Animals were divided into three groups each containing eight animals.

Group I: Rats received drinking water for 90 days from birth served as control (Euthyroid).

Group II: Rats received PTU for 90 days from birth (Persistent hypothyroid).

Group III: Rats received PTU for 30 days from birth and thereafter received only drinking water up to 90 days of age (Transient hypothyroid).

Animal sacrifice, serum collection, body and testes weight measurement and thyroid hormone estimation: Animal sacrifice, tissue processing and other related procedures were carried out as reported earlier (13). Testes from

five animals per group were processed for seminiferous tubule cell isolation and testes form rest three animals were processed for histology. The serum levels of T_3, T_4 and TSH were measured by commercially available ELISA kits (Monobind, Inc. Costa Mesa, CA, USA).

Isolation of seminiferous tubule cells (STCs): STCs were isolated by a two-steps enzymatic method (18) with a little modification. Briefly, testes were decapsulated, chopped and minced into finer pieces to disperse the seminiferous tubules. Resulting tubules were incubated in 50 mM PBS, pH 7.4, containing 0.05% (w/v) collagenase type-IV (GIBCO, Invitrogen corp., USA) at 32.5 ºC in a shaking water bath at 100 cycles per minute for 15 minutes. The dispersed seminiferous tubules were allowed to sediment and the supernatants were removed by aspiration. Then these suspensions were washed in PBS. This process was repeated thrice to ensure removal of the dissociated interstitial cells and remaining blood cells. Resulting suspensions were incubated in 50 mM PBS, pH 7.4, containing 0.05% trypsin at 32.5 ºC in a shaking water bath at 100 cycles per minute for 15 minutes. The ensued suspensions were centrifuged at 200 × g for 5 minutes and washed in PBS for three times and finally suspended in 50 mM PBS containing 10% (v/v) fetal bovine serum for 10 minutes. Crude cell suspensions obtained by this were filtered twice through 100 μm nylon meshes to remove cell aggregates and debris, and subsequently centrifuged at 200 × g for 5 minutes. The resulting pellets were resuspended in 4 ml of 50 mM PBS containing 10% (v/v) fetal bovine serum. 10 μl of this suspension was taken and to this 10 μl of 0.8% trypan blue solution was added and kept for 2 minutes. 10 μl of above suspension was taken in a hemocytometer and viable and total seminiferous tubule cells were counted in WBC counting chamber. After counting, the cell suspensions were centrifuged at 200 × g for 5 minutes and washed in 50 mM potassium phosphate buffer for three times, divided in to aliquots and kept at -80 ºC until further use. For the isolation of RNA, aliquots containing approx. 10-15 million cells were suspended in 1 ml of TRIZOL reagent. All procedures were carried out at 4 ºC in a sterile environment.

Sample preparation for biochemical assays: The seminiferous tubule cell suspensions (in 50 mM potassium phosphate buffer pH 7.4) inside the microfuge tubes were sonicated by placing them in ice bath and by giving three bursts of 15 second with each burst being followed by an interval of 30 seconds in a B. Braun Biotech International Sonicator. One tube containing approx. 1ml cell suspension centrifuged at 1000 × g for 10 minutes and the supernatant was used for the determination of the level of lipid peroxidation (LPx), lipid hydroperoxide (LOOH) and hydrogen peroxide (H_2O_2). The other tubes were centrifuged at 10,000 × g for 15 minutes and the supernatants (each about 1ml) were used for the determination of glutathione redox status and assay of CAT and glucose-6-phosphate dehydrogenase (G6PD) activities. About 200 μl of supernatant containing approx. 800-1000 μg crude protein was passed through 1 ml sephadex G-25 column to get rid of low molecular weight compounds, which was used for the assay of Cu/ Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), selenium dependent glutathione peroxidase (GPx1) and GR. Protein contents of the samples were determined by Bradford method (19) taking bovine serum albumin (BSA) as standard. All procedures were carried out at 4 ºC.

Determination of glutathione redox status and oxidative stress indices: Glutathione redox status was measured by following the method as described earlier (20). Oxidative stress index (OSI) was calculated as the ratio between GSSG and total GSH (OSI = 100× (2 × GSSG)/total GSH) (21). Endogenous lipid peroxidation levels in samples were estimated by monitoring the formation of thiobarbituric acid-reactive substances (TBARS) (22) in the presence of 0.02% (w/v) butylated hydroxytoluene to suppress endogenous peroxidation during heating. Results were expressed as nanomoles TBARS formed/

mg protein. FOX 2 method was used to measure the levels of lipid hydroperoxide (LOOH) in samples (23, 24). The LOOH content was expressed as nanomoles lipid hydroperoxide per mg protein. LPx and LOOH measurement were carried out in visible spectrophotometer (Model-106, Systronics, India). Hydrogen peroxide levels of different samples were measured according to Ruch et al. (25) and Pazdzioch-Czochra and Widenska (26) using homovanillic acid (HVA, Sigma, USA) in Hitachi 2500 spectrofluorimeter. The H_2O_2 content was expressed as nmol/mg protein.

Protein carbonyl content was determined using OxyBlot™ Protein Oxidation Detection Kit (Catalog# S7150, Millipore Corp., USA). Briefly, protein samples (5 µg/lane) were derivitized with dinitrophenyl hydrazine, fractionated by 10% SDS-PAGE, and electro blotted to PVDF membrane (0.45 µm, PALL Life Sciences). Membranes were blocked by blocking solution (1% BSA/PBST: phosphate buffered saline, pH 7.4, containing 0.05% tween-20). The dervatized proteins on the PVDF membrane were sequentially reacted with rabbit anti-dinitrophenyl (1:150 diluted with blocking solution) and horseradish peroxidase conjugated goat anti-rabbit IgG (1:300 diluted with blocking solution) antibodies and visualized by chemiluminescent ECL kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in Kodak X-ray film. Total carbonyl content was determined by densitometry using imageJ software developed by National Institute of Health, USA.

Determination of antioxidant enzyme activities: Activities of antioxidant enzymes were measured as described earlier (13, 20).

RNA isolation and semi-quantitative RT-PCR: RNA isolation and RT-PCR were done as mentioned earlier (20) with a little modification. Primer sequences (Table 1) of antioxidant genes (AOGs) were custom made by Integrated DNA Technology, USA. PCR cycle conditions were optimized, as 30 cycles for G3PDH and CAT, 32 cycles for SOD1 and GPx1, 34 cycles for SOD2 and 37 cycles for GR and electrophoresis was performed in 1.2% agarose gel.

Preparation of STCs homogenate and Western blotting: Approximately 30-40 million of STCs were homogenized in 50 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 104 mM PMSF, 100 µM E-64, 80 µM aprotinin, 100 µM leupeptin, 1% (v/v) Triton X-100 and 0.1% SDS to avoid protein degradation. Homogenates were centrifuged at 1000 × g for 20 min at 4 ºC. The resultant supernatant protein was estimated by Bradford method (19) using BSA as standard. About 80-150 µg of total cellular protein was resolved for detection of bands. All procedures of western blotting were performed with a little modification (27). We have used TBST (10 mM Tris-Cl pH 8.0, 150 mM NaCl and 0.1% Tween 20) instead of PBST as previously used. Rabbit polyclonal anti bodies used in different dilutions, as anti-G3PDH (1:1000), anti-SOD1 (1:5000), anti-SOD2 and anti-CAT (1:2000) were obtained from Imgenex India Pvt. Ltd., Odisha, India, except Anti-SOD2 antibody, which was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Horse radish peroxidase conjugated goat anti rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) used as 1:5000 dilution (1:10000 for SOD1). Their expression levels were measured by densitometry using imageJ software, NIH, USA.

Histology and Immunohistochemistry: All procedures were carried out as reported earlier (27) with a little modification. Before proceeding for endogenous peroxidase quenching, we have added on more step of antigen retrieval. In this step sections were submerged in 10 mM citrate buffer, pH 6.0 and heated in a microwave oven for 10 min to increase antigen accessibility. Rabbit polyclonal anti bodies, such as anti-SOD1, anti-SOD2 and anti-CAT were used as 1:100 dilutions, whereas biotinylated goat anti-rabbit IgG and streptavidin horse radish peroxidase conjugate were used as 1:200 dilutions. The seminiferous epithelium was staged according to the method of Leblond and Clermont (28). Only mature stages

(stage VII-VIII) were taken in to account as these are most affected by endocrine disruption and any hormonal changes (29). Photographs were captured in Olympus BX 53 microscope attached with MicroPublisher 5.0 CCD cooled camera, by Q-capture pro7 software.

Statistics: Quantitation of RT-PCR and Western blot bands were done using computer assisted densitometry ImageJ, Image Analysis Software, NIH, USA. Relative Integrated densities were determined as the ratio of AOGs band/G3PDH band. These density values and other data were subjected to one way analysis of variance (ANOVA) followed by Duncan's new multiple range test by using Statistica 6.0 software to find out the level of significance among mean values. A difference was considered significant minimum at $p < 0.05$ level.

Results

Body weight, testes weight and serum thyroid hormone concentration: The weight gain on day 91 postpartum was significantly ($p < 0.001$) reduced by 43% and 13% in animals of group-II and group-III in comparison to group-I, respectively. A significant reduction in the weight of testis (56%, $p < 0.001$) and gonadosomatic index (GSI) (23%, $p < 0.001$) was recorded in group-II animals in comparison to group-I. Testis weight and GSI of group-III animals were increased by 15% and 32% than group-I, respectively (Table 2). Serum T_3 and T_4 levels were significantly ($p < 0.001$) reduced in group-II by 48% and 78% in comparison to group-I, respectively. On the other hand, serum TSH level was several fold higher in group-II rats as compared to group-I. Serum T_4 level in group-III was significantly ($p < 0.05$) higher (8%) than group-I (Table 2).

Seminiferous tubule cells (STCs) count and viability: A mixed population of different types of seminiferous tubule cells mostly rich in germ cells (Spermatogonia, primary and secondary spermatocytes and spermatids) and some somatic cells (peritubular and sertoli cells) were isolated (Fig. 1a) and their viability was tested within 4 hours of isolation. The STCs viability was found to be 94-96% in all the groups. The STC number/ml was significantly higher (36%, $p < 0.001$) in group-III than group-I. However, the yield of STC number/ml in group-II was significantly low (33%, $p < 0.001$) in comparison to group-I (Fig. 1b).

Oxidative stress indices: A significant decrease ($p < 0.001$) in the levels of lipid peroxide (37%), lipid hydroperoxide (22%) and hydrogen peroxide (31%) in STC of group II were observed in

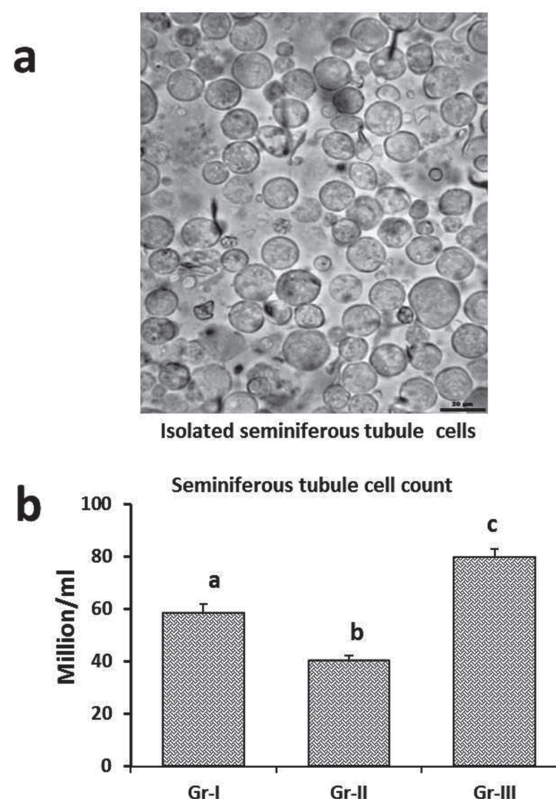

Fig. 1 (a) Isolated seminiferous tubule cells (STCs) from rat testis (20 µm scale bar and magnification ×400). **(b)** Cell number in group-I (euthyroid), group-II (persistent hypothyroid) and group-III (transient hypothyroid) rats. Data are expressed as mean ± S.D. of five animals. Data having different superscripts differ significantly ($p < 0.001$) from each other.

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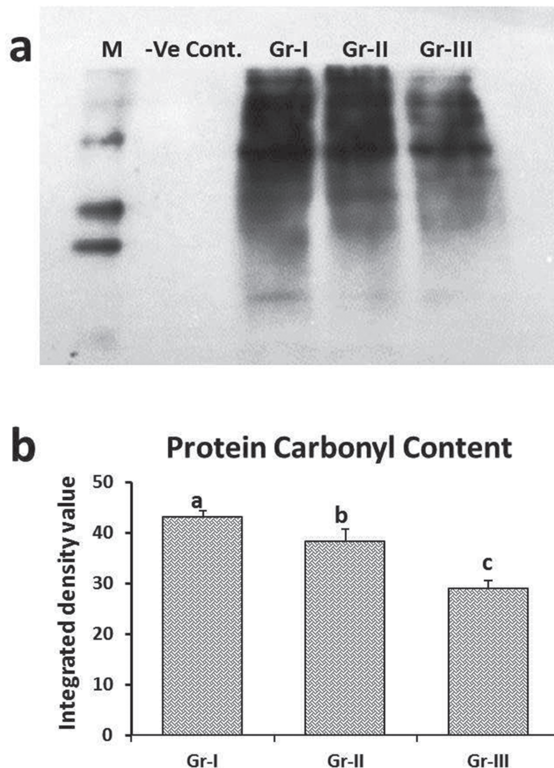

Fig. 2 Protein oxidation pattern of STCs in group-I (euthyroid), group-II (persistent hypothyroid) and group-III (transient hypothyroid) rats. (**a**) Western blot representation of protein carbonyl content, (**b**) integrated density values and **(M)** molecular wt. markers**.** Data are expressed as mean ± S.D of (n = 4) rats. Data having different superscripts differ significantly ($p < 0.05$) from each other.

comparison to group-I rats, respectively. Levels of hydrogen peroxide and lipid hydroperoxide in group-III were significantly lower ($p < 0.001$) by 36% and 24% in comparison to group-I, respectively (Table 3).

Densitometric quantification of derivatized protein immunoblots revealed a significant reduction in the total carbonyl contents of group-II (12%, $p < 0.05$) and group-III (33%, $p < 0.001$) than group-I (Fig. 2a and b).

Total, oxidized and reduced glutathione contents: Total GSH equivalent (T-GSH-eq) level in STCs was significantly higher in group-II (16%, $p < 0.05$) and group-III (35%, $p < 0.001$) rats in comparison to group-I. The level of GSSG in STCs of group-II (114%, $p < 0.001$) and group-III (204%, $p < 0.001$) was higher than group-I. The oxidative stress index (OSI) was significantly higher in group-II (81%, $p < 0.001$) and group-III (118%, $p < 0.001$) rats as compared to group-I (Table 3).

Expression of SOD1: The transcript level of SOD1 was increased significantly in group-II (31%, $p < 0.01$) and group-III (41%, $p < 0.01$) in comparison to group-I rats (Fig. 3 and 4a). SOD1 translated products increased significantly in group-II (38%, $p < 0.01$) and group-III (69%, $p < 0.01$) in comparison to group-I rats (Fig. 3 and 4b). SOD1 activity of group-II and group-III was significantly higher ($p < 0.001$) by 221% and 120% than group-I rats, respectively (Fig. 4c). An increased intensity of immunoreactions of SOD1 was recorded in all types of seminiferous tubule germ cells (spermatogonia, spermatocytes and spermatids) and somatic cells in the matured stages (stage VII-VIII) of group-II (Fig. 5e) and group-III (Fig. 5f) in comparison to group-I (Fig. 5d) rats.

Expression of SOD2: The transcript level of SOD2 remained same in all the three groups (Fig. 3 and 4d). The level of SOD2 translated products of group-II and group-III was significantly decreased ($p < 0.01$) by 66% and 71% than group-I rats, respectively (Fig. 3 and 4e). A significant reduction ($p < 0.001$) in SOD2 activity was noticed in group-II and group-III rats by 40% and 25% than group-I, respectively (Fig. 4f). Immunoreactions of SOD2 were not properly visualized in testicular sections of three groups, hence picture was not given.

Expression of CAT: Catalase transcripts level of group-II was significantly higher (35%, $p < 0.01$) than group-I and group-III (Fig. 3 and 4g). Level of CAT translated products of group-II was significantly lower (30%, $p < 0.001$) than group-I

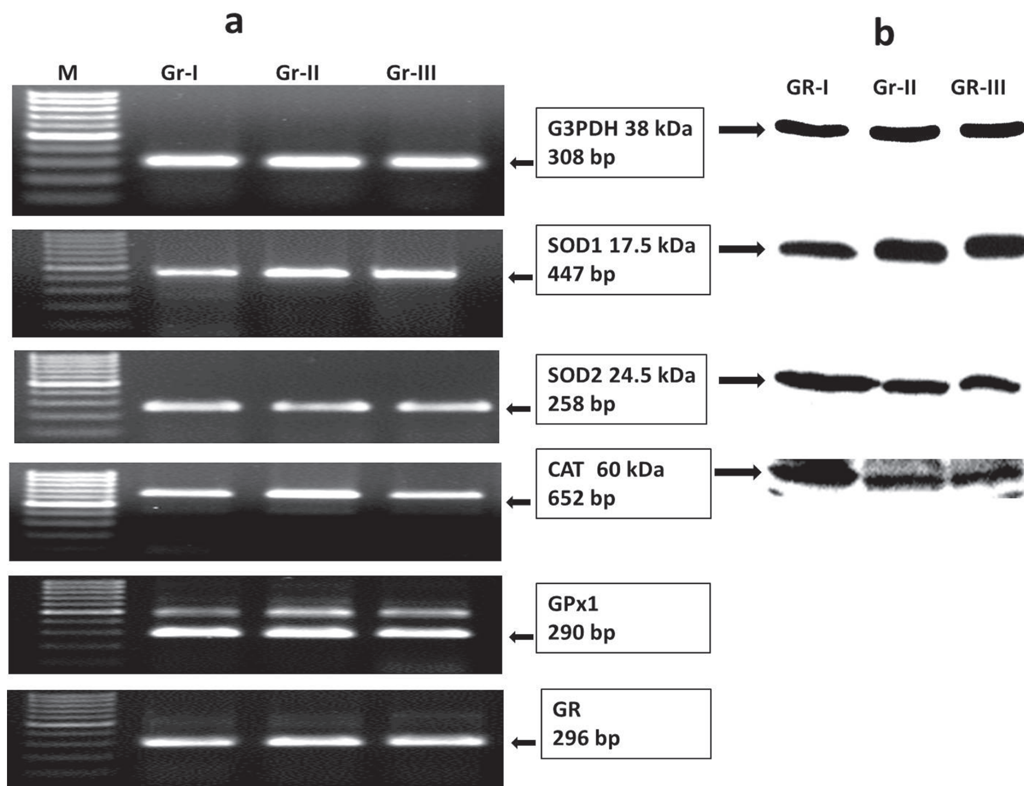

Fig. 3 Transcripts **(a)** and translated products **(b)** of antioxidant enzymes of STCs in group-I (euthyroid), group-II (persistent hypothyroid) and group-III (transient hypothyroid) rats. Representative pictures of RT-PCR in agarose gels **(a)** and western blotting (**b**) of antioxidant enzymes. (**M**) 100 bp DNA ladder.

and group-III rats (Fig. 3 and 4h). Catalase activity in group-II was significantly higher (40%, $p < 0.001$) than group-I and group-III rats (Fig. 4i). The intensity of immunoreactions of CAT in all types of testicular germ cells (spermatogonia, spermatocytes and spermatids) and somatic cells in the matured stages (stage VII-VIII) of group-II (Fig. 5h) and group-III (Fig. 5i) was low in comparison to group-I (Fig. 5g).

Expressions of GPx, GR and G6PD: There was no significant difference in transcript level of GPx1 among the three groups (Fig. 3 and 6a). A significant increase ($p < 0.001$) in the activity of total GPx was recorded in group-II and III by 58% and 47% than group-I, respectively (Fig. 6b). Transcripts level for GR was more or less same in all the three groups (Fig. 3 and 6c). However, its activity was significantly elevated ($p < 0.001$) in group-II and group-III rats by 59% and 80% than group-I, respectively (Fig. 6d). Glucose-6-phosphate dehydrogenase (G6PD) activity was significantly increased ($p < 0.001$) in group-II and group-III by 142% and 138% in comparison to group-I, respectively (Fig. 6e).

Discussion

Results of the present study on the induction of hypothyroidism by PTU, decrease in the number of seminiferous tubule cells (STCs) following persistent hypothyroidism and increase in the number of STCs due to transient

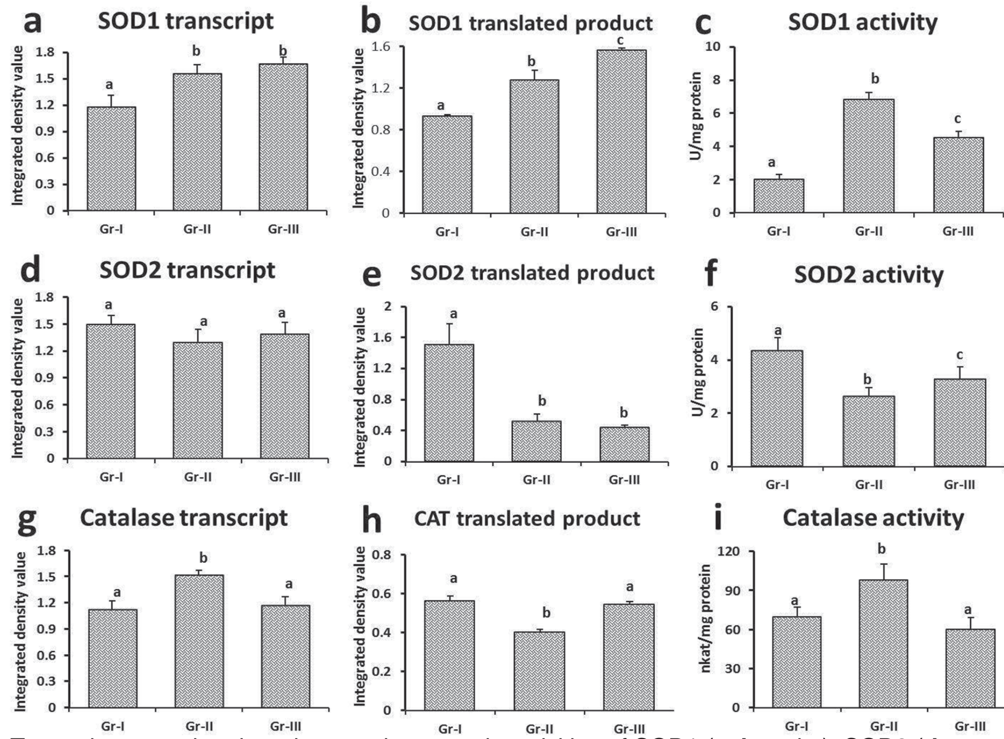

Fig. 4. Transcripts, translated products and enzymatic activities of SOD1 (**a**, **b** and **c**), SOD2 (**d**, **e** and **f**) and CAT (**g**, **h** and **i**) respectively in group-I (euthyroid), group-II (persistent hypothyroid) and group-III (transient hypothyroid). Data are expressed as mean ± S.D. of four animals for transcript and translate analysis, whereas five animals for enzyme activities. Data having different superscripts differ significantly ($p < 0.01$) from each other.

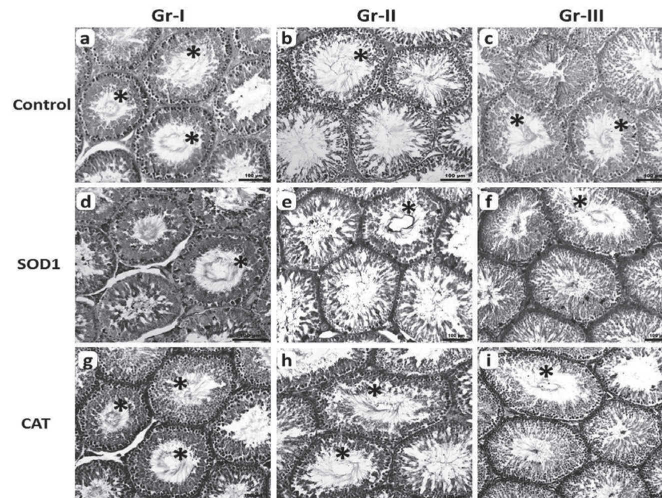

Fig. 5. Representative photomicrographs of transverse sections of testis showing SOD1 (**d**, **e**, and **f**) and CAT (**g**, **h** and **i**) immunoreactions in group-I (euthyroid), group-II (persistent hypothyroid) and group-III (transient hypothyroid). (**a**), (**b**) and (**c**) are negative controls for the respective groups. Sections marked by asterisk are identified as Stage VII-VIII. All figures are given with 100 µm scale bar and magnification is ×100.

Table 1. Accession number, nucleotide sequence, binding position, annealing temperature and products of RT-PCR primers (S = sense and AS = antisense).

Gene	Accession no.	Primer sequence (5'–3')	Binding position	Annealing temperature	PCR Product
G3PDH	DQ403053	S-TCC CTC AAG ATT GTC AGC AA AS-AGA TCC ACA ACG GAT ACA TT	352–371 659–640	48.0 ^{o}C	308 bp
SOD1	NM_017150	S-GCA GAA GGC AAG CGG TGA AC AS-TAG CAG GAC AGC AGA TGA GT	159–178 605–586	52.0 ^{o}C	447 bp
SOD2	BC070913	S-CTG AGG AGA GCA GCG GTC GT AS-CTT GGC CAG CGC CTC GTG GT	51–70 308–289	58.0 ^{o}C	258 bp
CAT	BC081853	S-GCG AAT GGA GAG GCA GTG TAC AS-GAG TGA CGT TGT CTT CAT TAG CAC TG	702–722 1353–1328	53.0 ^{o}C	652 bp
GPx1	S41066	S-CTC TCC GCG GTG GCA CAG T AS-CCA CCA CCG GGT CGG ACA TAC	367–385 656–636	56.8 ^{o}C	290 bp
GR	NM_053906	S-TCA CTG CTC CGC ACA TCC AS-CTC AAC ACC GCC AGC GTT CTC C	419–436 714–693	54.1 ^{o}C	296 bp

hypothyroidism are in confirmation with our previous report (13). Use of double enzymatic method has improved cell yield. Observed increase yield of seminiferous tubular cells in transient hypothyroid rats may be due to elevated GSH content. GSH is known to augment proliferation and differentiation of spermatogenic cells in rats (30).

It is apparent from the present study that reduction in thyroid hormone level during early part of the life has adverse impact on the expression of antioxidant enzymes of STCs. Two important observations were noted. First, the changes in the expression of some antioxidant enzymes due to neonatal hypothyroidism could not be reversed in adulthood on withdrawal of PTU treatment at the age of 30 day. Secondly, the adaptive responses of antioxidant enzymes to neonatal hypothyroidism in STCs were reflected at multiple steps, which includes transcriptional, translational and activity levels and it was enzyme specific.

Decline in general oxidative stress indices (LPx, LOOH, H_2O_2 and protein oxidation) in response to persistent and transient hypothyroidism can be linked with decrease in oxygen consumption and hypo metabolic state of seminiferous tubule cells. It has been reported earlier that oxygen consumption was decreased following hypothyroidism in testicular germ cells (31) and also in testicular slices of adult rats (12). Oxygen is reported to play a central role in the generation of reactive oxygen species in cells (32) and thyroid hormones are known to modulate oxygen metabolism of cells (33). However, oxidative stress index was elevated in both the groups of hypothyroid rats. It has been suggested that high intracellular GSSG may trigger cell death (34). GSSG is generally formed by the enzyme GPx which catalyses oxidation of cellular GSH by oxidants such as hydrogen peroxide or organic peroxides. Decrease in STCs following hypothyroidism might be mediated through physiological mechanisms of cell death as a consequence of high GSSG content. GSSG is capable of causing protein S-glutathionylation.

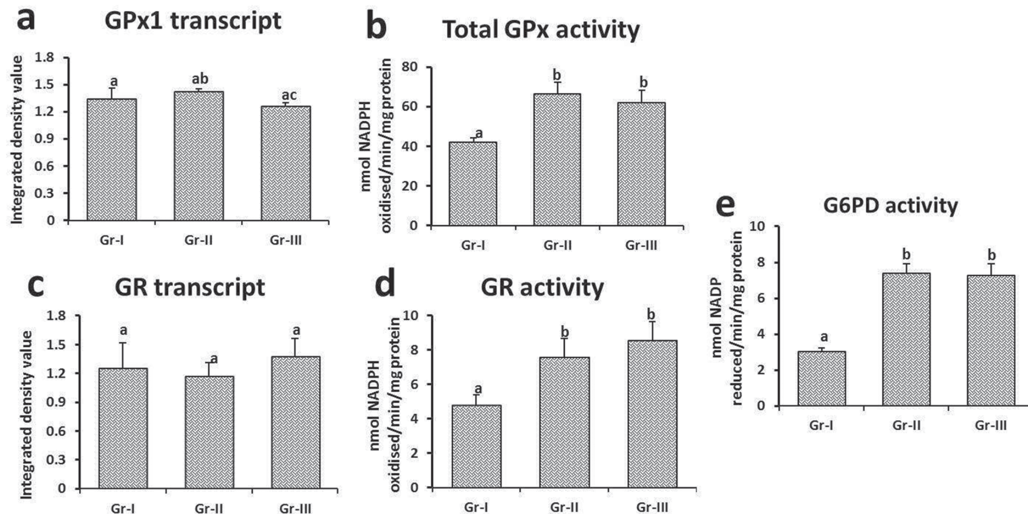

Fig. 6. Transcripts of GPx1 and GR (**a** and **c**) and enzymatic activities of GPx (**b**), GR (**d**) and G6PD (**e**) of group-I (euthyroid), group-II (persistent hypothyroid) and group-III (transient hypothyroid). Data are expressed as mean ± S.D. of four animals for transcript analysis and five animals for enzyme activities. Data having different superscripts differ significantly ($p < 0.05$) from each other.

Table-2. Effect of PTU-induced neonatal hypothyroidism on body and testes weight (g), gonadosomatic index (GSI; g/100 g body weight), serum total T_3, T_4 (ng/ml) and thyroid stimulating hormone level (TSH; µ IU/ml) of adult rat.

	Gr-I	Gr-II	Gr-III
Body and testes weight			
Initial (on date of birth)	6.48±0.20^a	6.48±0.18^a	6.49±0.17^a
Final (on date of sacrifice)	177.4±5.27^a	101.8±8.58^b	154.6±5.55^c
Decrease in body weight from control (%)	Nil^a	42.56±5.30^b	12.85±1.63^c
Testes weight	2.63±0.082^a	1.16±0.13^b	3.03±0.14^c
GSI	1.48±0.02^a	1.14±0.05^b	1.96±0.06^c
Serum analyses			
Total T_3	0.99±0.05^a	0.51±0.03^b	1.00±0.04^a
Total T_4	25.06±1.26^a	5.55±0.36^b	27.24±1.54^c
TSH	0.52±0.06^a	13.49±0.39^b	0.31±0.03^a

Data are expressed as mean ± S. D. of 8 observations. Data having different superscripts differ significantly ($p < 0.05$) from each other. Gr-I (90 day old control rats); Gr-II (90 day old rats with PTU treatment from day 1 postpartum to day 90 postpartum); Gr-III (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum).

Table 3 Effect of PTU-induced neonatal hypothyroidism on oxidative stress indices: lipid peroxidation (LPx; nmol TBARS/mg protein), lipid hydroperoxide (LOOH; nmol/mg protein), hydrogen peroxide (H_2O_2; nmol/mg protein) content and glutathione redox status: total (T-GSH-eq), reduced (GSH), oxidized (GSSG) glutathione content (micro molar) and oxidative stress index (OSI) of adult rat seminiferous tubule cells.

	Gr-I	Gr-II	Gr-III
Oxidative stress parameters			
LPx	0.54±0.08[a]	0.34±0.04[b]	0.49±0.07[a]
LOOH	18.58±1.17[a]	14.48±1.50[b]	14.08±1.74[b]
H_2O_2	7.94±0.72[a]	5.47±0.67[b]	5.09±1.02[b]
Glutathione redox status			
T-GSH-eq	5.58±0.68[a]	6.48±0.71[b]	7.55±0.61[c]
GSH	4.58±0.69[a]	4.38±0.74[a]	4.58±0.61[a]
GSSG	0.49±0.03[a]	1.05±0.10[b]	1.49±0.09[c]
OSI	18.09±2.72[a]	32.84±4.85[b]	39.55±3.70[c]

Data are expressed as mean ± S.D. of 5 observations. Data having different superscripts differ significantly ($p < 0.05$) from each other. Gr-I (90 day old control rats); Gr-II (90 day old rats with PTU treatment from day 1 postpartum to day 90 postpartum); Gr-III (90 day old rats with PTU treatment from day 1 postpartum to day 30 postpartum and left untreated up to day 90 postpartum).

Protein S-glutathionylation is involved in redox regulation of several enzymes as a consequence of post-translational modifications. For example, proteins, such as NfkB (35) and creatine kinase (36) are inhibited by protein S-glutathionylation, whereas, microsomal glutathione S-transferase (37) and metalloproteinases (38) are activated. Therefore the possibility of changes in levels of antioxidant enzymes in STCs due to PTU-induced neonatal hypothyroidism as a consequence of S-glutathionylation cannot be ruled out.

The differential effects of PTU-induced neonatal hypothyroidism on the expression of two types of SOD (SOD1 and SOD2) at different levels (activity, translational and transcriptional) were observed. The parallel up regulation of SOD1 in the above three levels implied a direct role of thyroid hormone in the regulation of SOD1 expression. Immunohistochemistry results for SOD1 also substantiated the findings. On the other hand, the transcripts of SOD2 remained unchanged but its translated products and activity decreased in hypothyroid rats than the control. It is reported that translocation of redox sensitive transcription factors into the nucleus is influenced by redox status of the cells (39). Therefore, the unchanged mRNA level of SOD2 in hypothyroid rats could be due to the oxidation of transcription factors. One of the prevailing modes of regulation of gene expression is the translational control, which explains inadequate correlation, often observed among transcripts, translates and activity level of proteins (40). It is now well established that translational ability of several proteins is modified by binding of RNA binding proteins (RBP) and small RNAs to the mRNA (41). SOD2 expression is regulated not only at transcriptional level but also at the translational level through RNA binding proteins in mammalian cells (42). The binding of proteins to RNA might have declined the SOD2 translated products and subsequently the activity. There was an elevation of CAT transcripts as well as activity in case of

persistent hypothyroid rat in comparison to euthyroid and transient hypothyroid, but in case of translated product the situation was just reversed. However, the inconsistency between the level of transcription and translation of CAT might be because of decreased mRNA stability or reduced translational efficiency (43) through binding of different transcription factors (44) and RNA binding proteins (42). Further, Van Remmen et al. (44) opined that CAT gene is under direct control of thyroid hormone as evident from the presence of thyroid response element (5'-AGGTCA-3') as an inverted palindrome sequence (5'-TGACTCTCAGAGGTCA-3') at -311 upstream of the ATG start codon within the CAT gene. Marked decrease in immunoreactions was observed for CAT in testicular sections of hypothyroid rats than euthyroid ones, further confirm its decrease at molecular level. Although transcript levels of GPx1 and GR did not change in response to hypothyroidism, activities of both the enzymes were elevated. It has been reported that T3 administration to euthyroid mice resulted in several fold increase in deiodinase-2 (D2) activity in brown adipose tissue despite a marked reduction in its transcripts (45). Recently, it has been observed that activities of catalase and GPx reduced in mice kidney following induction of diabetes despite augmentation of their respective transcripts (46). Observed elevation of GPx and GR in the present study could be a compensatory mechanism to maintain low levels of hydrogen peroxide and lipid peroxides at the cost of oxidation of GSH to GSSG. GR activity might have been elevated to maintain the GSH pool to sustain the elevated GPx level. Increased activity of G6PD in both persistent and transient hypothyroidism might be due to higher demand for NADPH which was used as reductant by GR to generate reduced GSH from GSSG.

Taken together, the findings of this study suggest that, PTU-induced neonatal transient or persistent hypothyroidism leads to permanent disruption of antioxidant defence system of seminiferous tubule cells in adulthood with altered redox status as evident from OSI. Compromised antioxidant defence status and elevated OSI in seminiferous tubule cells caused due to persistent hypothyroidism may be the major factors for decrease in number of seminiferous tubule cells, which may be linked to impaired fertility at adult stage.

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Purification, Characterization and Kinetic Properties of *Penicillium cyclopium* L-Asparaginase: Impact of L-asparaginase on Acrylamide Content in Potato Products and its Cytotoxic Activity

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Abstract

The present study was carried out for the purification and characterization of L-asparaginase produced by *Penicillium cyclopium*. Both food and medical applications were also carried in the present study. The molecular weight of enzyme was approximately 55 kDa as estimated by SDS-PAGE. The optimum temperature and pH of the enzyme were 37 ºC and 8, respectively with V_{max} of 3333U/mg and K_m of 0.3 mM. Studies for the substrate specificity indicated that, L-asparaginase has greater affinity towards L-asparagine. Metal ions such as Ca^{+2}, Fe^{+3}, Mn^{+2} and Hg^{+2} significantly affected the enzyme activity whereas presence of KCl and NaCl stimulated the enzyme activity. The impact of L-asparaginase on the acrylamide content reduction after high heat treatment in a model system as well as in potato based material was investigated. Addition of partial purified L-asparaginase enzyme followed by incubation of the mixture at 37 °C for 30 min led to 92 % reduction of acrylamide content. It showed potential candidate for food industry to reduce acrylamide content in starchy fried food commodities. The purified enzyme also inhibited the growth of three human cell lines including hepatocellular carcinoma (Hep-G2), breast carcinoma (MCF-7) and prostate carcinoma (PC3) with IC_{50} values of 14 µg/ml, 12.5 µg/ml and 37ìg/ml, respectively.

Keywords: Cytotoxicity, Acrylamide, L-asparaginase, *Penicillium cyclopium*, Purification.

Introduction

L-asparaginase is one of the known drugs for the treatment of cancer, especially acute lymphoblastic leukemia. In recent years, several bio-conjugation protocols have been developed to improve the pharmacokinetic and immunological properties of anti-leukemic enzyme, L-asparaginase. L-asparaginase (ASN) (EC.3.5.1.1; L-asparagine aminohydrolase) catalyzes the deamination of L-asparagine (Asn) to L-aspartate and ammonia. This enzyme has been isolated from a variety of sources; animals and plant cells, yeast, fungi, and bacteria. Unlike the normal cells, neoplastic cells cannot synthesize L-asparagine due the absence of L-asparagine synthetase. Therefore, they obtain the required asparagine from circulating pools. For this reason, intravenous injection of free enzyme results in selective neoplastic cell death, directly by the depletion of circulating asparagine levels. However, L-asparaginase from bacterial origin can cause hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis. The search for other L-asparaginase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects. It has been observed that eukaryote microorganisms like yeast and filamentous fungi have a potential for

L-asparaginase production (1). In this paper, an attempt has been made to purify this enzyme from *Penicillium cyclopium* which was further characterized along with its kinetics study.

Material and Methods

Microorganism, culture maintenance, inoculum preparation : The fungal strain of *Penicillium cyclopium* was kindly provided by the National Research Center, Cairo, Egypt. The production media and inoculum were prepared according to the method described by Sarquis *et al.,* 2004 (2)

Enzyme assay: L-asparaginase activity of culture filtrate was determined by quantifying the ammonia formation using Nessler's reagent (3). One unit (IU) of L-asparaginase activity is defined as the amount of enzyme which liberates 1µ mole of ammonia per minute (µmole/ml/min) under the standard assay conditions (3). The protein content was determined by the Lowry's method of protein estimation using bovine serum albumin as standard (4).

Purification of L-asparaginase : The purification was carried out using crude enzyme extract by the following steps at 4 ºC, according to method described by Fukumoto *et al.*,1963 (5).

Acetone Precipitation: The enzyme was precipitated in a sequential manner using acetone at concentration 60 % (v/v). The mixture was centrifuged at 6000 rpm for 30 min at 4 ºC and precipitate was and stored at 4 p C for further purification.

Sephadex G-100 gel filtration: The acetone fraction (40-60%) was applied to pre- equilibrated Sephadex G-100 column (2.5x30 cm) with a 0.05 M Tris buffer pH 8. The protein elution was done with the same buffer at a flow rate of 25 ml/h. The fractions (2 ml) were collected for protein as well as for enzyme activity. The active fractions were pooled, mixed, lyophilized, and used for investigating the properties and kinetics of the purified enzyme.

Molecular weight determination by SDS-PAGE: The SDS-PAGE was carried out to determine the molecular weight and purity of the isolated protein using Bio Rad Mini-protein II Dual -Slab apparatus according to Laemmle *et al.*, 1970 (6). 10-15 µl of protein sample containing about 10-100 µg protein were carefully loaded in the well on top of the gel. Electrophoresis was carried out at constant current of 25-30 mA per slab (10x8x0.2 cm) until the tracking dye migration and length of the gel were measured precisely.

Determination of Amino acids composition: The amino acid composition was determined using High Performance Amino acid analyzer LC3000 eppendrof Germany, at NRC.

Characterization of purified L-asparaginase

Determination of optimum pH & temperature: Optimum temperature and pH were determined by changing individually the conditions of the reaction mixture assay (temperature from 25 to 60 æ%C; pH from 3 to 7 using citrate phosphate buffer and pH from 8-9 using Tris-buffer) at different time intervals.

Thermal stability of L-asparaginase: Thermal stability for the purified enzyme was evaluated by measuring the residual activity of the enzyme samples incubated at various temperatures (from 30 to 80æ%C) in Tris–buffer (1 M, pH 8) for different time intervals (15, 30, 45 and 60 min).

Substrate concentration and determination of K_m and V_{max}: The kinetic constants K_m &V_{max} of the purified L-asparaginase were determined according to Lineweaver and Burk (7), using different concentrations (0.02-0.12mM) of L-asparagine as substrate. Reactions were performed in 0.2M Tris buffer (pH 8.0) at 37 ºC.

Effect of metal ions and EDTA on L-asparaginase activity: For determining the effect of metal ions on L-asparaginase activity, the purified enzyme was incubated with 10^{-3} M concentration of Na^+, K^+, Ba^{2+} , Ca^{2+} , Fe^{2+} , Hg^{2+}, Mn^{2+} and EDTA for 2 h at 30°C and the residual activity was then determined.

Application of L-aspararaginase

a- Application of L-asparaginase in potatoes: Potato chips were washed, peeled and cut into 2 mm thick chips, using a slicer. The potato chips were soaked for 10 min with 5 ml 0.5 % (w/v) glucose solution. Then, 5 ml of purified enzyme solution (12 ìg/ml^{-}) together with 15 % (w/v) of trichloroacetic acid were incubated in Tris–buffer (1 M, pH 8) at 37 ºC. The chips were then placed in the oil for 8 min at 190 °C**.** The quantification of acrylamide was performed by an Agilent 1100 model HPLC system (Waldbronn, Germany). The chromatographic separations were performed on Zorbax ODS column using the mobile phase (7% (v/v) methanol in 0.025 mol/L sodium dihydrogen phosphate) at a flow rate of 1 ml/min. The acrylamide was detected at 215 nm with continuous monitoring the peak spectra within the range of 190–350 nm (8). Control sample were also prepared, using untreated potato chips.

b-Application as antitumor activity: The cytotoxic effect of the enzyme samples were performed on breast cancer cell line (MCF7); hepatocellular carcinoma (Hep-G2) and prostate cancer cell lines (PC3) using Sulphorhodamine-B (SRB) assay according to method described by Vichai *et al*., 2006 (9). SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content. Cells ($3x10^3$ cells/well) were seeded in 150 ìl fresh medium and incubated for 24 h. Different concentrations of enzyme samples (0, 5, 12.5, 25, 50 µg/ml) were added to cells in triplicate and incubated for 48 h. The cells were fixed with 50 ìl cold trichloroacetic acid (10%) for 1 h at 4 ^{0}C followed by staining with 50 ìl 0.4 % SRB dissolved in 1 % acetic acid for 30 min at room temperature. The cells were washed with 1 % acetic acid and air-dried. The dye was solubilized with 100 ìl/well of 10M Tris base (pH 10.5). The absorbance was measured with an ELISA reader (Sunrse Tecan reader, Germany) at 570 nm. The means background absorbance was automatically subtracted and mean values of each drug concentration was calculated. The experiment was repeated three times. The data are expressed as the mean percentage of survival where,

Surviving fraction =O.D. (treated cells)/ O.D (control cell).

The IC_{50} values (*i.e.* the concentrations of resveratrol required to produce 50% inhibition of cell growth) were also calculated.

Results and Discussion

In an attempt to obtain pure enzyme, the most active partially purified enzyme fraction from acetone (40-60%) was applied on a pre-equilibrated Sephadex G-100 column (1.5 x 50 cm) with a 0.05 M Tris- buffer pH 8.0 at a flow rate of 5ml/5min (Table 1). The fractions were collected and examined for enzyme activity and protein content (Figure1). The specific activity of the enzyme increased with every step of purification until it reached 39480 IU/ mg after using sephadex G-100. The most active fractions (F9-F13) (total specific activity 39480 IU/ mg protein and purification fold of about 52.3) were pooled together, dialyzed against 0.01M Tris-HCl buffer (pH 8.0), and concentrated by lyophilization. The purity of the enzyme was analysed by SDS and native PAGE electrophoresis (Figure 2) revealed the presence of a signal distinctive protein band with an apparent molecular weight of 55 kDa which indicated the homogeneity of purified L-asparaginase from P*. cyclopium*.

The molecular weight was close to that of *Rhizopus sp* and *Aspergillus niger* (48 and 66 KDa respectively (10,11). Molecular weight of L-asparaginase purified from *Aspergillus terreus* was 94 KDa (12). Higher molecular weight of 216 KDa was also reported for L-asparaginase of mesophilic fungus *Cylindrocarpon obtusisporum* (13). The variation of L-asparaginase molecular weight may be ascribed to genetic differences (14). Amino acid composition shows the amino acid contents of the purified *Penicillium cyclopium* L-asparaginase. The purified enzyme was rich in proline, arginine, relatively higher amount of

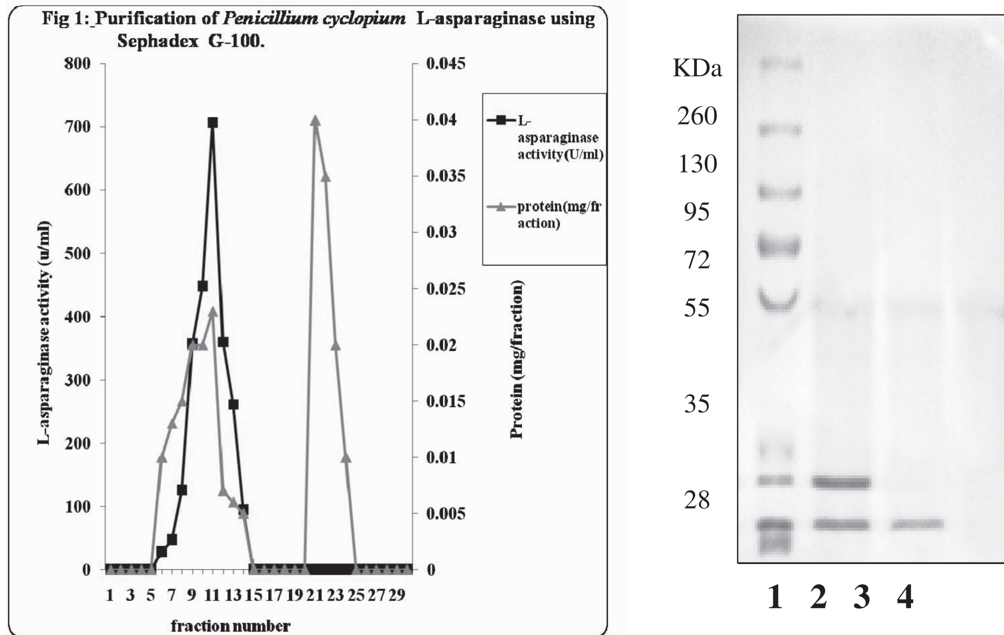

Fig. 1. SDS–PAGE analysis of the purified L-asparaginase, produced. The molecular marker, crude, partial purified and purified enzymes are located in lane 1, 2, 3, and 4, respectively.

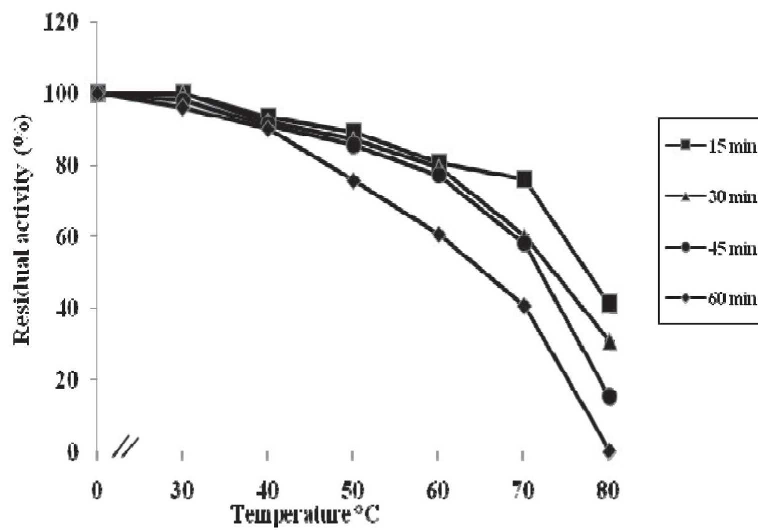

Fig.2. Thermal stability of the purified *Penicillium cyclopium* L-asparaginase activity

Table 1. Purification of *Penicillium cyclopium* L-asparaginase using Sephadex G-100 gel filtration

Treatments	Total protein (mg/fraction)	Recovered protein (%)	Total L-asparaginase activity (U)	Recovered activity (%)	S.E.A (U/mg Sprotein)	Purification fold
Crude enzyme	28	100	21080	100	750	1
Acetone 40-60%	1.2	4.3	2545	12	2120.8	3
SephadexG-100 (Fraction 9-13)	0.055	4.5	1992	78.6	39480	52.3

glutamic acid, aspartic acid, threonine, lysine, Phenylalanine and methionine. The purified L-asparaginase from *Aspergillus terreus* was rich in glycine and relative amount of glutamic acid, aspartic acid, lyscine, leucine and serine (15).

Characterization of L-asparaginase

Effect of pH on enzyme activity : The purified L-asparaginase exhibited maximum activity at pH 8.0. At pH 9.0 it retained only 29.3 % activity (Table 2). It clearly indicated that L-asparaginase from Penicillium *cyclopium* is pH dependent. These results coincide with that of Dhevagi *et al*., 2006 (16) who reported that the maximal L-asparaginase activity of *Streptomyces sp.* PDK7 was between pH 8.0-8.5 and the optimal L-asparaginase activity extracted from *Streptomyces gulbargensis* was 9.0 (17). Similar results were found by El-shafei *et al.,* 2012(18) who revealed that pH 8.0 was the optimal for L-asparaginase from *Penicillium brevicompactum*. L-asparaginase is one of the amidases that are generally active and stable at neutral and alkaline pH, whereas pH 5.0-9.0 were reported earlier to be optimum for amidase activity (19).

Effect of temperature on enzyme activity and stability: The effect of temperature on L-asparaginase activity was investigated and maximum enzyme activity was found at 37 ºC (Table 2). L-asparaginase *Aspergillus terreus* KIS2 (20) exhibited an optimum temperature at 37 ºC. Low L-asparaginase activity observed at higher temperatures may be attributed to partial denaturation (21, 22).

Table 2: Properties of purified *Penicillium cyclopium* L-asparaginase

Properties	Relative activity (%)
pH	
3	22.5±0.5
4	34.4±0.4
5	35.1±0.1
6	36.3±0.7
7	60.4±0.2
8	100
9	29.3±0.25
10	14.1±0.3
Temperature	
20	35±1.7
25	40±0.5
30	69.8±0.5
37	100
40	74.5±0.5
50	43.4±0.7
60	28±0.56
K_m	0.3 mM
V_{max}	3333U/ mg protein
Additives (Mm)	
Control	100
NaCl	101.5±0.3
KCl	106±0.8
$BaCl_2$	100
$CaCl_2$	95.5±0.3
$FeCl_3$	50.3±0.3
$HgCl_2$	0
$MnCl_2$	60.4±0.2
EDTA	45.8±0.4

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The enzyme was 100% stable for 30 min at 30ºC (Figure 2). However, after 30 min, the enzyme showed a slight decrease in its activity. About 24.5% of L-asparaginase activity was lost after incubation at 50 ºC for 60 min. A rapid decrease in its acivity (59%) was observed after incubation at 80 ºC for 15 min. On the other hand, purified L-asparaginase from *Aspergillus oculeatus* was stable for 2 h at 30 ºC (23). L-asparaginase purified from *Mucor hiemalus* showed higher stability as compared with other fungal enzymes as it retained stability after overnight incubation. The stability of an enzyme at physiological pH and temperature is a desirable characteristic for medical enzyme (24).

Kinetic parameter for the purified L-asparaginase: L-asparaginase of different microorganisms has different substrate affinities and probably plays different physiological roles in the enzyme activity. The substrate affinity of L-asparaginase to its substrate is related to its degree of effectiveness against tumor (24). The substrate affinity of L-asparaginase measured by K_m value and V_{max} were found to be 0.3 mM and 3333U/ mg protein respectively as determined by Lineweaver Burk plot (Figure 3). The low K_m noted for L-asparaginase of *Penicillium cyclopium* suggested the high affinity to substrate and thus its effectiveness towards tumor cells. In contrast K_m and V_{max} for *Mucor hiemales* L-asparaginase were 4.3 mM and 625 U/ ml (11). Slightly higher K_m (12.5mM) and a V_{max} of (104.06 U/ml) were observed for L-asparaginase of *Aspergillus aculeatus* (24). A low K_m value of 1mM was recorded by Raha, *et al.,* 1990 (13) for L-asparaginase of Cylindrocarpon *obtusisporum*.

Effect of different additives: On testing the effect of different metals and EDTA on L-asparaginase, the enzyme was highly sensitive to K^+, followed by Na^+ ions (Table 2). On the other hand Hg^{2+} had inhibitory effects on the activity of the purified enzyme, while Mn^{2+}, Fe^{3+} and EDTA had less inhibitory effect. Similar results were reported by El-shafei, *et al.*, 2012 using *Penicillium brevicompactum* (18).

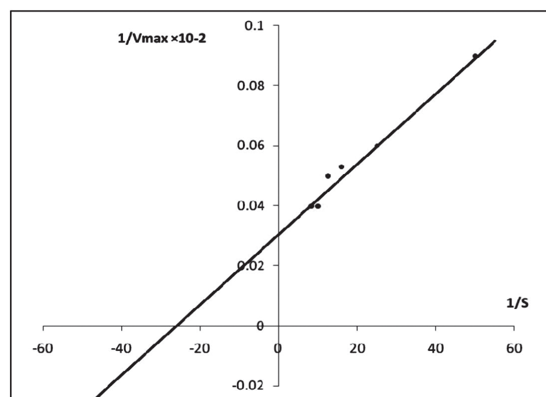

Fig. 3: Lineweaver –Burk plot for the purified *Penicillium cyclopium* L-asparaginase.

Application of L-asparaginase

a- Application of L-asparaginase on food: When the purified enzyme (12 ìg/ml enzyme, 30 min reaction time, reaction temperature 40°C and reaction pH 8.0) was tested for its effect on L-asparagine present in potato, released ammonia indicated the conversion of L-asparagine present in potato to L-aspartic acid. Acrylamide formation upon frying of potato chips treated with L-asparaginase was approximately (92%) lower than that measured for untreated potato chips (Figure 4). Acrylamide content in enzyme untreated potato chips was 8500 ìg/Kg while in the partially purified L-asparaginase treated potato chips it was 655.6 ìg/Kg. This reduction may be attributed principally to a reduction of L-asparaginase in the more external layer of the potato chips that could be reached by the enzyme, indicating the conversion of L-asparagine to L-aspartic acid and ammonia preventing formation of Millard reaction product and reduction in the high L- asparagine content in potato chips by L-asparaginase treatment (24).

b- Antitumor activity: Using MTT assay, the *in vitro* bioassay cytotoxic of *Penicillium cyclopium* enzymes on the growth of three human tumor cell lines; Hep-G2 [Human hepatocellular carcinoma cell line], MCF 7 [Breast cancer cell line] and PC3 [Prostate cell line] showed that the purified enzyme have antiproliferative activity

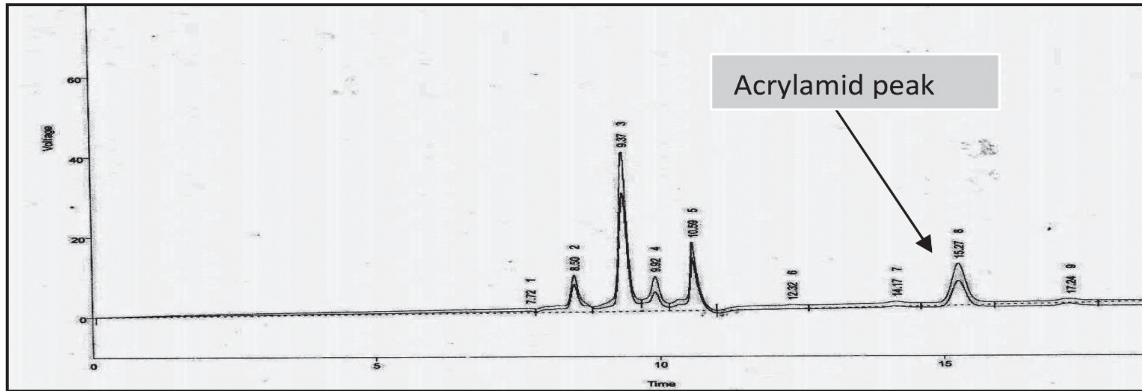

Fig. (4.A): HPLC chart of the enzyme untreated fried potato chips , showing the acrylamide peak at 15.27 min retention time.

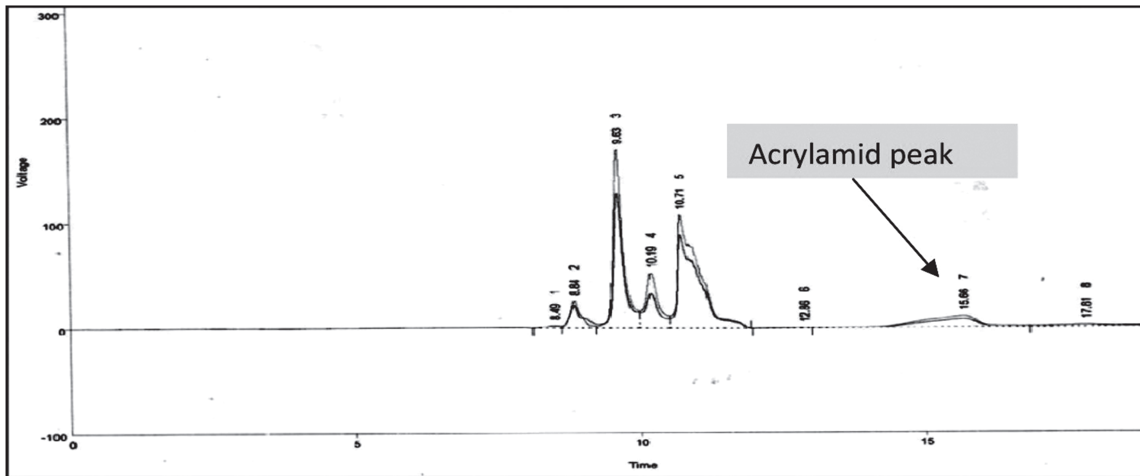

Fig. (4.B): HPLC chart of enzyme(partial purified) treated fried potato chips , showing the acrylamide peak at 15.66 min retention time.

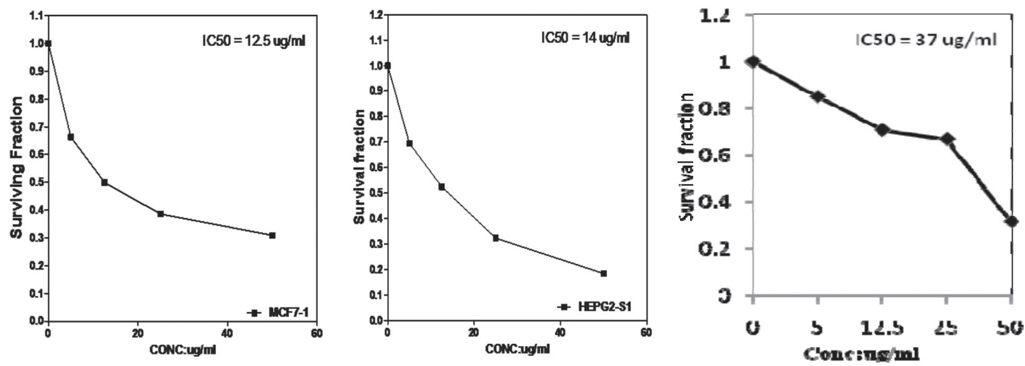

Fig. 5: Cytotoxic effect of *Penicillium cyclopium* purified enzymes on the growth of human tumor cell lines:

in different cell lines growth (Figure 5). The incubation of Hep-G2, MCF 7 and PC3 with gradual doses of pure L- asparaginase from *Penicillium cyclopium* led to a gradual inhibition in the cell growth as concluded from the low IC_{50} values of 14 , 12.5 and 37 ìg/ml respectively. Elshafei *et al*. (18) found that the incubation of Hep-G2 with gradual doses of *Penicillium brevicompactum* NRC 829 L-asparaginase (partialy purified and purified enzyme) led to a gradual inhibition in the cell growth as concluded from the low IC_{50} values of 76.4 and 43.3 ìg/ml, respectively. However L-asparaginase of *Aspergillus terreus* suppressed walker 256 ascites carcinoma in rats and Ehrlich asciles in susceptible Swiss mice (25).

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Enhancement of Steviol Glycosides in Stevia (*Stevia rebaudiana* Bertoni*)* Through Induction of Polyploidy

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Abstract

Mutation and manipulation of ploidy were tried on stevia (2n=22) to develop higher content of steviol glycosides. Different stevia mutants were developed using Colchicine using 0.25%, 0.50%, 0.75%, 1.0%, 1.50%, and 2.5 % and they were tested for their DNA content to ascertain the change in ploidy. Ploidy level was identified by flow cytometry analysis and steviol glycoside content in the leaves was determined by HPLC. Some polyploids showed two times increase in the percentage of stevioside as well rebaudioside-A, compared to control. Thus, induction of polyploidy in stevia confirmed the effectiveness of colchicines as a polyploidizing agent creating new variants with higher steviol glycosides (stevioside and rebaudioside-A) content contributing to crop improvement in stevia.

Key words : Stevioside, mixaploid, colchicine.

Introduction

The sugars along with sweetening qualities also have been found to contribute calories, which can lead to obesity, a risk factor for some chronic diseases such as diabetes mellitus, hypertension, cardiovascular diseases, etc. Hence, the craving for sweetness led man to discover several forms of alternative intense sweeteners which have made possible to offer consumers the sweet taste without the calories (1). The worldwide demand for high-potency sweeteners is expected to increase, especially with the new practice of blending different sweeteners.

Stevia rebaudiana Bertoni (2n=22) stands out among more than 150 species because of its intense sweetness. It is a perennial, endemic shrub belonging to the Asteraceae family (2, 3). Leaves of stevia contain around 10 sweetening glycosides, of which stevioside (5–10%), rebaudioside-A (2-4%), and rebaudioside- B, C, D and dulcoside are important. This sweetener does not metabolize in the human body, but pass through the digestive process without chemically breaking down. This property makes stevia safe for those who need to control their blood sugar level (4). Steviosides ($C_{38}H_{60}O_{18}$) are the major compounds (60-70% of the total glycosides content) and are assessed as being 110-270 times sweeter than sugar, responsible for the bitter aftertaste. The other compound rebaudioside-A($C_{44}H_{70}O_{23}$), is usually present as 30-40% of total sweeteners and has the sweetest taste, assessed as 180-400 times sweeter than sugar with no bitter aftertaste (5).

Development of new varieties of stevia with a higher content of Steviol Glycosides is the primary aim in this crop. Polyploids have successfully been induced earlier in stevia via colchicine and induction of polyploidy has been observed to improve desirable traits (6). Polyploid individuals have a higher content of stevioside than diploid plants and the selection of plants for commercial production could possibly increase the level of these compounds (7).

The main objective of this study was to identify the mutants with high steviol glycosides content through induction of polyploids in stevia using different colchicine treatment. Polyploids of *Stevia rebaudiana* were developed in 2012 using colchicine as the mutagen. The ploidy level was confirmed through flowcytometry at the University of Agricultural Sciences, Bangalore in 2012 and analysis of the leaf samples recorded higher Stevioside and Rebaudioside-A content through High Performance Liquid Chromatography (HPLC) (8).

Materials and Methods

Plant material: The *S. rebaudiana* plants were treated with colchicine (0.25%, 0.50%, 0.75%, 1.0%, 1.50%, and 2.5 %) at the Dept. of Plant Biotechnology, University of Agriculture Sciences, Bangalore in the year 2012. These polyploidy induced each plants were separately propagated by means of cuttings and planted in separate plots, the experiment was conducted during 2013-2014 at Sugandhavana, Department of Horticulture, University of Agricultural Sciences, Bangalore.

Preparation of sample: Leaves were harvested after 120 days of planting and separated from stevia stem. Leaves were dried at room temperature for 4 days and then made into fine powder in blender. The powder was sieved using 100 micron mesh. Then one gram of leaf powder was taken for extraction from each replication. 20 ml of HPLC grade methanol was added and kept on stirring in water bath at 60^0 for two minutes. Later filtered with whatman filter paper and repeated the process by washing it for 4 times in methanol. The filtrates were taken in a measuring flask and the volume of remaining extract was made to 100ml by adding HPLC grade methanol. The filtrate was again filtered using nylon membrane filter paper and the solution obtained was used for HPLC analysis.

HPLC analysis of Stevioside and Rebaudioside

HPLC condition: The analysis was performed using the high performance liquid chromatography (Shimadzu LC solution, Japan). Separation was performed on a column: NH_2, C18 column (250 mm × 4.6 mm). The mobile phase consisted of sonicated HPLC grade acetonitrile: water (75:25). The analysis was performed at 25^0 C with 1ml/min flow rate at detection wavelength of 210nm and Injection volume is set for 20µl (9).

Standard preparation: The samples of standard stevioside of pure form (95%) obtained from Natural Remedies India, and Rebaudioside-A (96%) obtained from Sigma Aldrich Pvt Ltd, USA. The reference stevioside compound of 2 mg quantity was dissolved in 1 ml of HPLC grade methanol to make stock solution of 2000 ppm (concentration of 2 mg ml^{-1}). From this stock solution, serial dilution of 1mg ml^{-1}, 0.5mg ml^{-1} and 0.25 mg $ml^{-1}$was made. Similarly reference rebaudioside-A stock solution and serial dilutions were made to obtain the standard calibration curve.

Analysis of samples: Extracted leaves samples were injected (20 µl) in port with the help of 25 µl syringe. Before injection the syringe was thoroughly rinsed with HPLC grade methanol and then with the sample. The run time was adjusted for 20 minutes throughout the experiment. Stevioside and rebaudiosides were identified in chromatogram with a peak by means of retention time.

Calculation

Stevioside and Rebaudioside content (100%) was calculated by using the formula (10):

$$\frac{\text{Area of sample x standard wt. (in mg) x sample dilution x Purity of standards}}{\text{Area of the standard x standard dilution x sample weight (in mg) x 100}} \times 100$$

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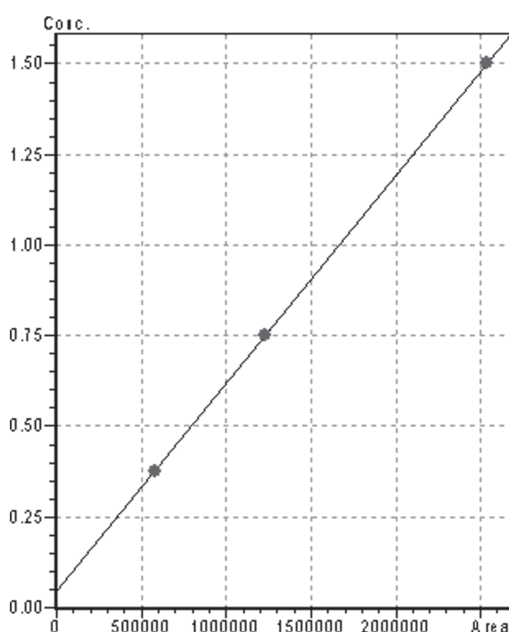
Results and Discussion

Calibration Curve

Fig. 1A. The calibration curve of stevioside standard:- linear regression



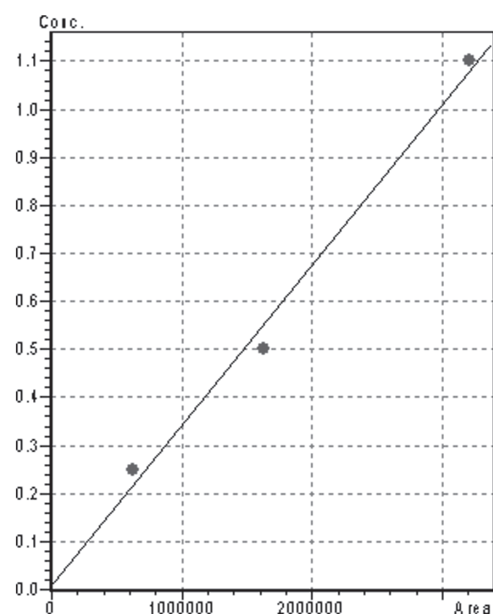


Fig. 1B. The calibration curve of rebaudioside standard:- linear regression

A particular peak with retention time of 8.2 minute for stevioside and 12.8 minute for rebaudioside-A was identified as the standard concentration peak of stevioside and rebaudioside-A. Calibration curve was prepared on graph by putting area on x- axis and concentration on y-axis and presented in figure-1A and 1B.

Quantitative analysis of diploids and polyploids of stevia for steviol glycosides

The stevioside and rebaudioside-A was estimated by High Performance Liquid Chromatography (HPLC), from untreated and different colchicine treated stevia plants. Highest stevioside content of 13.50% was observed in mixaploid- $T_{7,}$ followed by tetraploid- T_9 (11.77%). Highest rebaudioside-A content of 6.21% was recorded in tetraploid-T_9, followed by mixaploid-T_7 (5.94%), triploids and treated diploids, while the least was recorded in untreated diploid (Table-1, figure- 2A, 2B, 2C, 2D and 2E). Treated diploids showed higher stevioside content compared to that of untreated control diploid. Colchicine concentration may also influence the glycoside content because the variation was observed in plants of similar ploidy.

In the present study, ploidy level positively influenced the stevioside and rebaudioside content. Manipulation of ploidy is a valuable tool and has long been used in plant breeding programmes to improve agronomic yield, particularly biomass production. In addition, with the doubling of the gene products and increased dosage effect, polyploids provide a wider germplasm base than diploids for breeding purposes.

The colchicine treated plants showed higher stevioside content than the control, stevioside content in the leaves of different polyploids of stevia plants ranged from 5.57% to 14.98% (8). Leaves are the economic part where steviol glycoside synthesis takes place, improvement in leaf characters will have a direct influence on yield as well as glycoside content of the plant (11). Glycoside synthesis is reduced at

Table 1. Stevioside and Rebaudioside-A contents of *Stevia rebaudiana*

Treatments	Colchicine (%) used	Polyploidy level content (%)	Stevioside Content (%)	Rebaudioside-A
T_0	0.00	Untreated diploid	06.80	3.32
T_1	0.25	Treated diploid	08.50	4.83
T_2	1.00	Treated diploid	07.85	4.65
T_3	2.50	Treated diploid	07.98	4.25
T_4	1.50	Triploid	10.79	5.65
T_5	2.00	Triploid	11.01	4.94
T_6	2.50	Triploid	11.76	5.89
T_7	0.50	Mixaploid	**13.50**	5.94
T_8	1.00	Tetraploid	10.48	5.26
T_9	1.50	Tetraploid	11.77	**6.21**

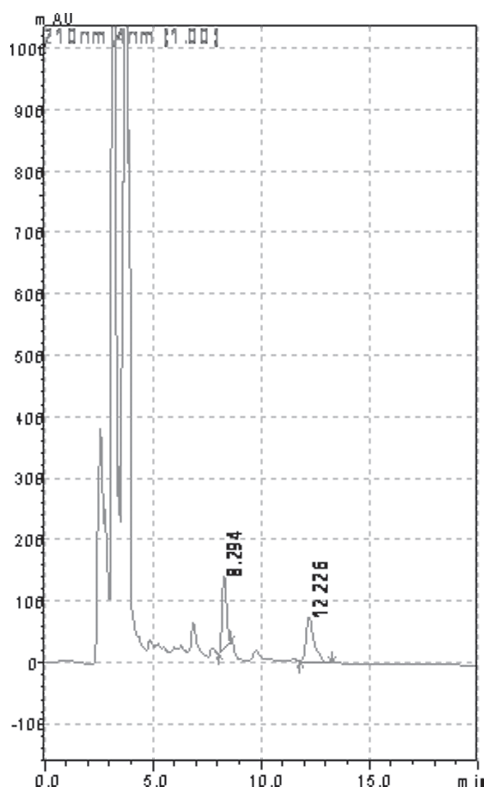


Fig. 2A. HPLC profile of Stevioside and Rebaudioside-A of tetraploid plants (T_9)

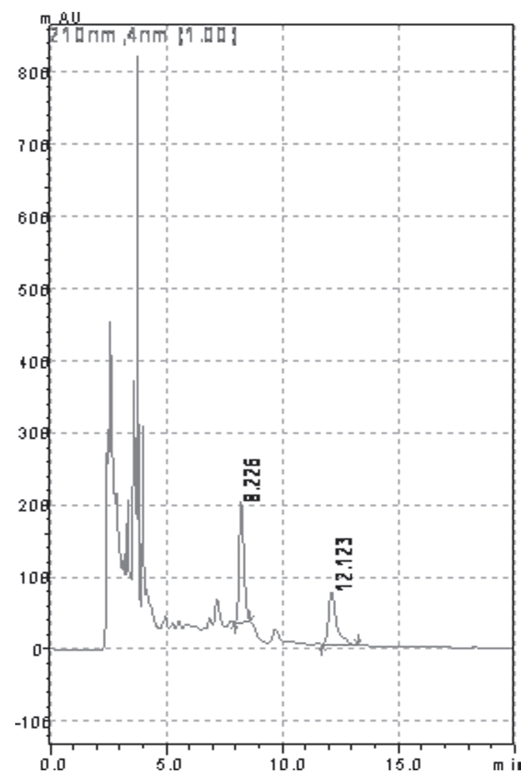


Fig. 2B. HPLC profile of Stevioside and Rebaudioside-A of mixaploid plants (T_7)

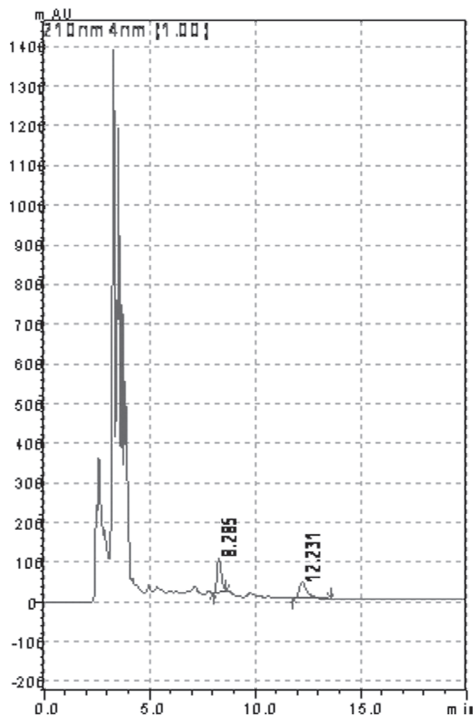


Fig. 2C. HPLC profile of Stevioside and Rebaudioside-A of untreated diploid plants (T_o)

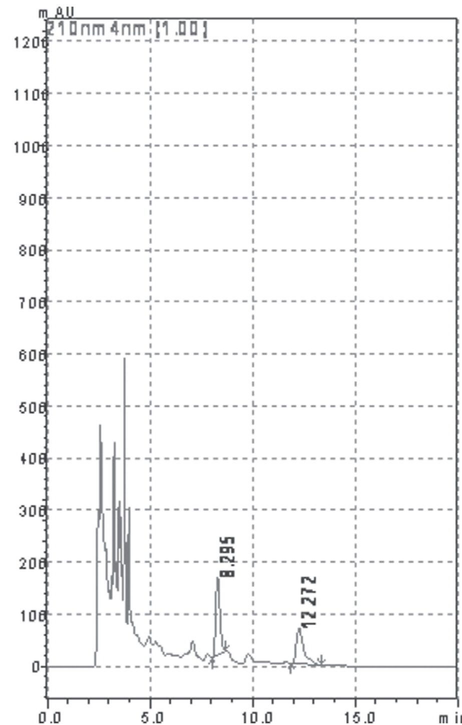


Fig. 2D. HPLC profile of Stevioside and Rebaudioside-A of triploid plants (T_6)

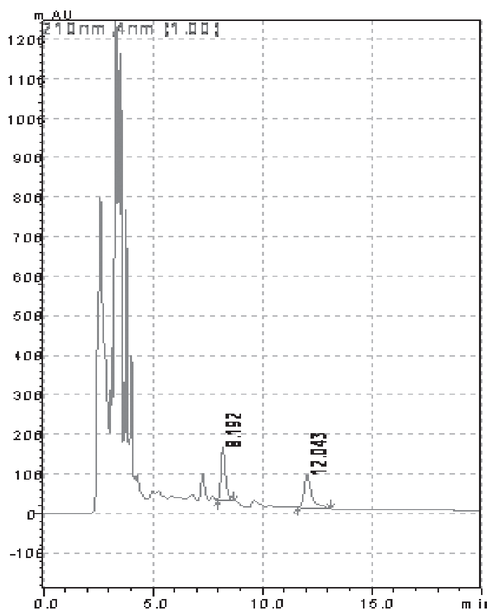


Fig. 2E. HPLC profile of Stevioside and Rebaudioside-A of treated diploid plants (T_3)

or just before flowering, delayed flowering in mixaploid allows more time for steviol glycoside accumulation. The results are in conformity with findings (12) in stevia.

According to a study conducted by different researchers that the stevioside and rebaudioside content varied from 2-10 % and 1-7.12% respectively (13). It varied from location to location, stage of harvest, method of extraction of steviosides, *etc*. Under Indian conditions stevioside concentration was about 9.08% of the dry weight of leaves (14). The glycoside quality of stevia is improved by changing the ploidy level. Stevioside content is influenced by both leaf surface and number of roots; however, the leaf surface has more influence on stevioside content than the number of roots (15).

Breeding of stevia at the ploidy level and induction of polyploidy will generate new variants and can be used in further crop improvement program in stevia with improved glycoside profile particularly rebaudioside-A which is most preferred glycoside has no bitter after taste.

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Antibacterial Screening of Root Extract of *Asparagus racemosus* Willd.

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Abstract

Asparagus racemosus Willd. is a medicinal plant belonging to the family Asparagaceae, found in tropical and subtropical regions of India. The present study has been attempted to explore the antibacterial activity of the root extracts of the plant against pathogenic bacteria. The plant material was defatted with petroleum ether and successively extracted with two solvents namely methanol and water using soxhlet extractor. Preliminary phytochemical screening of the extracts revealed the presence of terpenoids, saponin and glycosides. The antimicrobial activity of both the root extract of the plant was evaluated against *Actinomyces howellii, Bacillus cereus*, *Staphylococcus aureus* and *Klebseilla oxytoca*, in which the methanolic extract showed significant antibacterial activity as zone of inhibition. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) exhibited by methanolic extract against bacterial strains was found to be in the range of 12.5-25 mg/ml and 12.5-50 mg/ml respectively. The compounds that impart antimicrobial activity to this plant thus needs to be identified which could be further purified and characterized for commercial applications.

Key words: Antibacterial, *Asparagus racemosus,* phytoconstituents, saponin.

Introduction

Medicinal plants have been indispensable source of pharmaceuticals worldwide. Despite of continuous discoveries of new synthetic drugs, the demand of plant based medicines has never declined. The more reliability of herbal drugs and multi drug resistance of pathogens have encouraged researchers to explore new therapeutics from plants. Therefore, researchers are increasingly turning their attention to herbal medicines, seeking new leads to develop efficient drugs against microbial infections (1). In this regard, present study has been undertaken to explore the antimicrobial potential of an important medicinal plant *Asparagus racemosus* Willd.

Asparagus racemosus Willd., belonging to family Asparagaeceae (2), is a commercially important medicinal plant found in tropical and sub-tropical regions of India. The plant is basically known for its phytoestrogenic properties and traditionally been used for the treatment of various ailments (3). Antimicrobial activity of leaves and roots of the plant has also been reported against some microorganisms but the activity against numerous pathogenic microbes are yet to be explored. In the present study, an attempt has been made to test the antibacterial activity of root extracts of *A. racemosus* against four pathogenic bacteria viz. *Actinomyces howellii, Bacillus cereus*, *Staphylococcus aureus* and *Klebseilla oxytoca*. Also, the antibacterial activity of *A. racemosus* root extract has not been previously reported against *A. howellii and B. cereus*.

Materials and Methods

Plant material: The roots of the plant were collected from campus of Pt. Ravishankar Shukla University, Raipur (Chhattisgarh). The roots were washed thoroughly with distilled water and shade dried for six days, powered and stored in air-tight container till use.

Extraction of plant material: The powdered material was defatted with petroleum ether (Merck, analytical grade) and then subjected to successive soxhlet extraction at 60°C with methanol and distilled water. The extract were evaporated to dryness and kept at 4°C till use.

Phytochemical screening: The methanol and aqueous root extract was used for phytochemical screening. The dried extract was reconstituted in respective solvents and tested for the presence of alkaloid, flavonoid, tannin, saponin and glycosides.

Bacterial strains: Three gram positive bacteria viz. *Actinomyces howellii* MTCC 3048*, Bacillus cereus strain* McR 3, *Staphylococcus aureus* MTCC 3160 and one gram negative bacteria *Klebseilla oxytoca* ATCC 13182 was collected by School of Studies in Biotechnology, Pandit Ravishankar Shukla University, Raipur, Chhattisgarh, India. The inocula were prepared by inoculating the test organisms in nutrient broth and incubating them for 24 hours at 37°C.

Antibacterial assay: Antibacterial activity of both the methanolic and aqueous extract was evaluated by agar well diffusion method (4). The dried plant extract was dissolved into respective solvent to achieve a concentration of 200 and 400mg/ml. 100 µl of standardized inoculums was spread into nutrient agar plates and allowed to dry. A 6.0 mm diameter well was bored into nutrient agar plates using a sterile cork borer and 100 µl of root extracts was introduced into triplicate wells. For positive control 15 µg/ml streptomycin as standard antibiotic was used. The plates were allowed to stand for one hour for proper diffusion of extracts and then incubated at 37 °C for 24 hours. The zone of inhibition was recorded using HiAntibiotic zonal scale (Himedia).

Minimum inhibitory concentration (MIC): Minimum inhibitory concentration was determined by broth dilution method. A two-fold serial dilution of methanolic extract was made to a concentration ranging from 0.781 to 100 mg/ml using nutrient broth. Each dilution of extract was seeded with 100 µl of standardized inoculums of test bacterial strains. All the tubes were then incubated at 37°C for 24 hours. Least concentration of the extract where no visible turbidity observed was taken as the MIC.

Minimum bactericidal concentration (MBC): MBC were determined by assaying the test tubes resulting from MIC determinations. A loopful of the content of each test tube was streaked on a solidified nutrient agar plate which is then incubated at 37°C for 24 hours and observed for bacterial growth. The least concentration of the subculture with no visible growth was considered the minimum bactericidal concentration.

Statistical analysis: Means and standard errors (SE) of the samples were calculated and each treatment was carried out with three replicates. Mean differences were determined by using Duncan's multiple range test at 5% level of significance by SPSS 16.0.

Results and Discussion

Plant phytochemical constituents such as saponins, alkaloids, flavonoids, tannin and several aromatic compounds are secondary metabolites that serve as defense mechanisms against predation by many microorganisms, insects and other herbivores (5). This can partially explain the antimicrobial activity demonstrated by the medicinal plants. The preliminary analysis of phytochemicals (Table 1) revealed the presence of terpenoids, saponin and glycosides in the methanolic root extract of *A. racemosus* while only saponin and glycosides were found in aqueous extracts of the plant. Battu and Kumar (6) suggested that the antimicrobial properties in leaves of *A. racemosus* may be attributed to the presence of flavonoids. On the contrary, present results showed the absence of flavonoids in the root extract. Also, our findings are in agreement with previous reports of Wani et al., (7) and Nagamani et al., (8) who also reported absence of flavonoids in both alcoholic and aqueous root extract of *A. racemosus.* Thus according to our results flavonoids are possibly

Table 1. Phytochemical analysis of root extracts of *Asparagus racemosus*

Plant constituents	Methanolic extract	Aqueous extract
Alkaloid	-	-
Terpenoid	+	-
Tannin	-	-
Saponin	+	+
Flavonoid	-	-
Glycoside	+	+

not responsible for the antimicrobial properties exhibited by this plant. More detailed study is needed to reveal the exact compounds that impart antimicrobial properties to *A. racemosus*.

The antibacterial activity of the methanolic and aqueous root extract of plant was assessed against four pathogenic bacteria all of which were found to be susceptible against the methanolic extract. Aqueous extract did not showed any zone of inhibition against the tested bacterium at both the doses. The zone of inhibition observed by methanolic extract at a dose of 200mg/ml and 400 mg/ml of extract is depicted in Table 2. Gram negative bacteria *K. oxytoca* was more susceptible to methanolic extract followed by gram positive bacteria *S. aureus*. The antimicrobial activity of methanolic extract against *A. howellii* was more or less similar with that of *B. cereus*. Demonstration of antimicrobial activity against both Gram-positive and Gram-negative bacteria indicates the presence of broad spectrum antibiotic compounds (9). The antibacterial activity of leaves (6), roots (10) and whole plant (11) of *A. racemosus* has already been reported against various microorganisms. The extract has shown to inhibit the growth of

Table 2. Antimicrobial activity of methanolic root extract of *A. racemosus* against pathogenic bacteria

Microorganisms	Diameter of zone of inhibition in mm			
	Antibiotic (15µg/ml)	Control	Methanolic Extract (200mg/ml)	Methanolic Extract (400mg/ml)
Actinomyces howeii	26.66 ± 0.33b	6.00 ± 0.00	10.66 ± 0.66ab	14.33 ± 0.33c
Bacillus cereus	22.00 ± 0.00c	6.00 ± 0.00	10.00 ± 0.00c	13.00 ± 0.00d
Staphylococcus aureus	27.00 ± 0.00a	6.00 ± 0.00	11.33 ± 0.66ab	15.66 ± 0.33b
Klebsiella oxytoca	24.00 ± 0.00d	6.00 ± 0.00	12.33 ± 0.33a	16.66 ± 0.33a

*All values are expressed as Mean ± Standard error with three replicates for each experiment. Means with the same letter are not significantly different at $P \leq 0.05$.
*Well diameter- 6mm

Table 3- Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of methanolic root extract of *A. racemosus*

Microorganisms	MIC (mg/ml)	MBC (mg/ml)
Actinomyces howeii	12.50 ± 0.00	12.50 ± 0.00
Bacillus cereus	25.00 ± 0.00	50.00 ± 0.00
Staphylococcus aureus	25.00 ± 0.00	50.00 ± 0.00
Klebsiella oxytoca	25.00 ± 0.00	50.00 ± 0.00

*All values are expressed as Mean ± Standard error with three replicates for each experiment.

Actinomyces howellii and *Bacillus cereus* as well and thus the plant root extract can now be used in curing various ailments caused by these microorganisms.

Determination of MIC and MBC was carried out using methanolic extract as aqueous extract was not found effective against the tested microorganisms. From the results of MIC and MBC of methanolic extract, presented in Table 3, it may be concluded that the three bacteria viz. *B. cereus, S. aureus* and *K. oxytoca* have high MIC (25 mg/ml) and MBC (50 mg/ml). For *A. howellii*, MIC and MBC was recorded as 12.5 mg/ml which was found much lower than for other pathogenic bacteria. Our results demonstrated that the roots extract was active against these bacteria at a concentration of 12.5 mg/ml and 25 mg/ml respectively. The low MIC and MBC against *A. howellii* shows the efficacy of plant extract against gram positive bacteria as well as it could be tested for this activity against other microorganisms that cause dental infections.

Conclusion

Antibacterial properties of the metabolites from plants have recently gained interest of researchers as the herbal medications have always been the most reliable source of treatment to mankind. Emergence of multidrug resistance in microorganisms has increased the need of identification and validation of new classes of therapeutics from the inexhaustible source of medicinal plants. In this regard the present study was an attempt to explore the efficacy of *Asparagus racemosus* plant root extract against some pathogenic microorganism which could provide leads for further studies on purification of the active principle involved and its practical effectiveness.

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Study of Leptin Gene Polymorphism In Surti and Jaffarabadi Buffaloes by PCR-RFLP

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Abstract

The present research work was undertaken with the objective to study leptin gene polymorphism using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) in Surti and Jaffarabadi buffaloes. Fifty Surti buffaloes maintained at Livestock Research Station under Navsari Agricultural University, Navsari, Gujarat and 50 Jaffarabadi buffaloes from field areas of Junagadh district of Gujarat. Genomic DNA was isolated from 5ml whole blood sample by phenol chloroform method. From the purified genomic DNA, a 522 bp region spanning over a part of intron 2 and exon 3 and a 94 bp region of exon 2 of the leptin gene was amplified using two different set of primers. The 522 and 94 bp PCR products were digested using *BsaA*I and *Kpn2*I restriction enzymes, respectively. For the 522 bp PCR leptin fragment, three *BsaA*I digestion patterns were found in both the breed groups indicating three genotypes; the frequency of AA, AB and BB genotypes were observed as 0.42, 0.52 and 0.06 in Surti and 0.42, 0.44 and 0.14 in Jaffarabadi buffaloes, respectively. In case of 94 bp PCR products, the *Kpn2*I digestion revealed two alleles (A and B) and two genotypes; The frequency of BB and AB genotypes were 0.96 and 0.03; 0.94 and 0.06 in Surti and Jaffarabadi buffaloes, respectively. We found that the 522 bp leptin gene fragment was fairly polymorphic, while the 94 bp leptin gene was less polymorphic in both buffalo populations under study.

Key words: Jaffarabadi buffaloes, Leptin gene, PCR-RFLP, Surti buffaloes

Introduction

Leptin is a 16-kDa protein that is synthesized by adipose tissue and is involved in regulation of feed intake, energy balance, fertility and immune functions (1). It has been shown that leptin gene influences milk production performance (2,3) and reproduction (4) in cattle. It also modulates appetite, energy expenditure and reproductive axis by signalling the status of body energy stores to brain via leptin receptors (5). In cattle, leptin also plays an important role in lactogenesis in mammary gland (6). *Leptin* is one of the important candidate genes in buffalo to understand genetic variation among different breeds which influence milk production, milk composition, fertility, etc. It is located on chromosome 4 in cattle (7, 8) and chromosome 8 in buffalo (9). *Leptin* has 3 exons separated by 2 introns, of which only exon 2 and exon 3 are translated into the proteins. The PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) is one of the most commonly used technique for tracing the inheritance of genes and gene markers. RFLP can detect a SNP that eliminates a restriction site in a strand of DNA (PCR fragments) on digestion with restriction enzyme. If a restriction site is present, the DNA strand is cleaved, resulting in the strand decreasing in size and thus showing up as two different bands on a gel. Differences

in the length of the fragments generated as a result of mutation like insertions, deletions and base substitutions can be detected using PCR-RFLP.

Keeping in view these facts, the aim of present study was to investigate distribution of the allelic and genotypic frequencies of this gene in Surti and Jaffarabadi buffaloes.

Meterials and Methods

Ethical approval: The experimental and plan of study was duly approved by Institution Animal Ethics Committee of Veterinary College, Navsari Agricultural University, Navsari, Gujarat, India.

Resource population: Present study was conducted on 50 Surti buffaloes maintained at Livestock Research Station under Navsari Agricultural University, Navsari, Gujarat and 50 Jaffarabadi buffaloes from field areas of Junagadh district of Gujarat. About 5-7ml of the blood was collected from each animal from the jugular vein in sterile vacutainer containing 0.5 M EDTA (pH 8.0) solution as an anti-coagulant. After collection of blood, the vacutainers were shaken gently to facilitate proper mixing of blood with the anti-coagulant. The vacutainers were then kept immediately in icebox containing ice and gel cool packs; and transported to the laboratory.

DNA extraction: Genomic DNA was isolated from 5ml whole blood sample of 50 Surti and 50 Jaffarabadi buffaloes by phenol chloroform method according (10) with necessary modifications (11). The spectrophotometer (ND-2000c) and 0.8 % agarose gel electrophoresis methods were used to determine quantity and quality of extracted DNA.

Genotyping of SNPs by PCR – RFLP: From the purified genomic DNA, a 522 bp region spanning over a part of intron 2 and exon 3 and a 94 bp region of exon 2 of the leptin gene was amplified using two different set of primers (Table 1). PCR was carried out for both the fragments in a final reaction volume of 25µl consisting 12.5µl of 2X PCR assay buffer containing 4.0 mM $MgCl_2$, 0.05 unit/µl of *Taq* DNA polymerase, 200 µM dNTPs mix, 1µl of each primer containing 10pmol/µl, 3µl of genomic DNA (30ng/µl) and 7.5µl nuclease free deionised water.

The amplification of the 522 bp region was carried out using a thermal cycler, pre-programmed with initial denaturation for 5 min at 95 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 45 s; and a final extension at 72 °C for 10 min. The amplification program for the 94 bp fragment of exon 2 consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 45 s; and a final extension at 72 °C for 10 min. The 522 bp and 94 bp fragments were separated on a 2.5 % and 3.0 % agarose gel, respectively at a constant voltage 80 V for 60 min in 0.5X TBE buffer and documeted using Gel Documentation System.

The 522 and 94 bp PCR products were digested using *BsaA*I and *Kpn2*I (Table 2, Fermentas Life Sciences) restriction enzymes, respectively. Each digestion contained 15µl PCR products, 2µl 10x buffer, 1µl restriction enzyme and 2µl autoclaved water. In restriction enzymatic digestion, the 522 and 94 bp PCR fragments were incubated and inactivated at 37 °C and 65 °C for

Table 1. Primer sequences used to amplify fragments of the leptin gene.

Primer sequence	Fragment size	References
L1: 5'-GTCTGGAGGCAAAGGGCAGAGT-3'(22) L2: 5'-CCACCACCTCTGTGGAGTAG-3' (20)	522 bp	[12]
L5: 5'-ATGCGCTGTGGACCCCTGTATC-3' (22) L6: 5'-TGGTGTCATCCTGGACCTTCC-3' (21)	94 bp	[13]

7 min and 10 min, respectively in thermocycler. The fragments were separated using 3 % agarose gel electrophoresis and documeted using Gel Documentation System. Determination of gene and genotype frequencies and χ^2 test were carried out using POP Gene 1.31 software.

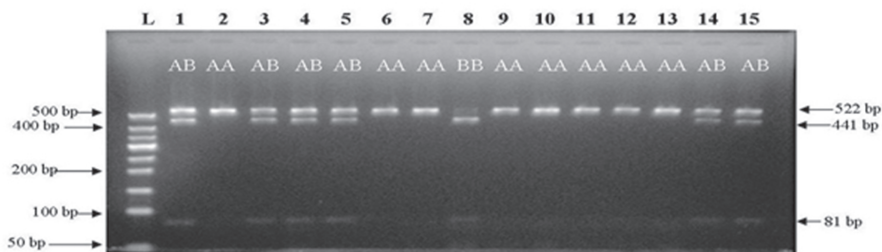
Results and Discussion

The extracted DNA by phenol chloroform method showed a good quality by spectrometry and PCR resulted in clear bands. For the 522 bp PCR leptin fragment, three *BsaA*I digestion patterns were found in both the breed groups indicating three genotypes; an intact 522 bp fragment as AA genotype; 441 and 81 bp fragments as BB genotype; and 522, 441 and 81 bp fragments as AB genotype (Plate 1).

These three types of patterns confirm frequency of allele A and B as 0.68 and 0.32 in Surti buffalo and 0.64 and 0.36 in Jaffarabadi buffalo, respectively. The frequency of AA, AB and BB genotypes were observed as 0.42, 0.52 and 0.06 in Surti and 0.42, 0.44 and 0.14 in Jaffarabadi buffaloes, respectively (Table-3). Frequency of the 'A' allele was higher than the 'B' allele in the present study. Both the breeds follow Hardy Weinberg's law of equilibrium.

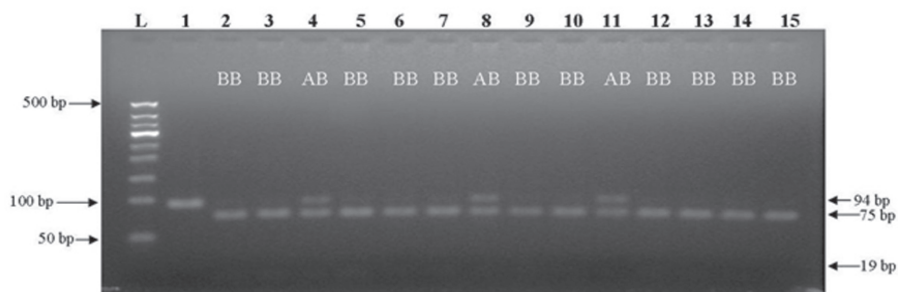
In case of 94 bp PCR products, the *Kpn2*I digestion revealed two alleles (A and B) and two genotypes (BB: 75 bp and 19 bp bands; AB: 94, 75 and 19 bp bands) in both the buffaloes studied (Plate 2). The genotype AA (94 bp) was absent in both the buffaloes studied. The frequency of

Plate 1: RE (BsaAI) digestion of leptin 522 bp PCR products.



Lane: L– 50 bp ladder, 1 to 7-RFLP products of Surti buffalo, 8 to 15- RFLP products of Jaffarabadi buffalo.

Plate 2. RE (Kpn2I) digestion of leptin 94 bp PCR product.



Lane : L– 50 bp ladder, 1– 94 bp PCR product, 2 to 7– RFLP products in Surti buffaloes, 8 to 15- RFLP products in Jaffarabadi buffaloes.

Table 2. Restriction enzymes and their restriction sites

Restriction enzymes	Restriction site
*BsaA*I *(Ppu21*I*)*	5'...Y A C G T R…3'3'...R T G C A Y…5'
*Kpn2*I	5'…T C C G G A…3'3'…A G G C C T…5'

Table 3. Genotypic and allelic frequency in *leptin* 522 bp and 94 bp locus in Surti and Jaffarabadi buffaloes

Buffaloes	Locus	Genotype	Observed number of genotype	Expected number of genotype	Genotype frequency	Allele frequency A	Allele frequency B	Chi square test
Surti	L522	AA	21	23.01	0.42	0.68	0.32	1.71
		AB	26	21.97	0.52			
		BB	3	5.01	0.06			
	L94	AA	0	0.01	0.00	0.02	0.98	0.01
		AB	2	1.97	0.04			
		BB	48	48.01	0.96			
Jaffarabadi	L522	AA	21	20.36	0.42	0.64	0.36	0.15
		AB	22	23.27	0.44			
		BB	7	6.36	0.14			
	L94	AA	0	0.030	0.00	0.03	0.97	0.03
		AB	3	2.939	0.06			
		BB	47	47.03	0.94			

BB and AB genotypes were 0.96 and 0.03; 0.94 and 0.06 in Surti and Jaffarabadi buffaloes, respectively (Table-3).

Choudhary *et al.*(14) reported lower frequency of 'A' allele (522 bp) in all crossbreds, Holstein Friesian, Jersey, Hariana, Sahiwal, Gir and Nimari cattle breeds but in present study the frequency of 'A' allele was higher in both the buffaloes. Our findings are similar to Azari *et al.* (15) who reported 0.39, 0.50 and 0.09 genotypic frequencies for AA, AB and BB genotype in Mazandarani native buffaloes. However present findings conflicts with the results of Kale *et al.* (16) who reported the single restriction pattern (only AA genotype) in 522 bp PCR products of *leptin* with *BsaA*I restriction enzyme in Murrah, Surti and Bhadawari breeds of riverine buffaloes.

The present finding is the first report of 94 bp leptin gene fragment of exon 2 polymorphism in indigenous buffaloes. However several researchers like Buchanan *et al.* (13); Liefers *et al.* (17); Choudhary *et al.* (14); Almeida *et al.* (18); Alashawkany et al. (19); Nassiry et al. (20); Kulig et al. (21); Oztabak et al. (22) observed the polymorphism in different cattle breeds. Choudhary *et al.* (14) reported only BB genotype in indigenous cattle (Hariana, Sahiwal, Gir and Nimari). The present findings are in agreement with Nassiry *et al.* [20] who reported the BB and AB genotypes in Sarabi, Golpayegani, Brown Swiss cattle whereas the AA, AB and BB genotypes were observed in the Taleshi, Sistani and Holstein cattle breeds. The present results conflict with findings of Kale *et al.* [16] who reported only AA genotype in Murrah, Surti and Bhadawari breeds of riverine buffaloes.

In the present study, 50 Surti and Jaffarabadi buffalos were selected randomly to determine genetic polymorphism of the leptin gene. To characterize leptine gene, polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) method were used. We found that the 522 bp leptin gene fragment was fairly polymorphic, while the 94 bp leptin gene was less polymorphic in both buffalo populations under study. The results showed that PCR-RFLP is an appropriate tool for detecting genetic polymorphism. The data generated in the present study can be correlated with production data and effectivly utilized for the formulation of suitable breeding strategies for the genetic improvement of the buffalo population.

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Validation of Simple Sequence Repeats Marker System in Different Genomic Groups of *Musa* sp.

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Abstract

An investigation was done to validate Simple Sequence Repeats (SSR) marker in 50 Musa accessions. All the accessions with exclusively AA genome showed only one allele of 733 bp, while accessions having only the BB genome lacked 733 bp allele but had the an allele of 652 bp. A total of 12 ABB natural hybrids screened, among these hybrids two accessions Kach Kela and Klue Tiparod possessed only 652 bp which has confirmed the presence of B-genome and remaining 10 had both 652 bp and 733 pb specific bands. Of the 7 AAB natural hybrids screened, 5 had both alleles whereas two hybrids namely Pisang Kelat and Nendran which possessed only allele of 733 bp confirming the presence of A genome but lacked allele of 652 bp which was exceptional. All the 21 AB natural hybrids possessed both alleles of 733pb and 652 bp thus confirming the presence of A and B genome respectively. In this experiment 5 AA and 5 BB accessions used as reference groups exhibited either an allele of 733 bp or 652 bp alone, according to their genome composition *i.e.* AA or BB genome. Hence, it could be concluded that the unique allele of 733 bp was specific to the presence of A genome while the allele of 652 bp was specific to the presence of B genome.

Key words: *Musa*; A-genome; B-genome; SSR.

Introduction

Banana (*Musa* spp.) is one of the most important staple crops in the World and is widely cultivated in the tropics and sub-tropics. It ranks fourth after rice, wheat, and maize in terms of gross value of production (1). Banana belongs to the family Musaceae, in the order Scitaminae. Cultivated bananas are mostly intra- or inter-specific hybrids of two wild species, *M. acuminate* (A genome) and *M. balbisiana* (B genome; 2n = 22; (13). Wild bananas are diploid, while cultivated bananas may be diploid, triploid or (rarely) tetraploid. The evolution of banana occurred through polyploidisation and the accumulation of somatic mutations (15).

Simmonds and Shepherd (13) devised a system of classifying *Musa* into genomic groups based on scores for a range of morphological features. A plant's morphology can be altered by environmental factors, and such a system of morphological classification would be inconsistent. Additionally, some characteristics are only expressed at maturity and these traits can only be measured after 18–24 months in field plantations. Therefore, easier, more stable, and more reliable techniques are needed to determine genomic groups in *Musa* (9).

Simple sequence repeat (SSR or microsatellite) markers are useful as they are highly informative, co-dominant, adaptable to high-throughput genotyping, and are simple to maintain and exchange between laboratories. SSRs are preferred over other DNA markers for the characterization and assessment of genetic variability, since they are also highly polymorphic, detect high levels of allelic diversity, reproducible, easy to interpret, assayed by PCR (16), and amenable for automation (14).

Based on this background, we have attempted to validate one SSR marker that was selected from our previous study (11) in 50 *Musa* genotypes belonging to different genomic groups (i.e., AA, BB, AB, AAB and ABB).

Materials and Methods

Plant material and DNA isolation: Fifty accessions (Table 1) of banana were used for genetic analysis. These genotypes belonged to different genomic groups (i.e., five each of the AA genotype and the BB genotype, 21of the AB genotype, seven of the AAB genotype and 12 of the ABB genotype). All Musa accessions used in this study were maintained at the Banana Germplasm Collection of the Indian Institute of Horticultural Research (IIHR), Bangalore, India. DNA isolation was done from fresh, young leaf tissue (2.0 g) using a modified CTAB method (10). DNA was quantified using a UV-spectrophotometer at 260 nm and its integrity was examined by 1.0% (w/v) agarose gel electrophoresis.

PCR amplification: The selected SSR marker Ma GSS8 (F: GAC AAA GCC GGC AAA AGT AG; R: CGG TAG GCA ACT CGT TCA AT) were used for amplification of all 50 *Musa* genotypes. Each PCR amplification reaction (25 μl) contained 2 mM MgCl2, 16 mM (NH4)2SO4, 67 mM Tris HCl pH 8.8, 0.01% (v/v) Tween-20, 0.1 mM of each dNTP, 0.5 μM of the forward and reverse primers, 50 ng genomic DNA, and 0.3 Units of TaqDNA polymerase (Bangalore Genei, Bangalore, India). PCR reactions were carried out in a thermal cycler (Primus 96; Peqlab Biotechnologies GmBH, Erlangen, Germany) using the following amplification conditions: an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step of 5 min at 72°C. The PCR amplification products were separated on 5% (w/v) high-resolution agarose gels [2:1 (w/w) High Resolution Agarose (Sigma, St. Louis, MO, USA) and normal Seakem LE agarose (Lonza, Rockland, MD, USA)] containing 0.5 μg ml –1 ethidium bromide (Ribaut et al., 1997). Gels were photographed on a UV-transilluminator (UVPro-Platinum; UVITEC, Cambridge, UK). Fragment sizes were calculated using the software provided by the manufacturer (UVPro Platinum 2.0 Version 12.9 for Windows) by comparison with standard DNA size markers.

Results

In the previous work (11), we had developed the 26 SSR markers based on a survey of genomic sequences and analyzed in banana. Among, 26 SSR markers we were analyzed, one SSR marker (Mag-SS8) had shown an allele of 733 bp in the AA and AB genotypes and an allele of 652 bp in the BB genotypes. Hence, in this study SSR marker Mag_SS8 was chosen for validating its specificity among the different genomic groups of *musa* sp.

This primer tested in 50 accessions of natural hybrids of banana genotypes representing various genome combinations including 5-AA (*M. acuminata* ssp Calcutta-4, *Musa acuminata* ssp .*malaccensis*, Erachi vazhai, Tongat and Pisang lilin,) 5- BB (Bhimaithia, *Musa balbisina* Tani, *Musa balbisiana,* Amturkela, Fruitless variety and Beeheekela), 21-AB (Bathesa paro, Chundilla Poovan, Adukkan Kunnan, Kunnan, Valia Kunnan, Vannetu Kunnan, Vaddakkan Kadali, Chakkara Kadali, Elakki bale, Njali poovan, Ney poovan, Hoo bale, Safed velchi, Putta bale, Mittili Bale, Kappu Kadali, Bile Kadali, Kodapanilla Kunnan, Hu Bale, Elakki Mitiga and Budi Mitiga), 7- AAB (Rasthali, Rajapuri, Foconoh, Palayan kodan, Karibale, Pisang Kelat and Nendran,) and 12- ABB (Karpooravally, Kallu Monthan, Kach Kela, Klue Tiparod, Bagner, Jurmoney, Karim Bontha, Pidi Monthan, Cuba, Bungan, Birbutia and Shanbale)

All the accessions with exclusively AA genome showed only one allele of 733 bp, while accessions having only the BB genome lacked 733 bp allele but had the an allele of 652 bp. Among interspecific hybrids of banana possessed both an alleles of 733 bp and an allele of 652 bp. A total of 12 ABB natural hybrids

screened, among these hybrids two accessions Kach Kela (ABB) and Klue Tiparod (ABB) possessed only 652 bp which has confirmed the presence of B-genome and remaining 10 had both 652 bp & 733 pb specific bands (Figure 1). Of the 7 AAB natural hybrids screened, 5 had both alleles whereas two hybrids namely Pisang Kelat (AAB) and Nendran (AAB) which possessed only allele of 733 bp confirming the presence of A genome but lacked allele of 652 bp which was exceptional (Figure 2). All the 21 AB natural hybrids possessed both alleles of 733pb and 652 bp thus confirming the presence of A and B genome respectively (Figure 3). In this experiment 5 AA and 5 BB accessions used as reference groups exhibited either an allele of 733 bp or 652 bp alone, according to their genome composition *i.e.* AA or BB genome (Table 2).

Hence, it could be concluded that unique allele of 733 bp was specific to the presence of A genome while the allele of 652 bp was specific to the presence of B genome. This SSR marker could be used for analyzing the genome composition among the hybrids or new collections.

Discussion

The present study aims at molecular differentiation of A and B genome in *Musa* in this regard SSR marker i.e., MaGSS_8 (11) was selected for screening the *Musa* natural hybrids representing various genomic combinations including 5 AA, 5 BB, 21 AB, 7 AAB and 12 ABB genotypes.

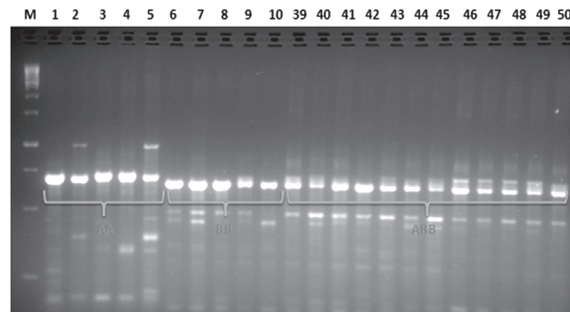

Fig.1. Gel profile of Ma_GSS8 marker for ABB accessions (Table 1)

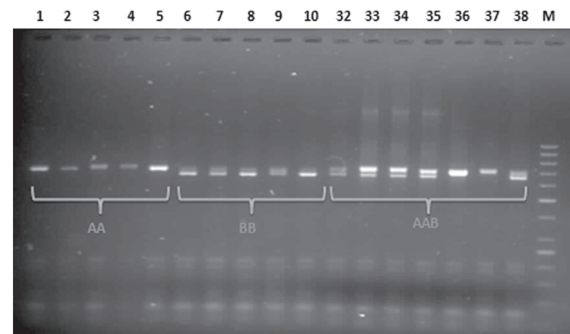

Fig.2. Gel profile of Ma_GSS8 marker for AAB accessions (Table 1)

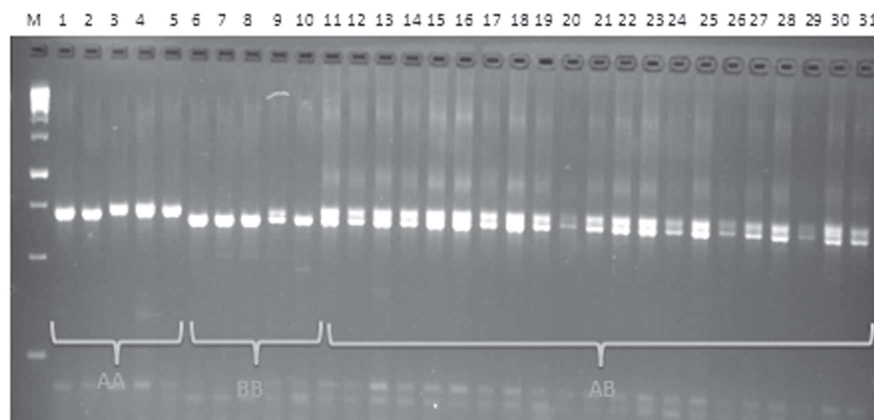

Fig.3. Gel profile of Ma_GSS8 marker for AB accessions (Table 1)

Table1. Details of banana (Musa sp.) accessions used in this study.

No.	Accession name	Source	Genome
1	*M. acuminate* Calcutta-4	IIHR	AA
2	*M. acuminata* ssp. *malaccensis*	IIHR	AA
3	Erachi Vazhai	IIHR	AA
4	Tongat	IIHR	AA
5	Pisang Lilin	IIHR	AA
6	Bhimaithia	IIHR	BB
7	*M. balbisina*	IIHR	BB
8	Amturkela	IIHR	BB
9	Fruitless variety	IIHR	BB
10	Bee Hee Kela	IIHR	BB
11	Bathesa Paro	IIHR	AB
12	Chundilla Poovan	IIHR	AB
13	Adukkan Kunnan	IIHR	AB
14	Valia Kunnan	IIHR	AB
15	Vannetu Kunnan	IIHR	AB
16	Vaddakkan Kadali	IIHR	AB
17	Chakkara kadali	IIHR	AB
18	Elakki Bale	IIHR	AB
19	Njali Poovan	IIHR	AB
20	Ney Poovan	IIHR	AB
21	Hoo Bale	IIHR	AB
22	Safed Velchi	IIHR	AB
23	Putta Bale	IIHR	AB
24	Mittili Bale	IIHR	AB
25	Kappu Kadali	IIHR	AB
26	Bile Kadali	IIHR	AB
27	Kodapanilla Kunnan	IIHR	AB
28	Hu Bale	IIHR	AB
29	Elakki Mitiga	IIHR	AB
30	Budi Mitiga	IIHR	AB
31	Rasa Kadali	IIHR	AB
32	Rasthali	IIHR	AAB
33	Rajpuri	IIHR	AAB
34	Foconoh	IIHR	AAB
35	Palayam Kadan	IIHR	AAB
36	Karibale	IIHR	AAB
37	Pisang Kelat	IIHR	AAB
38	Nendran	IIHR	AAB
39	Karpooravalley	IIHR	ABB
40	Kallu Monthan	IIHR	ABB
41	Kach Kela	IIHR	ABB
42	Klue Teparod	IIHR	ABB
43	Bogner	IIHR	ABB
44	Jurmoney	IIHR	ABB
45	Karim Bontha	IIHR	ABB
46	Pidi Monthan	IIHR	ABB
47	Cuba	IIHR	ABB
48	Bungan	IIHR	ABB
49	Birbutia	IIHR	ABB
50	Shanbale	IIHR	ABB

The ancestral species *M. acuminata* and *M. balbisiana* evolved in vastly different environments and contributed several agronomic traits towards the present genetic composition of the various *Musa* cultivars. For example, genes for hardiness, drought tolerance, greater disease resistance, improved nutritional value, and increased starchiness were contributed by the B genome of *M. balbisiana* (12).

Many studies on genetic diversity in banana have used molecular markers such as randomly amplified polymorphic DNA (RAPDs; (9), simple sequence repeats (SSRs) (4,7), or genomic in situ hybridization (GISH) (8). Simple sequence repeat (SSR or microsatellite) markers are useful as they are highly informative, co-dominant, adaptable to high-throughput genotyping, and are simple to maintain and exchange between laboratories. SSRs are preferred over other DNA markers for the characterization and assessment of genetic variability, since they are also highly polymorphic, detect high levels of allelic diversity, reproducible, easy to interpret, assayed by PCR (16), and amenable for automation (14).The SSR markers can be used to determine a plants genomic status especially in breeding programmes that involve interploidy crosses.

The amplification profile of SSR marker *i.e.*, MaGSS_8 clearly differentiated the A and B genomes in all natural hybrids by generating a allele of 733 bp for presence of A genome and an allele of 652 bp for presence of B genome with few exceptions. Out of the seven AAB natural hybrids screened two hybrids namely Pisang Kelat (AAB) and Nendran (AAB) had only allele of 733 bp was confirmed presence of A genome but lacked allele of 652 bp which is specific to B-genome. In ABB types two natural hybrids Kach Kela (ABB) and Klue Tiparod (ABB) possessed only 652 bp was confirmed the presence of B-genome, lacked in 733 bp allele which is specific to the A genome.

The absences of the B specific allele (652 bp) in 2 AAB hybrids may be due to the high composition of A genome than B genome where

Table 2. List of genotypes and its expression by Ma_Gss8 marker

Sl. No	Accession name	Genome	A	B
1	*M. acuminata* Calcutta-4	AA	+	-
2	*M. acuminata* ssp *.malaccensis*	AA	+	-
3	Erachi Vazhai	AA	+	-
4	Tongat	AA	+	-
5	Pisang Lilin	AA	+	-
6	Bhimaithia	BB	-	+
7	*M. balbisina*	BB	-	+
8	Amturkela	BB	-	+
9	Fruitless variety	BB	-	+
10	Bee Hee Kela	BB	-	+
11	Bathesa Paro	AB	+	+
12	Chundilla Poovan	AB	+	+
13	Adukkan Kunnan	AB	+	+
14	Valia Kunnan	AB	+	+
15	Vannetu Kunnan	AB	+	+
16	Vaddakkan Kadali	AB	+	+
17	Chakkara Kadali	AB	+	+
18	Elakki Bale	AB	+	+
19	Njali Poovan	AB	+	+
20	Ney Poovan	AB	+	+
21	Hoo Bale	AB	+	+
22	Safed Velchi	AB	+	+
23	Putta Bale	AB	+	+
24	Mittili Bale	AB	+	+
25	Kappu Kadali	AB	+	+
26	Bile Kadali	AB	+	+
27	Kodapanilla Kunnan	AB	+	+
28	Hu Bale	AB	+	+
29	Elakki Mitiga	AB	+	+
30	Budi Mitiga	AB	+	+
31	Rasa Kadali	AB	+	+
32	Rasthali	AAB	+	+
33	Rajpuri	AAB	+	+
34	Foconoh	AAB	+	+
35	Palayam Kadan	AAB	+	+
36	Karibale	AAB	+	+
37	Pisang Kelat	AAB	+	-
38	Nendran	AAB	+	-
39	Karpooravalley	ABB	+	+
40	Kallu Monthan	ABB	+	+
41	Kach Kela	ABB	-	+
42	Klue Tiparod	ABB	-	+
43	Bogner	ABB	+	+
44	Jurmoney	ABB	+	+
45	Karim Bontha	ABB	+	+
46	Pidi Monthan	ABB	+	+
47	Cuba	ABB	+	+
48	Bungan	ABB	+	+
49	Birbutia	ABB	+	+
50	Shanbale	ABB	+	+

+ denotes presence of a band................. - denotes absence of band

single set of 'B' chromosomes are present. Lack of A specific allele (733 bp) in 2 ABB hybrids also may be due to high composition of B genome than A genome, where a single set of 'A' chromosomes are present. This may be due to lack of marker amplification in some genotypes is a common observation in *Musa* as reported by Crouch et al., 1998; 1999) and may reflect divergence in the sequences flanking the microsatellite loci, leading to the production of null alleles, or completely inhibits amplification (3).

These studies validate the SSR marker MaGss8 that are specific to the A and B genomes in *Musa.* Markers for the B genome can be used to distinguish cultivars containing a double A or B, such as AAB from those with two B genomes, such as BB and ABB. The SSR marker analysis provides a quick and reliable system for genome identification in *Musa* that could facilitate genome characterization and manipulation in breeding lines. This SSR marker will also helps in selection of plants on the basis of genomic composition which could be done at the nursery stage from a few grams of leaf tissue, overcoming problems in *Musa* breeding that has a long seed to seed cycle (18-24 months), and the large space required for field testing (9).

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TCP Solubilization by Growth Promotory Endophytic *Acinetobacter calcoaceticus* TM8 from Tomato

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Abstract

The endophytic strain, TM8 isolated from tomato fruit, was identified as *Acinetobacter calcoaceticus* TM8 on the basis of morpho-biochemical characteristics and 16S rRNA gene sequence analysis. The strain with IAA, siderophore, ACC deaminase and phosphate solubilizing activity presented a direct mechanism for plant growth promotion. The strain possessed a remarkably high phosphate solubilising activity. The intent of the study was *in vitro* optimization of phosphate solubilizing activity *in vitro* and to assess its ability to solubilise P_i in broad range of pH, temperature and incubation period. Central composite design (CCD) and response surface methodology (RSM) were adopted to optimize phosphate solubilization *in vitro* and to investigate the mutual interactions between pH, temperature and incubation period. The maximum phosphate solubilization of TCP was 273.50 g mL^{-1} under the optimum conditions: pH 7.3, temperature 37.6^{0}C and incubation period of 7 days. The strain has ability to solubilize TCP even at alkaline pH. The strain with multiple PGPR traits could be exploited for developing *Acinetobacter* based bio-fertilizer.

Key words : *Acinetobacter calcoaceticus*, CCD, Endophytic bacteria, PSB, PGPR, Tomato, RSM

Introduction

With unprecedented population growth, there has been increasing pressure on land to meet growing demand of food. More fertilizers are being applied in order to maximize yield. Among NPK fertilizers, phosphorus is the second most important macro-nutrient next to nitrogen. It is required for all major developmental processes and reproduction in plants. Phosphate anions are the only form of phosphorus that plants can assimilate. Despite high amount of phosphorus present in the soil, most of this is insoluble, and is unavailable to plants (1). The insoluble phosphorus is present as either an inorganic mineral or in organic forms (2). In neutral/ alkaline soils, Ca^{2+} content of the determines the availability of inorganic P while in acidic soils Fe^{3+} and Al^{3+} controls the P solubility. When phosphate is applied as fertilizer, it is rapidly immobilized in soil owing to fixation. The cultivated plants use approximately 20-30% of the applied phosphate, and the rest is lost, eventually causing water eutrophication (3). Phosphate solubilizing bacteria (PSB) and fungi (PSF) can enhance availability of Pi from soil improving growth and development of plants (4, 5). *Acinetobacter* is widely distributed in soil, and possess high phosphate solubilizing activity, and other PGPR traits (6-8). In addition to rhizospheric PGPR, plant growth promoting endophytic bacteria (PGPE) has been proved effective against several crop diseases (9). Hence, attempts were made to characterize plant growth promontory endophytic (PGPE) bacteria, *Acinetobacter calcoaceticus* from tomato fruits.

Materials and Methods

Microbiological sample

***Isolation and characterization of bacterial isolate*:** Tomato fruits were sampled from local vegetables growing areas of Dehradun, Uttarakhand (India) during August-September, 2013. Sample (25 gm) was homogenized in a blender with 225 ml of 0.1 % tryptone water for 2 min. 10-15 ml of homogenate was incubated in modified tryptone-Soy Broth (mTSB) at 37^{0}C for 16-20 h for pre-enrichment. 1 ml of this broth was then diluted serially with Luria-Bertani (LB) and plated onto LB agar plates. Subsequently, these plates were incubated for 24 h at 37^{0}C. Following this, the bacterial colonies were streaked on MacConkey agar. Gram-negative bacterial isolate that grows on MacConkey agar but does not ferment lactose was selected. The pure culture of the bacterial isolate was prepared, and pot inoculation experiment was performed to re-isolate endophytic strain, and its confirmation as endophytic strain.

Pot inoculation experiment and identification of endophytic bacteria: TM8 strain was grown in nutrient broth in a gyrorotatory shaker at 200 rpm at 30°C for 48 h. Subsequently, bacterial cells were precipitated by centrifugation at 5,000×g for 5 min. The pellet was suspended in broth at concentration of 10^8 CFU /ml. Tomato seeds of cultivar Arka were surface-sterilized with 0.1 % $HgCl_2$ followed by quick dip in 70% alcohol for 30 s and several changes with sterile water. The disinfected seeds were immersed in the bacterial suspension overnight. The treated seeds were sown in pot. The pots were kept in a greenhouse for 45 days with the day/night temperatures at 28-35°C/15-20°C under natural light condition. Pots were maintained near filled capacity during growth. Endophytic bacteria was re-isolated from tomato pods. Pods were surface sterilized with immersion in 70% ethanol for 30 s followed 0.1% $HgCl_2$ treatment for 3 min, and then washed thoroughly by giving several changes with sterile distilled water. Inner portion of pods were aseptically removed, and then added to 100 ml of sterile saline (0.85%), and blended for 2 min in a Waring blender, serially diluted, and 100 ul of culture was spread onto mAc medium and incubated at 30°C for 48 h. Following incubation, bacterial colony was transferred to a differential carbohydrate medium (SR) for 2 h incubation at 30°C(10). The selected isolate from a SR medium was designated as TM8 strain and examined by various morpho-biochemical test (11). The strain was gram-negative, pleomorphic rods or diplococcic, nitrate reductase negative, catalase positive, oxidase negative, and was presumpti-vely identified as *Acinetobacter spp*., and further verified by partial 16S rDNA sequence analysis.

PCR amplification, sequencing and phylogenetic analysis*:* Genomic DNA was isolated from presumptive screened Acinetobacter strain TM8, using a fast DNA kit (Q-Biogene). Partial 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) using primer sets: 27F (5'-AGAGTTTGAT CCTGGCTCAG-3'), and 1492R (5'-GGTTACCT TGTTACGACT T-3'). 50 µL reaction mixture 100 ng of total DNA, 2U of Taq polymerase, 0.2 mM of dNTPs 3.0 mM of $MgCl_2$ and 0.4 µM of each primer. The PCR amplification was carried out using the initial denaturation step of 10 min at 94°C, followed by 30 cycles 1 min 94^{0}C, 30s 55°C, 72°C for 1 min. The reaction was completed at final extension temperature 72^{0}C for 10 min. The presence of PCR products was determined by electrophoresis of 10 µL of the reaction product in a 1.5% agarose gel. Purified PCR products were sequenced using ABI Big Dye Terminator chemistry v3.0 (Applied Biosystems). The 16SrRNA sequence was aligned and compared with other 16SrRNA genes in the GenBank by using the NCBI BLASTn program (http://www.ncbi. nlm.nih.gov/BLAST). A distance matrix was generated using the Jukes-Cantor corrected distance model. The phylogenetic trees created using NJ (Neighbor Joining) method. The 16S rRNA gene sequence of the isolate TM8 has been deposited in GeneBank using BankIt submission tool, and obtained accession number KF686821.

***In vitro* screening of bacterial isolate for plant growth promoting (PGP) traits**

***a. IAA production* :**The bacterial suspension (0.5 optical density, approximately 1×10^7 CFU/ml) was inoculated to 50 ml nutrient broth (NB) contained in 250 ml Erlenmeyer flask with 0.5 g/l tryptophan and incubated in a gyrorotatory shaker (180 rpm) at 30°C for 96 h in dark. The bacterial cultures were centrifuged at 6,000 ×g for 10 min at 4 °C. 2 ml supernatant was mixed 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production. Optical density of the resulting pink colour was read after 30 min at 530 nm. The concentration of IAA in culture was measured with the help of standard curve of IAA.

***b. Siderophore production*:** Siderophores production was assayed on Chrome azurol S (CAS) agar medium. Freshly grown bacterial cultures are inoculated as spot on CAS agar plates and incubated at 28°C for 24-48 hours. After incubation, siderophore production was confirmed by the presence of yellow- orange colour zone around bacterial colonies.

c. ACC deaminase activity: Screening for ACC deaminase activity of isolate was done based on their ability to use ACC as a sole nitrogen source. The isolate was first grown in 5 ml of TSB medium incubated at 28°C at120 rpm for 24 h. The cells were harvested by centrifugation at 3000× g for 5 min and washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) and spot inoculated on petri plates containing modified DF (Dworkin and Foster) salts minimal medium (without nitrogen source) containing 3.0 mM ACC or 0.1 M $(NH_4)_2SO_4$ as a N source. The 3 mM ACC were filter sterilized with 0.2 ìm membrane filter and was stored at -20°C before the assay. Prior to inoculation, the ACC solution was thawn and properly added to sterile DF medium. Plates containing only DF salts minimal medium without ACC as negative control and with $(NH_4)_2SO_4$ (0.2% w/v) as positive control. The plates were incubated at 28°C for 72 h. Growth of isolates on ACC supplemented plates was compared to negative and positive controls and was selected based on growth by utilizing ACC as nitrogen source.

***d. Qualitative estmation of TCP solubilization*:** Qualitative estmation of TCP solubilization was performed using Pikovskaya (PVK) agar medium. An aliquot (10 ìl) suspension (~ 1×10^7 CFU ml^{-1}) from overnight grown culture was inoculated as spot on agar plates. A clear zone around each spot during 1 week at 28°C was considered positive evidence of phosphate solubilization.

***e. Quantitative estimation of phosphate solubilisation*:** Quantitative estimation of phosphate solubilization was undertaken in PVK broth containing 0.5% Tricalcium phosphate (TCP). The flasks containing broth were inoculated by 1 ml of bacterial suspension (10^5 CFU ml^{-1}), *Acinetobacter calcoaceticus* strain TM8. The strains were harvested by centrifugation at 10,000 rpm for 10 min. In the culture supernatant, soluble phosphorus was estimated by the vanadomolybdophosphoric yellow colour method (11). The values obtained with the uninoculated controls were always subtracted from their respective treatments.

Optimization for enhanced phosphate solubilization: The effect of temperature and pH were studied by incubating the cultures over a range of temperature, 15.8-56.2°C and pH 6.0-8.7 for incubation period 1-9 days. The effect of pH on phosphate solubilization was studied by growing the bacterium at 36°C in the medium with varying pH made in citrate phosphate buffer, Tris-HCl, and glycine-NaOH buffer for maintaining pH of 5.3-7, and 8, and 8.7 respectively. The optimal levels of culture conditions (pH, temperature and incubation period), and the interaction of culture conditions on phosphate solubilizing activity, were analyzed by central composite design (CCD). In this study, a three-factor, five-level CCD with 20 runs was employed. Tested variables (pH, temperature and

incubation period) were denoted as A, B and C respectively, and each of them was assessed at five different levels as shown in Table-1. Data from CCD were analyzed with the following second-degree polynomial equation:

$$Y= \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{22}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \quad \text{....Eqn (1)}$$

where Y represents the predicted response; β_0 is an intercept; β1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are cross-product coefficients; β_{11}, β_{22} and β_{33} are quadratic coefficients; and X_1,X_2 and X_3 are input variables. The combined effect of pH, temperature and incubation period - as in-dependent variables, and phosphate solubilizing activity- as dependent variables, was investigated by the response surface methodology. The second order polynomial (regression) equation was used to estimate quadratic surface response. ANOVA for response surface quadratic model was conducted to determine the significance of model and regression coefficients.

Design-Expert 9 (Stat-Ease Inc., Minneapolis, USA) was used for designing experiments as well as for regression and graphical analysis of the experimentally obtained data.

Results and Discussion

Isolation and biochemical characterization: Morphological and biochemical analysis indicated that TM8 strain is gram negative, non-motile, coccobacili, catalase positive, oxidase negative, lactose negative and sorbitol negative (Table 2), and therefore, it was presumptively identified as *Acinetobacter* sp. Further characterization of strain was done through partial sequencing of 16S rRNA gene. PCR amplification of 16S rRNA gene resulted in a 1.5 kb amplification product in electrophoresis separation (Fig.1).

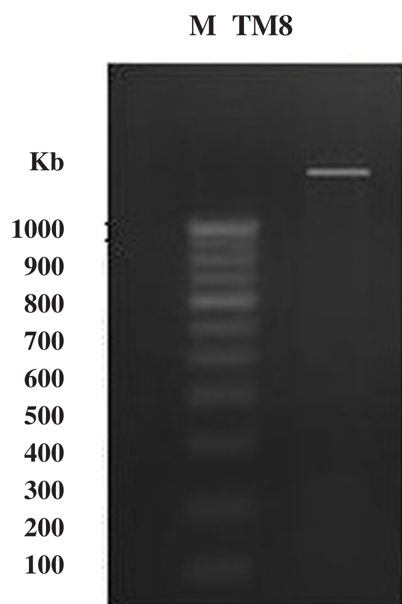

Fig. 1. Electrophoresis in a 1.5% agarose gel of PCR amplified 16S rDNA of *Acinetobacter* species TM8. M, molecular weight marker (100-bp DNA ladder); TM8, *Acinetobacter calcoaceticus* TM8

The partial16S rRNA gene sequence analysis of the strain showed 100% identity with that of several *Acinetobacter* reference strains of GenBank sequence database. The phylogenetic tree clearly indicated that bacterial strain TM8

Table 1. Original and coded levels of the independent variables

Independent variables	Coded levels -1.682	-1.000	0	1	1.682
A : pH	5.3	6	7	8	8.7
B: temperature (^{0}C)	15.8	24	36	48	56.2
C:Incubation Period (Day)	1.64	3	5	7	8.36

Table 2. The biochemical and plant growth promoting traits of *Acinetobacter* sp. TM8 strain isolated from tomato fruit, Dehradun, India

Morphology	Coccobacilli
Motility	Non motile
Gram stain	-
Oxidase	-
Glucose	+
Xylose	+
Mannitol	-
Sucrose	+
Lactose	-
Sorbitol	-
Catalase	+
Oxidase	-
Methyl Red	-
Voges–Proskauer	-
Nitrate reduction	-
Citrate utilisation	-
Indole	-
Decarboxylation of lysine	-
Urea, Christensen	-
H_2S on TSI	-
Gelatin liquefaction	-
Plant-growth-promoting traits	
IAA production	+
Siderophore production	+
ACC deaminase	+
Phosphate solubilization	-

+ indicates presence; – indicates absence of the morpho-biochemical/or plant growth promoting traits

strain was closely related to strains within the *Acinetobacter calcoaceticus* species (Fig.2). The strain has been designated as *Acinetobacter calcoaceticus* TM8, and assigned NCBI accession number KF686821.

***In vitro* screening of Plant Growth Promontory traits of PGPE bacteria:** The *Acinetobacter* spp. has been reported to be part of the epiphytic community of tomato leaves and also as endophytes (13). The results of *in vitro* plant growth promotion analysis showed that strain TM8 possessed indole acetic acid, siderophore and ACC deaminase production and phosphate solubilising activity (Table 2). The strain TM8 showed of indole acetic acid (50 mgl^{-1}) at 0.5g/l of tryptophan in nutrient broth supplemented with tryptophan. The ability of the bacterial strain to produce siderophores was evaluated on CAS agar medium. The strain TM8 was screened for ACC deaminase on DF salt minimal medium with ACC as the sole nitrogen source. The strain grew well on DF salt minimal medium with either ACC or ammonium sulphate as the sole nitrogen source that indicated strain as ACC positive. Formation of yellow-orange halo zone around colonies confirmed siderophore production. On PVK agar medium, halo-zones around the growing colony revealed phosphate solubilising activity of the strain. PGPB may promote plant growth directly usually by either facilitating resource acquisition or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogenic agents on plant growth and development, that is, by acting as biocontrol bacteria(1). Therefore the strain with IAA, siderophore, ACC deaminase and phosphate solubilising activity presented a direct mechanism for plant growth promotion. Studies have suggested that the bacteria that most effectively protect plants against a wide range of different stresses produce both IAA and ACC deaminase (14). Siderophores as well as membrane receptors that able to bind the Fe-siderophore complex facilitate iron uptake by microorganisms (15. Therefore, phosphate solubilizing ability together with the production of several compounds involved in plant growth promotion make our strain good candidate for development *Acinetobacter* based biofertilizers.

Optimization for Phosphate solubilisation: With a view to optimize culture conditions for enhanced phosphate solubilization by bacterium, central composite design was used to analyze the effect of three culture variables, i.e. pH, temperature and incubation period in 20 experimental trials at five levels-the actual and

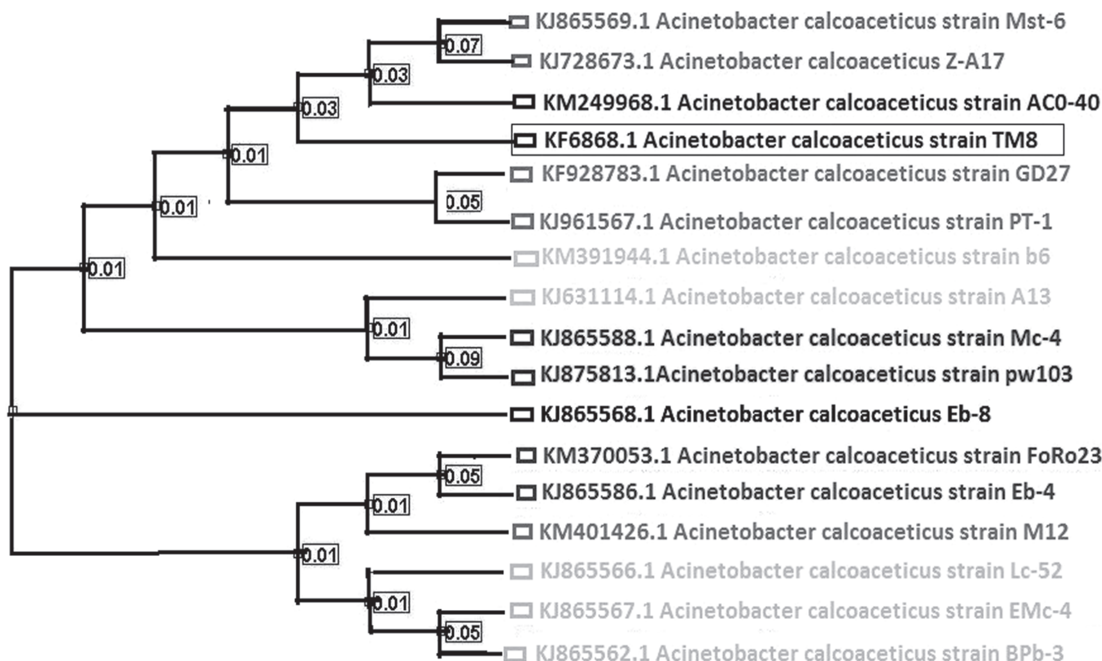

Fig. 2. Neighbor Joining tree of 16s rDNA partial sequences of *Acinetobacter calcoaceticus* TM8 [NCBI accession number KF 686821]

the coded levels are shown in Table 3. The F-value of the model was 28.41 and the value of Prob > F < 0.0001, suggesting that the model was highly significant (Table 4). The Linear term C (incubation period), and quadratic terms of A^2 (pH) and B^2 (temperature) were significant for phosphate solubilization (P< 0.05). Interactive term A*C (pH incubation period) was also significant for phosphate solubilization (P<0.05). "The "Lack of Fit F-value" of 2.82 implies that it is not significant relative to the pure error. There was a 14.00% chance that a "Lack of Fit F-value" this large could occur due" to noise. The model was found to be adequate for prediction within the range of variables employed. The coefficient of determination (R^2) was calculated as 0.9624 for phosphate solubilization activity. The pred-R^2 of 0.7528 was in reasonable agreement with adj-R2 of 0.9285. The adequate precision, the signal to noise ratio of 15.48, suggested an adequate signal. Therefore, the model can be used to navigate design space. The estimate regression coefficients are presented in table 5.

$$R1=245.26 + 4.73*A + 1.83*B + 14.93*C + 14.38*AB + 29.38*AC+ 13.13*BC-72.39*A^2-70.63 B^2 + 11.57*C^2$$

The regression Equation (2) was presented as response surface plot in Fig.3. Our present study reveals maximal predicted mean response for phosphate solubilization 276.98 µg ml^{-1} at pH 7.3, temperature 37.6^{0}C and incubation period 7 days. Validation of the statistical model was conducted by running test experiments using predicted culture condition. Under these optimized conditions, the mean value of phosphate solubilization was 273.50 µg ml^{-1}, which agreed well with the predicted response.

Table 3. Experimental design and results of CCD

Factors				Observed Phosphate solubilizing activity (µg/ml)	Predicted Phosphate solubilizing activity (µg/ml)
Run	A: pH	B: Temperture (^0C)	C:Incubation period (Days)		
1	-1	-1	1	100	94.06
2	0	0	0	250	245.26
3	0	0	0	240	245.26
4	1.682	0	0	30	44.07
5	1	1	1	220	192.17
6	1	-1	-1	90	71.15
7	1	-1	1	125	133.51
8	-1.682	0	0	60	28.00
9	-1	1	1	70	95.21
10	1	1	-1	65	77.30
11	0	0	1.682	300	3030
12	0	-1.682	0	50	42.04
13	0	0	0	275	245.26
14	0	0	-1.682	265	252.84
15	0	1.682	0	50	48.21
16	-1	-1	-1	115	149.2
17	-1	1	-1	100	97.86
18	0	0	0	220	245.26
19	0	0	0	240	245.26
20	0	0	0	245	245.26

Phosphate solubilization *in vitro* by microbes is largely influenced by the carbon (C) and nitrogen (N) source. *Acinetobacter* sp. showed maximum phosphate solubilization on glucose followed by galactose, maltose, lactose, mannitol, sucrose and sorbitol (15). Yeast extract nitrogen source was remarkably effective as a nitrogen source to support phosphate solubilization in *Acinetobacter* sp. (17, 18). The principal mechanism for mineral phosphate solubilization is the production of organic acids. The PSB solubilises inorganic phosphates by several mechanisms, such as organic acids production, gluconic, ketogluconic, oxalic, succinic *etc.* (19)., phosphatase enzymes, mainly acid phosphatases. Phosphate solubilization in *Acinetobacter* is enhanced by organic acid production (6).

Phosphate solubilization was also related to the proton (H^+) excretion accompanying NH^{4+} assimilation (20).. The report of Fan et al. (7) in *Acinetobacter* indicated that $(NH_4)_2SO_4$ was found to have significant effects on phosphate solubilization. Soil inoculation with phosphate

Table 4. ANOVA for response surface quadratic model

Source	Sum of squares	Df	Mean square	*F* Value	p-Value Prob > *F*
Model	156235.63	9	17359.51	28.41457	<0.0001[a]
A-A	305.06	1	305.06	0.499338	ns
B-B	45.76	1	45.76	0.074909	ns
C-C	3043.16	1	3043.16	4.981139	0.04968666[b]
AB	1653.13	1	1653.13	2.705884	0.131003981
AC	6903.13	1	6903.13	11.29924	0.007224851[b]
BC	1378.13	1	1378.13	2.255756	0.164019207[ns]
A^2	75529.21	1	75529.21	123.6285	<0.0001[a]
B^2	71885.63	1	71885.63	117.6645	<0.0001[a]
C^2	1930.62	1	1930.62	3.160092	ns
Residual	6109.37	10	610.94		
Lack of Fit	4509.37	5	901.87	2.818357	ns
Pure Error	1600.00	5	320.00		
Cor Total	162345.00	19			

ns-non-significant (P<0.05)
CV = 15.90 % ; [a] *F* > *F* 0.01 1% level significant; b *F* > *F* 0.05 5% level significant

Table 5. Estimated coefficients for the experimental design

Factor	Coefficient Estimate	df	Standard Error	Confidence interval at 95% Low	High	VIF
Intercept	245.26	1	10.08	222.80	267.72	
A-A	4.73	1	6.69	-10.18	19.63	1
B-B	1.8	1	6.69	-13.07	16.73	1
C-C	14.93	1	6.69	0.025	29.83	1
AB	14.38	1	8.74	-5.10	33.85	1
AC	29.38	1	8.74	9.90	48.85	1
BC	13.13	1	8.74	-6.35	32.60	1
A^2	-72.39	1	6.51	-86.90	-57.89	1.02
B^2	-70.63	1	6.51	-85.13	-56.12	1.02
C^2	11.57	1	6.51	-2.93	26.08	1.02

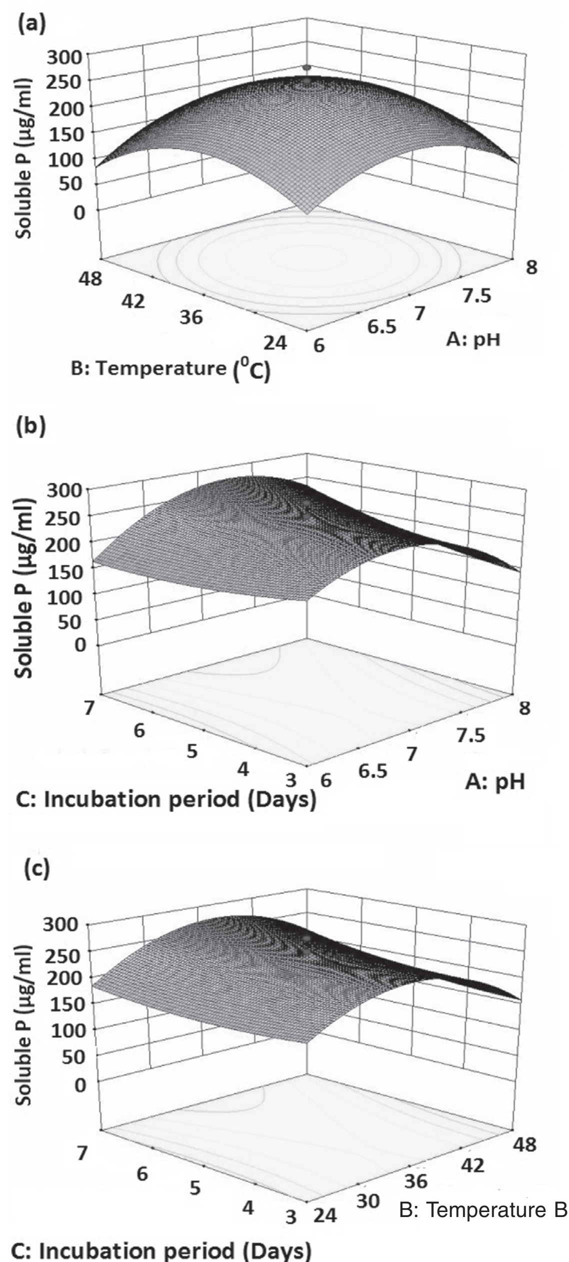

Fig. 3. Phosphate solubilization activity of *Acinetobacter calcoaceticus* TM8 : a. incubation temperature and pH interactions; b. incubation period and pH interactions; c. incubation period and temperature interactions.

solubilizing bacteria (PSB) has been shown to improve solubilization of fixed soil P and applied phosphates resulting in higher crop yields (21). Phosphate solubilizing bacteria faced with a myriad of environmental stresses while growing in the soil The ability to withstand the adverse environmental conditions such as high salinity, high/low pH, and high temperature is significant not only for rhizobacterial survival in tropical agricultural soils but also to be used as biofertilizer (22).

Quantitative estimation of phosphate solubilization by the bacterium in PVK broth containing TCP showed high phosphate solubilization (Table 3). In addition to P-solubilization, PSB also produced the other secondary metabolites like IAA and siderophore. *Acinetobacter calcoaceticu*s activates GAs biosynthesis pathways (23).

In vitro inhibition *of Fusarium oxysporum* under iron-limited conditions was demonstrated bythe siderophore-producing *Acinetobacter* strains (16). Several reports indicate sidophorogenic nature of *Acinetobacter calcoaceticus* (16, 24). The tomato is one of the world's most important vegetables, with an estimated total production of about 159.347 million tonnes in 2011 (FAOSTAT 2011). The application of P_i fertilizers is unsustainable and majority of which becomes immobilized and unavailable to plants. Phosphate solubilizing bacteria play a critical role in enhancing Pi acquision by plants. Plant Growth Promoting Endophytic bacteria (PGPE) and Plant Growth Promoting Rhizobacteria (PGPR) is a promising approach for enhancing the growth of tomato plants.

Conclusion

The study presents TCP Solubilization by Growth Promotory Endophytic *Acinetobacter calcoaceticus* TM8 from tomato. This report: (1) demonstrates the ability of strain to possess different PGPR traits, i.e. IAA, ACC deaminase, siderophore production and phosphate solubilising activity; and (2) demonstrates high

phosphate solubilising activity in broad range of pH, temperature and incubation period. The limitation of the present study is that results of *in vitro* studies on PGPR traits are to be tested under field conditions that would be necessary for understanding potential of inoculants under different soil and agro-ecological conditions.

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An efficient *in vitro* regeneration protocol from cotyledon and cotyledonary node of cluster bean (*Cyamopsis tetragonoloba* L. Taub)

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Running title – Efficient in vitro regeneration protocol

Abstract

Clusterbean (*Cyamopsis tetragonoloba* L. Taub) is a hardy crop and considered as green gold due to galactomannan content. Healthy seeds of clusterbean genotype GG-2 were surface sterilized and placed on medium containing B5 vitamins, 3% (w/v) sucrose, and 0.8% (w/v) bacteriological grade agar. The explants (cotyledon and cotyledonary nodes) obtained from seedlings of cluster bean (GG-2) were cultured in Murashige and Skoog's (MS) and B5 media supplemented with different concentrations of growth regulators NAA, BAP and 2,4-D. Successful shoot inductions of GG-2 were established in B5 medium supplemented with NAA (2 mg/l) + BAP (2 mg/l). The cotyledonary nodes cultured on BA showed higher shoot morphogenesis capability compared to the cotyledons. The highest overall average shoot morphogenesis percentage (34%) for GG-2 genotype was observed at 20μM BA. Subsequently, in the media supplemented with BA (14.4 μM) and IAA (12.3 μM), the cotyledonary node explants of cluster bean cultivars exhibited the highest percentage of multiple shoot formation. Highest percentage (74%) of rooting of in *in vitro*-regenerated shoots was recorded in B5 medium containing 3 mg/l IBA, 2 mg/L BA and 1 mg/l GA_3 in the cluster bean genotype GG-2. The present regeneration system is efficient and can be used for genetic transformation studies.

Key Words: *Cluster bean (Cyamopsiste-tragonoloba L. Taub), Direct Morphogenesis, Regeneration*.

Abbreviations: *NAA - 1-Naphthaleneacetic acid, BA - 6-Benzylaminopurine, MS medium - Murashige and Skoog's medium, IBA - Indole-3-butyric acid, TDZ - Thidiazuron, IAA - Indole-3-acetic acid.*

Introduction

Legumes are economically cheaper and a vital source of nutrients such as protein, dietary fiber, carbohydrates, minerals and vitamins which represent a significant food component of the human diet in various areas of the world, particularly in the developing countries (1). Owing to this, legumes are domesticated for the production of food, feed, forage, fiber, industrial and medicinal compounds (2).Cluster bean (*Cyamopsis tetragonoloba* L. Taub) is a major arid legume and is traditionally cultivated under poorly endowed situations with least management and after care (3). It has got a sobriquet of green gold due to its galactomannan content and industrial utility. It is one of the most crucial Kharif legume crops and is well adapted to arid and semi-arid regions of the world. India accounts for 80% of the total cluster bean produced in the world enabling its export to more than 65 countries recording an export turnover of 1126 crore rupees during 2006-2007 (4). Owing to its unique biochemical properties,

cluster bean gum is used in a wide range of industries from paper, cosmetic manufacture, to mining and explosives and therapy for a number of health problems like hypercholesterolemia, hyperglycemia and obesity (5).

Although cluster bean has attained the status of a commercial crop, an efficient high frequency regeneration protocol through tissue culture is essential for various in vitro manipulations such as hybrid embryo rescue and development of transgenics for desirable genetic traits such as biotic or abiotic resistance and/or to create new cultivars for improved production and post-harvest marketing. Such a system requires an efficient, high frequency plant regeneration, and the selection of fertile transgenic plants from various explants. The first step in the development of such a system requires a reliable plant regeneration protocol. This report presents an improved system for plant regeneration from cotyledonary nodes and cotyledons of cluster bean.

Materials and Methods

Plant material: Healthy seeds of cluster bean genotype Gujarat Guar-2 (GG-2) obtained from the Center Of Excellence for Research on Pulses, Sardarkrushinagar Dantiwada Agriculture University (SDAU), Sardarkrushinagar, Gujarat, India was used in all the experiments to establish and optimize the regeneration. The growth habit of cluster bean genotype GG-2 is early maturity and determinate growth. Its grain is pinkish, bold, and attractive. Its yield potential is 1.5 t/ha which is 17% higher yield than Gujarat Guar-1 (GG-1). GG-2 is very popular in Gujarat and adjoining states of Gujarat in India, since it has higher gum content (31%) and exhibits high level of resistance to bacterial blight, root rot and *Alternaria* blight (6).

Sterilization of Seeds and Germination: Healthy seeds were soaked in running tap water for 15 min, rinsed in 70% alcohol for 10 min and surface sterilized with 0.1% aqueous mercuric chloride solution for 15 min followed by rinsing five times with sterile distilled water on the hood of laminar flow to remove all traces of mercury. Five seeds of the genotype were aseptically germinated by removing its seed coat in a conical flask (100ml) containing 30 ml of MS medium(3% sucrose and 0.8% agar)under aseptic conditions with 16h photoperiod condition.

Shoot multiplication and elongation: Aseptically grown seedlings were used as a source of cotyledon and cotyledonary node as explants. The cotyledon and cotyledonary node explants were cultured with their abaxial surface touching the medium. Explants were cultured on MS medium (7) containing B5 vitamins (8), 3% (w/v) sucrose, and 0.8% (w/v) bacteriological grade agar. MS salts supplemented with B5 vitamins was used as basal medium and fortified with either alone or with different concentrations and combinations of growth regulators (Tables 1, 2). These cultures were incubated at 24^0 C under a 16h photoperiod for further growth and development of appropriate size of micro-shoots.

Rooting and acclimatization: To induce rooting, the elongated (>5 cm) micro-shoots were transferred to half strength MS medium containing B5vitamins and supplemented with different concentration of indole-3-butyric acid (IBA), BA and GA_3 (Table 3). Approximately 4-5-weak-oldplantlets with healthy roots were washed thoroughly with tap water to remove adhering medium and transferred to thermocol cups containing sterilized sand, soil and vermiculite (3:2:1 ratio). The transplanted plantlets were irrigated on alternate days with distilled water and weekly with Hoagland's solution (9). Hardening of plantlets was carried out in an incubator maintained at 24-26^{0}C; 16 h photoperiod. They were transferred to net house later.

Results and Discussion

In this study, GG-2 seeds germinated within 24 h were placed on medium containing 3% (w/v) sucrose, and 0.8% (w/v) bacteriological grade agar under aseptic conditions and it took 8-days to develop into seedlings of the appropriate size. Direct shoot morphogenesis was observed from cotyledons and cotyledonary node explants from

2.5-week-old cultures on MS (7) medium containing B5 vitamins (8), 3% (w/v) sucrose. The source of the explants played an important role in deciding callus induction frequency. 2,4-D was observed to be the most efficient source of auxin for callus induction (Table 1). Callus induction in legumes using 2,4-D as an auxin source have been reported with 2 mg/l concentration as the most suitable for callus induction (10; 11; 12; 13).

The morphogenic response of cotyledon and cotyledonary node explants of cluster bean with respect to different concentrations and combinations of NAA, BAP and 2,4-D are shown in Table 1.NAA (1 mg/l) failed to induce any morphogenic response from cotyledons and cotyledonary nodes while its higher dose (2 mg/l) induced rooting directly from cotyledons but not from cotyledonary nodes. 2,4-D (2 mg/l) and BAP (1 mg/l) on the other hand, induced callusing from cotyledon and cotyledonary nodes in cluster bean while NAA (1 mg/l) in combination with BAP (1 mg/l) caused only swelling of the explants. 2,4-D (2 mg/l) and BAP (1 mg/l) combination caused callus proliferation. The callus however, proliferated well on MS medium supplemented with NAA (2 mg/l) + BAP (2 mg/l). However, Singh (14) observed the best response on MS medium supplemented with 1.5 mg/l BAP with maximum number of shoots per explants and also on MS medium containing 1 mg/l TDZ. Genotypic differences were observed in callus initiation response of various cluster bean genotypes at different media compositions indicating that this character is genetically controlled.

BA or kinetin, both were used to find out the effect for direct shoot morphogenesis from cotyledons and cotyledonary nodes after 2.5-weeks of culture. The explants developed white friable callus at the cut ends on culturing in kinetin containing medium. This callus induction on kinetin indicates that it is a potent dedifferentiation agent for cluster bean. Similar findings were also reported by several authors (15; 16). The effect of six concentrations and combinations of kinetin and BA were recorded on cotyledon and cotyledonary node explants (Fig. 1). The cotyledonary nodes cultured on BA showed higher shoot morphogenesis capability compared to the cotyledons. For cotyledonary explants, the average shoot regeneration frequency of the GG-2 genotype ranged from 0.5 to 2.0% on media supplemented with BA. The highest overall average shoot morphogenesis frequency for GG-2 genotype was 34% observed at 20μM BA (Fig. 1).

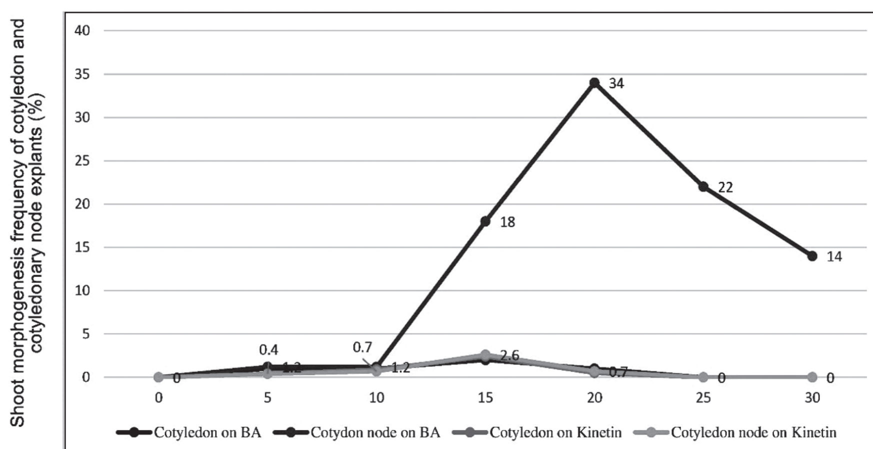

Fig. 1. Effect of various concentrations of kinetin and BA on shoot regeneration frequency of cotyledon and cotyledonary node explants of cluster bean genotype GG-2. Each data point represent the overall mean of percentage shoot morphogenesis observed from three replicates of 50 explants.

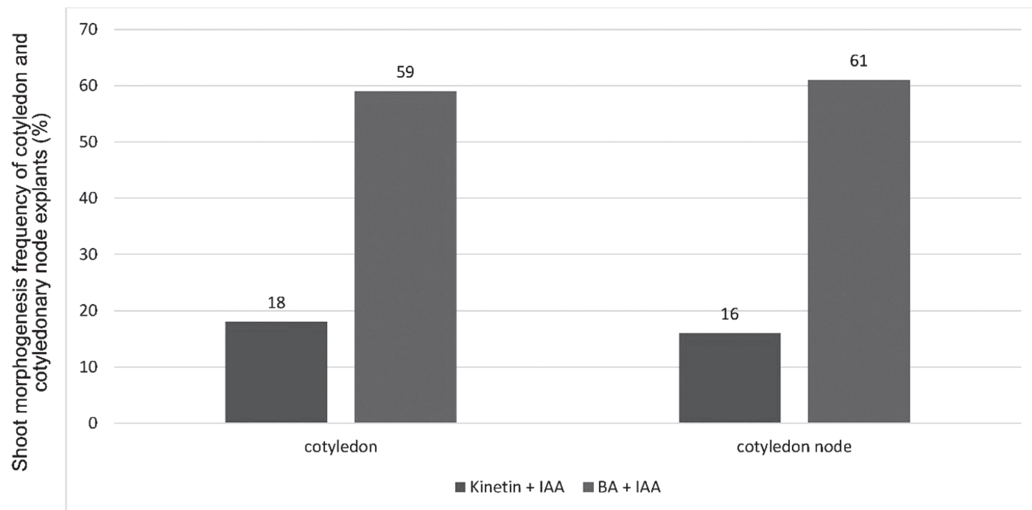

Fig. 2. Effect of kinetin and BA in combination with IAA on shoot differentiation from cotyledon and cotyledonary nodes of cluster bean genotype GG-2.

Table 1. Effect of different concentration of NAA, BAP, and 2,4-D with different combinations on the morphogenic response of cotyledon and cotyledonary node explants of clusterbean genotype GG-2.

Combination of growth hormone	Morphogenic response of cotyledon and cotyledonary node explants in cluster bean	
	Cotyledon	**Cotyledonary node**
MS basal medium	No response	No response
B5 vitamin + NAA (1 mg/l)	No response	No response
B5 vitamin + NAA (2 mg/l)	Adventitious root formation from explant	+
B5 vitamin + 2,4-D(1 mg/l) + BAP (0.5 mg/l)	+	No response
B5 vitamin + 2,4-D (2 mg/l) + BAP (1 mg/l)	++	++
B5 vitamin + NAA(1 mg/l)+ BAP (1 mg/l)	Swelling of explant	Swelling of explant
B5 vitamin + NAA (2 mg/l) + BAP (1 mg/l)	+	++
NAA(2 mg/l) + BAP (2 mg/l)	+++	+++

+, low amount of callus formation; ++, medium amount of callus formation; +++, good amount of callus formation.

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The addition of IAA showed a synergistic effect with kinetin or BA to enhance the frequency of shoot morphogenesis. The addition of IAA to BA containing medium increased the formation of multiple shoots, and also shootregeneration frequency to59-61%, when compared with kinetin and IAA combination (Fig. 2).

Although, kinetin in combination with various auxins showed the ability to induce shoot morphogenesis, its presence in the medium has been found to be detrimental for further shoot growth. The addition of IAA in combination with BA increased the formation of multiple shoots. The explants cultured on BA plus IAA did not show any callus formation at the base of the explants. The combination of BA and IAA was vastly superior compared to other growth regulator combinations, and data are presented in Table 2 (Fig. 3).

On media supplemented with BA (14.4 μM) and IAA (12.3 μM), the cotyledonary node explants of cluster bean cultivars showed the highest frequencies of multiple shoot formation. Highest percentage (74%) of in vitro root induction was observed in B5 medium containing 3 mg/l IBA, 2 mg/l BA and 1 mg/l GA_3 in the cluster bean genotype GG-2(Table 3).These data indicate that IBA is a potent root inducer which is also reported by many (17).

Conclusions

In this study, MS medium with B5 vitamins supplemented with 2,4-D (2 mg/l) yielded

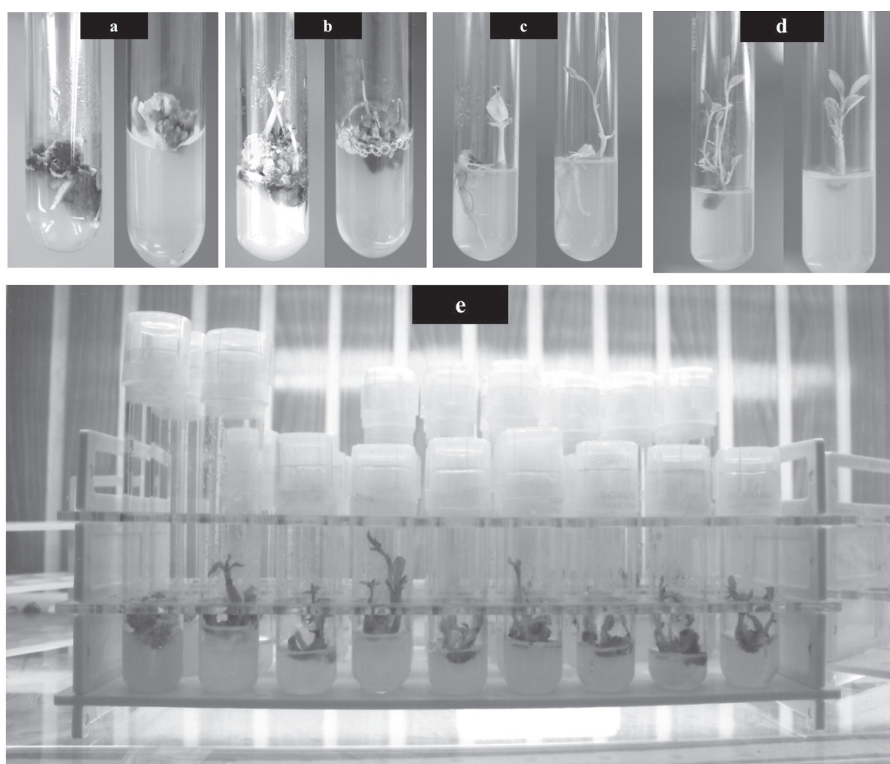

Fig. 3. Callus initiation from cotyledon and cotyledonary node explants and shooting, plant regeneration from callus of genotype GG-2 of Chusterbean (Cyamopsis tetragonoloba L. Taub). (a) In Callus formation from cotyledon and cotyledonary node explants, (b) Shoot initiation from callus on B5 medium supplemented with NAA (2 mg/l) + BAP (2 mg/l), (c) Shoot elongation from callusing, (d) Root induction on B5 medium containing 3 mg/L IBA, 2 mg/L BA and 1 mg/L GA3 and (e) Multiple shoots development on media supplemented with BA (14.4 μm) and IAA (12.3 μM).

Table 2. Effect of different concentration of BA and IAA on shoot morphogenesis in cluster bean genotype GG-2.

Concentration of growth hormones (μM)		Shoot morphogenesis frequency (%)
BA	**IAA**	15
6.5	5.5	24
8.5	7.5	50
14.4	12.3	88
18.4	14.4	67

Table 3. Effect of different concentrations of IBA, BA and GA3 on root regeneration in cluster bean genotype GG-2.

Concentrations of IBA, BA And GA_3			Root regeneration frequency (%)
IBA	**BA**	**GA_3**	
0.5	1	1	21
1	1.5	1	37
1	1.5	1	49
2	2	1	54
2	1.5	1	59
3	2	1	74

maximum callus induction from cotyledonary explants. Callus however, proliferated well on MS medium fortified with 2 mg/l each of NAA + BAP. NAA induced only callusing from cotyledonary nodes in cluster bean genotypeGG-2. When NAA (2 mg/l) was combined with BAP (1 mg/l), good quantity of callus was observed in cluster bean genotype GG-2, while 2,4-D (2 mg/l) induced callus from cotyledonary node explants. IBA also acts as potent root inducer. We have established a promising and reproducible protocol for efficient in vitro shoot regeneration from cotyledon and cotyledonary node explants of cluster bean. The described regeneration system may be adapted for genetic transformation studies in cluster bean.

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Understanding the Role of Iron and Zinc in Animals and Crop Plants from Genomics Perspective

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Abstract

The micronutrients iron (Fe) and zinc (Zn) play an important role in the metabolism of both animals and plants. The deficiency of these micronutrients, therefore, has a direct effect on their growth and metabolism. In order to enhance the level of micronutrients in crop plants, it is necessary to understand the genetic makeup and regulation of their transporter genes. The genetic improvement of crop plants is an option to attain nutritional security along with food security. In this review, we have described the impact of Fe and Zn on animal and crop plants, the need to improve the mineral contents (Fe and Zn) in crops with a special focus on common bean as a model for understanding the mineral uptake and the approaches towards deciphering the micronutrient contributing genes.

Keywords: Iron, Zinc, Common bean, Transporters, Genomics

Introduction

Micronutrients play a vital role in plant and animal metabolism. In case of human beings almost 49 micronutrients are known to be required to carry out metabolic activities. Deficiency of even one of the micronutrients affects the metabolism that may have adverse impact on human health (1, 2, 3, 4). To feed the rising human population, crop plants act as major source of providing nutrition. Hence, food security as well as nutritional security is essential to keep human population healthy. In many of the under developed and developing countries, nutritional security is a major concern that is having direct impact on human healthcare (5, 6, 7). Adequate quantities of micronutrients is very important for both plant and animal physiology as well as metabolism. Among all micronutrients, deficiencies of iron (Fe) and zinc (Zn) along with vitamin A accounts for almost two-thirds of the childhood deaths in the world. Since past two decades, the concept of hidden hunger which pertains to deficiency of certain vitamins particularly vitamin A and micronutrients despite eating enough calories has been well hypothesized (9). This has led to an increasing awareness as well as demand of micronutrient rich foods. Among micronutrients, Fe and Zn are of particular interest, as both of them are the essential micronutrients for all higher organisms. Hence, we will focus on these two micronutrients in detail.

Impact of Fe and Zn on Human health : Fe and Zn are the most important micronutrients that are necessary for proper metabolism in case of human beings. According to WHO, 30% of world's population is affected by Fe deficiency

and more than 2 billion people around the globe are affected by Zn deficiency (http://www.who.int/nutrition/topics/ida/en/index.html). In adult women and men, Fe represents approximately 35 and 45 mg/kg of body weight respectively (10, 11, 12). About 60-70% of body Fe is present in hemoglobin in circulating erythrocytes whereas another 10% of essential body Fe is present in the form of myoglobin, cytochromes and iron-containing enzymes. The remaining 20-30% of surplus Fe is stored as ferritins and hemosiderin in hepatocytes and reticuloendothelial macrophages, in healthy individuals (13). Fe is required by human for a number of highly complex processes that continuously take place on a molecular level and that are indispensable to human life, e.g. the transportation of oxygen in human body in which Fe is an important prosthetic group in both protein carriers; myoglobin and hemoglobin. Fe is the most important cofactor for the production of red blood cells, conversion of blood sugar to energy and also plays an important role in production and functioning of various enzymes. It is also essential for the production of fresh cells, important biomolecules like amino acids, hormones and neurotransmitters (http://www.nelsonsnaturalworld.com/en-gb/uk/our-brands/spatone/iron-essentials/role-of-iron-in-the-body). The non-vegetarian diets like egg yolk, red meat serve as essential sources of Fe for humans, whereas mushrooms, green leaved vegetables (spinach), chickpeas, soybean, kidney beans and lentils, are some of the critical plant based Fe sources. (http://foodscience-techn.blogspot.In/2012/08/the-importance-of-iron-in-humanbody.html). According to WHO report, the worldwide statistics reveals that most affected individuals by the deficiency of Fe among the human population comprises of children and women. According to the latest figures released by the Department of Health, about 91% of women aging between 16-64 years do not get their *recommended daily allowance* (RDA) of Fe in their diets. As a result of this, one third of all women, and two in every five women under the age of 34 years have low iron intakes that affects their health. In addition, 6% of women have been found suffering from clinical anemia, which is really a major concern [The National Diet & Nutrition Survey: adults aged 19 to 64 years (2003), Carried out in Great Britain on behalf of the Food Standards Agency and the Departments of Health by the Social Survey Division].

Zn being the second micronutrient in concentration to Fe, is equally important for human health. It is found in cells throughout the body and needed for proper working of defensive (immune) system, plays an important role in cell division, cell growth, wound healing, and the breakdown of carbohydrates. It is also needed during pregnancy, infancy and childhood for proper growth and development. Zn also protects the prostate gland from prostatitis and prostatic hypertrophy in males. It helps in maintaining sperm count and mobility and normal levels of serum testosterone. Although the rich sources of Zn are meat based foods which include beef, pork, lamb, fish, and chicken, yet, plant based sources like whole grains, legumes especially common bean also possess sufficient amounts of Zn but lower compared to animal based food products (http://www.nlm.nih.gov/medlineplus/ency/article/002416.html). Deficiency of Zn leads to frequent infections, hypogonadism in males, loss of hair, poor appetite, problems with the sense of taste and smell, skin sores, slow growth, trouble seeing in the dark, and delay in wound healing (http://www.nlm.nih.gov/medlineplus/ency/article/002416.html). Many clinical trials have shown that Zn supplementation results in growth improvement in children, lower rates of diarrhea, malaria, pneumonia, and reduction in child mortality (Zinc Investigations' Collaborative Group, 1999), (14). Approximately 8 lac children die per year due to zinc deficiency (World Health Report, 2002) (15).

Role of micronutrients in plants: In plants, Fe and Zn play an important role in maintaining proper metabolic and physiological cellular processes. These transition metals have unpaired electrons that promote their involvement in oxidation-reduction reactions (16). Fe is

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essential for chlorophyll biosynthesis, nitrogen fixation, DNA replication, reactive oxygen species (ROS) scavenging, and electron transport chain in both mitochondria and chloroplasts (17, 18). Chlorosis (low chlorophyll content) of young leaves is the most obvious visible symptom of Fe deficiency (19) and its deficiency also triggers the oxidative stress (20, 21). Fe is an essential cofactor for the functioning of a number of proteins and enzymes involved in metabolic processes of chloroplast and mitochondria. In case of plants many Fe transporters have been identified that help in translocation of Fe from soil to plant organs (22). Like Fe, Zn, a divalent cation, also plays diverse roles in different cellular processes (23). The metabolism of proteins, nucleic acids, carbohydrates, and lipids is dependent on Zn to a large extent (24, 25). Tight regulation of the Zn concentration is essential for its uptake by the plants (23). Inactive RNAses and starch accumulate in plants as a result of Zn deficiency. This indicates that RNA degradation could be regulated as well as overcome by the availability of Zn in the cells (26). Fe and Zn transport overlap in plant biological systems and deficiencies of both prevail when plants are grown in alkaline soil. Currently, cultivation of plants able to withstand and grow under alkaline conditions accounts for 30% of the world's cultivated soils may greatly benefit agriculture (27).

Common bean as a model system to understand Fe/Zn availability and uptake : *Phaseolus* is a diploid genus having 2n = 2x = 22 chromosomes. The genome size of *P. vulgaris* (580 Mbp/haploid genome) is comparable to that of rice (490 M bp / haploid genome) (28). The levels of duplication in common bean and the amount of highly repeated sequences are generally low. Most loci are single copy as revealed by the mapping experiments (29, 30, 31). Gene families tend to be small. The traditionally large families such as resistance gene analogs and protein kinases are of moderate size in common bean (32, 33). According to the USDA Nutrient Database, a bowl of cooked beans (100 g) provides 2 mg Fe and 1 mg Zn to an individual and depending on the age and gender, a normal individual requires 3 to 23 mg Fe and 2.5–10.9 mg Zn per day (34). One of the major challenges for general public is to meet the daily dietary requirements of Fe and Zn. It has been estimated that around two billion people suffer from iron deficiency, a major contributor to anaemia (35-36). Legumes in general contain appreciable quantities of Fe and other mineral elements which make common bean a good model for understanding the crop dynamics. The per capita consumption of legumes has decreased in India over the years, which seems the possible cause of increasing Fe deficiency, illustrating the importance of legumes in diet. Common bean is the most important grain legume for direct human consumption. The mobilisation of Fe and Zn from the soil is a preliminary need of the plants to ensure their availability for uptake. However, it has been observed that the genetic makeup of the genotypes also determine the content of Fe and Zn in seeds. We evaluated 51 diverse genotypes of common bean that were collected from foot hills of Himalayas falling in Jammu and Kashmir. We observed a great variation in Fe as well as Zn content (unpublished data). Similar studies have been carried out earlier in various cereal crops (wheat, barley, rice and maize) as well as legumes (bean, chickpea), indicating that there is enough genotypic variation in their germplasm which can be availed by breeders for nutritional improvement (37). In terms of both utilization and uptake of Zn, there is wide variation in plant genotypes with respect to their Zn deficiency tolerance. Nutritional traits are mainly stable across the environment, despite some reported genotype by environmental (G x E) interactions making the combination of high micronutrient traits with high yield possible. Variation in Zn efficiency is closely related to variation in Zn uptake capacity. This variation has been found in wheat cultivar (38). A comparative study has been carried out on rye and wheat which shows that rye has higher Zn accumulating efficiency than wheat cultivars. It is not necessary that genotype having higher Zn uptake ability has

higher Zn concentration in leaves, shoots or grains (39). Genetic ability of rye to absorb Zn from soils is higher, whereas availability of Zn in plant is lower (40). In wheat, Zn-inefficient genotypes have greater Zn concentrations in leaves or grains than Zn-efficient genotypes (41, 42). Wild varieties of common bean have a higher ability to accumulate iron (71-280 mg kg-1 compared to a mean Fe content of 100 mg kg-1 in cultivated varieties) and Zn (24-38 mg kg-1 compared to 17 mg kg-1) (43).

On the basis of acquiring Fe, plant species are distinguished into two types. Strategy I plants, include dicots like *Arabidopsis*, common bean and non-graminaceous monocots. Strategy II plants, which are graminaceous monocots such as rice, corn and wheat, use a chelation strategy for primary acquisition of Fe from the soil. Common bean, being a dicot comes under the strategy I plants similar to *Arabidopsis* category and thus will go through morphological modifications under Fe deficiency, which include enhanced development of lateral roots and differentiation of specialized transfer cells, introduction of physiological responses including increased acidification of the rhizosphere and secretion of phenolics and organic acids to chelate Fe. Reduction to ferrous from ferric on the root surface is an obligatory process for Fe acquisition from soil (44). This reduction is performed by Fe-deficiency inducible, plasma membrane-bound Fe (III) reductase. In *Arabidopsis*, this enzyme is encoded by the AtFRO2 gene (45). Like AtFRO2, expression of AtFRO3 is also strongly induced upon Fe deficiency, which suggests that the gene has a similar function (46).

Genomics approaches to explore micronutrient contributing genes : Exploring the genetic diversity of the germplasm is the initial step for identifying the micronutrient contributing genes. Various molecular and genomic tools can be put to use for identifying these genes. These techniques may include the use of molecular markers, saturating metabolic pathways and subsequently identifying the genes involved. Some strategies towards this approach include increasing the efficiency of the biochemical pathways in edible tissues or redistribution of micronutrients among different tissues. Yet, there can be some strategies which rather than increasing production or accumulation may involve addition of some factors which increase the bioavailability of these micronutrients. A number of genes have been identified in different plants that are responsible for transport and homeostasis of various micronutrients. Several molecular systems such as formation of specialized secondary structure of mRNA, customized use of stop codons and specialized molecules as metal-activated transcription factors help to regulate the translation and stability of those genes. Mutants of selective transporters help in studying the actual role of those elements in over all cellular processes and metabolism. The best strategy to know the difference in concentration of one or more elements is to study the ionome of the particular plant (47). Mutation in one or more genes affects the ionome by inhibiting transport of not only that particular element, but by inducing changes in whole ionome. These changes can be seen by characterizing the phenotype that demonstrates the complex physiological interactions or by high throughput elemental screening. Large ionomic screens of mutagenized populations of *Arabidopsis thaliana* grown on soil and *Lotus japonica* grown in liquid cultures were performed for analysis of element/s (48, 49). In both the studies, variations in concentrations of many elements were observed in addition to the element for which transporter was mutated. Similar results were observed in case of *irt1-1 Arabidopsis* mutant (50). This is due to the complex gene regulatory network responsible for efficient nutrient transport connected with each other in between cells.

Transporters and Transcription Factors : Transporters like IRT (Fe regulated transporter), NRAMP (the natural resistance associated macrophage protein) family are involved in Fe transport whereas ZIP family transporters are

involved in Zn transport in plants. These transporters are identified in various crop plants and show different functions (Table 1). Extensive work has been carried out using omics based approaches to understand transport, homeostasis and regulation of mineral nutrients. In case of plants, a number of mineral transporters have been identified (97, 98, 99, 100). Several transporters involved in Fe/Zn uptake and translocation have also been identified and characterized. ZRT/IRT-like proteins, ZIP like transporters are needed for uptake of Fe and Zn (101). AtIRT1 and AtIRT2 are involved in Fe uptake (51, 102, 103) while as AtZIP1, AtZIP2, AtZIP3 and AtZIP4 for Zn uptake into the roots (86). From the roots, both the mineral elements need to be transferred to xylem where from they are further transported to other above ground parts of the plant. Both in xylem as well as phloem, Fe and Zn are most likely chelated by nicotianamine (NA) during transport (104). Some concentration of these micronutrients (Fe/Zn) remains in roots as a stock and are used in case there is more increasing demand from the above ground parts. From the vacuolar pool, remobilization of minerals is assisted by NRAMP protein transporters (105). The metal homeostasis is regulated by metal-responsive transcription factors. One such transcription factor viz FER gene of tomato has been cloned, which encodes a basic helix loop-helix (bHLH) protein that is expressed at the root tip independently from Fe supply and is required for induction of Fe mobilization in tomato (106). The FER-like regulator of Fe uptake gene in *Arabidopsis* has also been cloned and is known as FIT1, FRU or AtbHLH29 (107, 108, 109). The amount of Fe and Zn can be increased by understanding the genetic variation of the associated trait. Studies reveal that a wide range of genetic diversity and variation is available in bean, rice, wheat, maize and cassava germplasm which allows breeding for nutritional improvement (37, 110, 111, 112, 113). Higher amounts of Fe and to some extent Zn, were found in aromatic rice genotypes as compared to non-aromatic genotypes, as such aromaticity can be an easy selection marker (114). The linkage amount of the genes and QTLs largely contributing for aroma and Fe/Zn transport are needed to be established to have nutritionally improved aromatic rice. In wheat, a highly significant positive correlation between the concentrations of Fe and Zn across different genotypes has also been observed (114). This suggests that common mechanisms regulate Fe and Zn accumulation in wheat. The improvement of crop plants for enhanced Fe and Zn contents through molecular breeding is only possible when a well-established knowledge on the genetic basis of Zn and Fe content is available (115). QTL's for Fe, Zn and Ca as well as loci affecting antinutrients have been identified in beans (116). In this study, they identified two unlinked QTLs for iron content and one for Zn content. The two QTLs associated with Fe content explained ~ 25% of variance whereas Zn QTL explained 15% of the variance (117). A QTL for Zn concentration and a marker assisted breeding program resulting in an increase in Zn content by 11.7% and 15.3% respectively, in a RIL (recombinant inbred lines) population of navy bean has been found (118). Recent studies have been carried out which show that only a single dominant gene might be the reason for higher Zn concentrations in the seed (119, 120). Nearly 17.8% of the variability has been described for a single QTL of seed Zn (118). A recombinant inbred population was also developed and it was found that the QTL responsible for improving Zn accumulation in bean is located on chromosome number 9. Afore mentioned studies show that plant breeding programmes can be useful to improve Zn accumulation in crops. Genetic variability can result in an increase of 80% iron content and 50% Zn content in common bean. Twenty three members of the ZIP (ZRT, IRT like protein) family of metal transporters and three transcription factors of the bZIP family have been reported in *Phaseolus vulgaris* L. recently. Expression patterns of seven genes were characterized in two bean genotypes (G19833 and DOR364) under two types of Zn treatments. Four genes (*PvZIP12, PvZIP13*, *PvZIP16*, and *PvbZIP1*)

Table 1: Transporters for Fe and Zn found in various crops

S.No.	Fe Transporter	Function	Source	Reference
1	AtIRT1	Fe (II) influx; primary iron uptake from soil	*A. thaliana*	(51,52,53,54)
2	AtNRAMP1	Transport Fe, Mn & Cd	*A. thaliana*	(55, 56)
3	AtNRAMP3	Transport Fe, Mn & Cd, mobilization of vacuolar Fe stores, export of vacuolar Mn into photosynthetic tissues of adult plants	*A. thaliana*	(55,56, 57)
4	AtNRAMP4	Mobilization of vacuolar Fe stores, export of vacuolar Mn into photosynthetic tissues of adult plants	*A. thaliana*	(56 57, 58)
5.	AtNRAMP6	Cd toxicity	*A. thaliana*	(59)
6	AtATM3	Export of Fe–S from mitochondria	*A. thaliana*	(60, 61)
7	LeNRRAPM1	Distribution of Fe in the vascular parenchyma upon Fe deficiency	*Solanum lycopersicum*	(62)
8	MbNRAMP1	Fe, Mn, and Cd trafficking	*Malus baccata*	(63)
9	OsIRT1/OsIRT2	Fe transportation	*Oryza sativa*	(64)
10	OsNRAMP1		*Oryza sativa*	(65)
11	OsNRAMP5	Uptake and transport Mn, Fe, and Cd	*Oryza sativa*	(66)
12	AhNRAMP1	Fe transporter and responsible for Feacquisition and distribution	*Arachis hypogaea*	(67)
13	OsYSL2	Influx of metal–NA complexes; translocation of Fe from root to shoot; loading of Fe in seeds	*Oryza sativa*	(68, 69)
14	OsYSL18	Influx of Fe (III)–PS complexes; facilitates xylem-to-phloem transfer of Fe	*Oryza sativa*	(70)
15	AtYSL1/ AtYSL2/ AtYSL3	Influx of Fe(II)–NA complexes; remobilization of transition metals during senescence and seed set; iron uptake from xylem	*Oryza sativa*	(71, 72, 73)
16	AtIRT2	Fe influx into cortical vesicles	*A. thaliana*	(74)
17	AtNAP14	Iron influx into plastids	*A. thaliana*	(75)
18	ZmYS1	Influx of Fe (III)–PS complexes; primary iron uptake from soil	*Zea mays*	(76, 77,78, 79,80, 81)
19	HvYS1		*Hordeum vulgare*	
20	OsYSL15		*Oryza sativa*	
21	MxIRT1	Ferrous transporter	*Malus xiaojinensis*	(82)

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Zn Transporter:				
1.	AtIRT3	Increased accumulation of Zn in shootsand Fe in roots, translocate Fe & Zn in Plant	*A. thaliana*	(83)
2.	TdZIP1	excess accumulation of Zn incells, thus generating a toxic cytosolic environment	*Triticum* aestivum	(84)
3.	AtZIP1 to AtZIP5, AtZIP9 to AtZIP12,	enhance Zn acquisition under deficient Zn conditions in Arabidopsis	*A. thaliana*	(85, 86)
4.	GmZIP1	highly selective for Zn, and might play a role in the symbiotic relationship between soybean and *Bradyrhizobium japonicum*	*Glycine max*	(87)
5.	ZmZLP1	May be responsile for transport of Zn from ER to Cytoplasm	*Zea mays*	(88)
6.	AtHMA2,AtHMA3 and AtHMA4	Zn uptake from the soil	*A. thaliana*	(89)
7.	AtMTP1	Stores Zn in the vacuole of predominantly leaf tissue.	*A. thaliana*	(90, 91)
8.	OsZIP4	Zn^{2+}-HCO_3 co-transporter	*Oryza sativa*	(92)
9.	OsZIP1/ OsZIP3	functional Zn transporters	*Oryza sativa*	(93)
10.	AtFPN1	Iron efflux across plasma membrane; loading ofFe into xylem	*A. thaliana*	(94)
11.	AtFPN2 (AtIREG2)	Influx of transition metals into vacuole; sequestration oftoxic metals during Fe deficiency	*A. thaliana*	(95, 96)

showed differential expression for Zn treatment in tissues. *PvZIP12* and *PvZIP13* show higher expressions in G19833 than DOR364. *PvZIP12* was the highly expressed in vegetative leaves under the Zn deficient condition. *PvZIP16* was highly expressed in leaf tissue, especially at flowering stage grown in the Zn deficient treatment, whereas *PvbZIP1* was the highly expressed in leaf and pod tissues. Mapping of these 23 *Pv*ZIP genes and three bZIP genes on the DOR364 × G19833 linkage map was also reported and it has been found that *PvZIP12*, *PvZIP13*, and *PvZIP18*, *PvbZIP2*, and *PvbZIP3* were located near QTLs for Zn accumulation in the seed. It was also found that *PvZIP12* is a good candidate gene for increasing seed Zn concentration and understanding the role of ZIP genes in metal uptake, distribution, and accumulation of zinc in *P. vulgaris* based on the expression and mapping results (121). In case of rice, a clear candidate gene has been identified, contributing for tolerance to P-deficiency (122). In another recent study, carried out by Tan and coworkers, a ferrous transporter (MxIRT1) from one of the species of apple is transferred to rice which enhanced the efficiency of rice for Fe and Zn uptake (123). Hence, in order to enhance the content of Fe and Zn in food crops, genetic engineering for transferring efficient transporters is a viable option.

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NEWS ITEM

SCIENTIFIC NEWS

Scientists are India's pride, salute their untiring efforts: PM Modi on National Science day

Prime Minister, Shri Narendra Modi has remembered the dedication, determination and untiring efforts of our scientists on National Science Day.

"National Science Day is an occasion to remember the dedication, determination and untiring efforts of our scientists. They are India's pride. He has mentioned that Science and technology will determine India's future, just as it has played a crucial role in bringing our Nation to where we are today. The Government is fully committed to encouraging research, innovation and excellence in science, particularly among our young minds. On National Science Day we salute the brilliance and long-lasting contribution of Sir C. V. Raman to the field of science.

Buffalo Clone 'APURVA' from urine cells - Scientists at National Dairy Research Institute in Karnal, Haryana, have produced a clone calf of a Murrah buffalo using somatic cells from its urine. This is the first time any animal has been cloned from cells isolated from its urine. If the technique succeeds and the clone survives, it may set a benchmark in cloning research and will help increase milk production in the country, as Murrah buffaloes produce more milk than any other variety of buffalo. In 2009, NDRI, the premium dairy research institute in India, became the first in the world to clone a buffalo. Since then it has produced seven clones, of which three have died. The first clone survived only six days, but a female calf born in 2012 has survived and even delivered a calf. Scientists have named the newborn calf as Apurva, which means "the unprecedented one". Apurva was born on 5 February, and its DNA fingerprints were confirmed in the second week of March, 2015.

New Beneficial algal species discovered - Two new bloom-forming algal species were discovered recently off the west coast of India. These two species, **Ulva paschima Bast, Cladophora goensis Bast** have excellent carbon capture properties — ability to absorb carbon dioxide from the atmosphere and reduce global warming — and are also promising candidates for use as biofuels, Some key features of the algae were both of the newly discovered species are endemic and bloom-forming. As they are endemic, their cultivation is not going to cause any environmental harm. i.e., they are not bio invasive species. Bloom forming indicates spontaneous growth. There is no need for fertilizers/pesticides or any expensive cultivation systems such as photo bioreactors for their cultivation. These can grow sporadically at shorelines and can sequester CO_2. These newly discovered algae have profound sequence differences from previously discovered algae.

Molecule with a Potential for Cancer Chemotherapy - The role of IP7, a lesser known molecule, have revealed that it has potential to be developed as a drug for the prevention of stroke and cancer chemotherapy. ATP molecule is known as a storehouse of energy and fuels the physiological activities. Another equally important but lesser known molecule is IP7, which too carries high energy and regulates many processes in the cell. This molecule consists of a sugar inositol and seven phosphate groups. Its levels in a cell are approximately thousand fold lower than

those of ATP. Studies conducted by scientists at the Centre for DNA Fingerprinting and Diagnostics (CDFD) in Hyderabad have revealed that IP7 plays a crucial role in DNA repair, blood clotting and protein synthesis by ribosomes. The team led by Dr. Rashna Bhandari, Group Leader, Laboratory of Cell Signalling, CDFD found that mice with lower levels of IP7 show reduced blood clotting. Inadequate levels of IP7 led to reduction in another phosphate-rich molecule called polyphosphate (a long chain of phosphate groups linked to each other). In mammals, polyphosphate is predominantly found in platelets and helps in strengthening blood clots during their formation. Polyphosphates housed inside platelets break up during clotting.

Unravelling the 'blood rain' mystery - Since 1896, reports have been coming in of sporadic instances of red coloured rain over parts of Kerala and Sri Lanka. A recent study by Indian and Austrian scientists has led to the discovery of the cause of the 'Blood Rain' phenomenon to be dispersal of spores of micro algae. The rain colours red even laundry left in the open to dry and reminds one of human blood. Many reasons were attributed to this mysterious phenomenon, some irrational, like a divine spell, and alien involvement. The Huffington Post reported in 2012 that this was caused by extraterrestrial life (aliens). The recent study, published in the journal, Phylogenetics and Evolutionary Biology, confirmed that the red colour in the rain was caused by the presence of spores of a European species of green microalgae, *Trentepohlia annulata* that was reported previously only from Austria.

Candidate malaria drug possible to cure with single dose - A library of half a million compounds that Astra Zeneca possessed were tested for almost three and half years. An automated process rapidly tested these molecules, looking for ones that could kill the malaria parasite, *Plasmodium falciparum*, at the stage in its complex lifecycle when it infects red blood cells. *P. falciparum* is responsible for causing the most dangerous forms of disease. The most promising compound to emerge from this quest, however, had poor solubility and also affected a molecule involved in maintaining the heart's rhythmic beating, was identified as per the publication of Nature Communications.

A smartphone dongle to diagnose HIV, syphilis - A palm-sized dongle connected to a smartphone will soon be able to diagnose HIV and syphilis with a good accuracy. The device, which was recently field-tested on 96 patients in Rwanda, had high sensitivity and specificity for both HIV and syphilis. The results are published in the journal of *Science Translational Medicine* (February, 2015). Sensitivity and specificity for both the infections are comparable to the lab-based ELISA. In the case of HIV, the sensitivity was 100 per cent and specificity was 87 per cent. For syphilis, the sensitivity was 92-100 percent and specificity was 79-92 percent.

OPPORTUNITIES

ICMR Centenary – Post Doctoral Research Fellowship Scheme - ICMR Postdoctoral Research Fellowship Scheme is being instituted to foster high quality research opportunities to promising fresh PhD/ MD/MS holders in the cutting edge areas of basic science, communicable and non communicable diseases, and reproductive health including nutrition at ICMR Institutes / Centers. Special focus will be on fundamental research in areas of science and other priority areas identified by ICMR from time to time. ICMR intends to offer fifty (50) such fellowships every year for working in ICMRInstitutes/Centers with state-of-art R&D facilities. These fellowships will be allocated by Director General, ICMR, depending upon the needs of the Institute/Centre. Eligibility: Fresh PhDs/MD/MS within three years of completing their PhD/MD/MS and also those who have submitted PhD/MD/MS thesis. ICMR Postdoctoral Research Fellowship is open to Indian nationals only.

IIT, Bhubaneswar- Post Doctoral Fellowship Programme - Candidates preferably below 35 years having a Ph.D. Degree and willing to carry out advanced research in the fields of Basic Sci-

ences; Earth, Ocean & Climate Sciences; Electrical Sciences; Infrastructure; Mechanical Sciences; Humanities, Social Sciences & Management may apply for this position. Consistently good academic record followed by a Ph.D. degree in the relevant fields in Engineering/Science/ Humanities. **Only those candidates, who have received the Ph. D. degree within the past two years, will be considered.**

Department of Biotechnology, IIT Roorkee-PDF position - The Department of Biotechnology at Indian Institute of Technology (IIT) Roorkee invites applications from outstanding and enthusiastic researchers for Pdf position under the mentor ship of Dr.Naveen K Navani (Associate Professor, Department of Biotechnology) focused on 'Development of Aptamer – Gold nano particles based system for detection of Toxic shock syndrome (TSS) Toxin-1'.

IISER Pune Postdoctoral Research Associate - Applications are invited for Postdoctoral Research Associate (PRAs) positions at the Indian Institute of Science Education and Research (IISER), Pune, India. These positions Are open for candidates with 0 5 years of experience after the submission of their PhD thesis. Candidates should provide evidence of having carried out high quality research. Appointments will be made for a year at a time with a maximum tenure of three years. Benefits include a monthly stipend of 40,000 INR (30,000 INR for those who have submitted their PhD thesis and have not yet been awarded a PhD), 20% house rent allowance (or housing provided by the institute) and a contingency grant of 1,00,000 INR per year.

Postdoctoral Opportunities at NCBS - NCBS has a small but vibrant program for researchers who have a PhD degree. We seek to enhance this program substantially. The purpose of the research program is to bring the best young scientists to a stimulating intellectual environment where the best facilities are available. We expect that this will result in excellent science and the opportunity for maturing to move on to independent positions in the best places in India and abroad. There are several funding modes for these postdoctoral programs. These programs do not lead to a continuing appointment and have a fixed maximum term. Applicants will be expected to identify a host laboratory and submit a preliminary application written in conjunction with the host faculty member.

Fulbright-Nehru Postdoctoral Research Fellowships - These fellowships are designed for Indian faculty and researchers who are in the early stages of their research careers in India. The Postdoctoral Research Fellowships will provide opportunities to talented faculty and researchers to strengthen their research capacities. Postdoctoral fellows will have access to some of the finest resources in their areas of interest and will help build long-term collaborative relationships with U.S. faculty and institutions. These fellowships are for eight to twenty-four months. Applications are invited in the following fields only: Agricultural Sciences; Applied Economics; Education Policy and Planning; Energy Studies; International Security and Strategic Studies; Public Health Policy and Management; Public Policy (limited to Science and Technology Policy); Science and Technology (limited to bioengineering, climate change sciences, computer and mathematical sciences, and neurosciences); Study of India (limited to language/literature/linguistics, history, and visual and performing arts); Study of the United States (limited to language/literature/ linguistics, history, and visual and performing arts); Urban and Regional Planning; Women's and Gender Studies.

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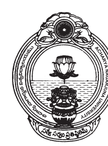

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MS in Pharmacy from USA

1st semester at Alliance - JNTUH in India and remaining courses & research in USA at the University of the Pacific, California, USA.

University of the Pacific, USA has entered into collaboration with JNTUH & Alliance Institute, India, for offering Masters (MS) program in Industrial Pharmaceutics. In this program students take courses in the first semester at Alliance-JNTUH and after successful completion of first semester at Alliance and fulfilling admission, TOEFL and visa requirements, students can go to USA to complete remaining courses and research at Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific. Upon successful completion of the requirements, University of the Pacific will award Master's degree.

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About Alliance: Alliance, located conveniently in the heart of Hyderabad, trains industry-ready graduates by bridging education with industry needs in pharmaceutical sciences. Alliance's visionary management built state of the art facilities and laboratories to provide quality education meeting national and international standards.

Collaboration with JNTUH, India: Alliance is having collaboration with **Jawaharlal Nehru Technological University, Hyderabad (JNTUH)**, which is a premier institution with academic and research–oriented programs, offered through the constituent and affiliated colleges. Alliance's syllabi, academic regulations and course structure are **approved by the JNTUH. JNTUH awards the degrees after fulfilling the degree requirements.**

Collaboration with University of the Pacific, USA: University of the Pacific, ranks in the top 100 among the 3000 national universities in the United States. Alliance has entered into research collaboration with Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific.

Alliance students have an option to do research work at the University of the Pacific to fulfill requirements for MS degree in India. Pacific faculty teaches Alliance students via live online classes. Pacific is also interested to offer admissions to Alliance students based on their performance at Alliance.

Programs offered :

* MS in Industrial Pharmaceutics
* MS in Pharmaceutical Analysis & Quality Control
* MS in Drug Development & Regulatory Affairs

For admissions, application forms and additional information visit online at
www.jntuh.ac.in/alliance or www.allianceinstitute.org.

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