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Genotypic Differences in Some Physiological and Biochemical Parameters Symptomatic for Nickel (Ni) Induced Stress in Groundnut (*Arachis hypogaea L.*)

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Abstract

An excess concentration of nickel (Ni) in soil significantly affects the growth and yields of crop plants. Physiological functions and antioxidative enzyme status of the plants are also altered by this stress. The present study aimed to evaluate nickel induced physiological and biochemical changes in a set of thirteen groundnut genotypes to identify the nickel tolerant genotype. Accumulation of free proline and the activities of Catalase (CAT) and Ascorbate Peroxidase (APX) significantly increased in Ni treated plants. Our results indicate that all the genotypes were differing in their growth parameters, concentrations of free proline, electrolyte leakage and antioxidative enzymes. Ni sensitive genotypes correlated with decrease in shoot: root ratio, increase in electrolyte leakage and decrease in antioxidative enzymes. In contrary tolerant genotypes showed better shoot: root ratios, high free proline content, less electrolyte leakage, optimum CAT and APX activities. The study leads in grouping the genotypes into three categories, i.e. tolerant genotypes- Abhaya, Anantha, Greeshma, ICGV-91114 and Dharani; moderately tolerant- K-1375, K-6 and TG-47 and sensitive genotypes- JL-24, Narayani, Prasuna, Rohini and TPT-4 for Ni induced stress.

Key words: Nickel (Ni), proline, Anti oxidative Enzymes – Catalase (CAT), Ascorbate Peroxidase (APX), Ion leakage.

Introduction

Heavy metals enter to the biosphere through natural weathering process (1). A number of the heavy metals are essential for growth and development of the plants. Limited quantities of heavy metals are non toxic to plants, but when exceeding them critical concentrations, these metals become more toxic to the plants. They enter and accumulate in various organs of the plants through the root system of plants (2, 3). Heavy metals affect the plants in two ways. Firstly, they alter plant metabolism by influencing the reaction rates and kinetic properties of enzymes. Secondly, they increase the generation of reactive oxygen species (ROS). In response to metal stress plant develop resistant mechanism to avoid or tolerance to the mental stress (4).

Nickel (Ni) is an essential micronutrient for plants in small quantities (5) and increased levels in soil lead to crop yield loss and human health hazards (6). Random urbanization and other human activities like mining, burning of coal, oil, phosphate fertilizers and pesticides have contributed to a significant increase of soil Ni

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concentrations (7, 8). The toxicity of this metal has been attributed to its negative effect on growth, photosynthesis, mineral nutrition, sugar transport, water relations, chlorosis, necrosis and wilting (9, 10, 11, 12). Seed germination is the first physiological process in the soil, but germination was highly affected by Ni and this metal was positively associated with proteins that inhibit the germination and chlorophyll production (13). Ni toxicity was reported to plants decreases shoot and root growth and reduction in leaf area (14) and also chlorophyll content (15,16, 17). Many other studies indicated that the toxicity of Ni is associated with oxidative stress in plants (10, 18, 19, 20).

In recent years, it was reported that most of the metal hyper accumulators are oil seed crops of the *Brassicaceae* family and have been studied significantly for phytoremediation of contaminated sites (21). Groundnut (*Arachis hypogaea* L.), is cultivated as an important food and cash crop in tropical, sub-tropical and warm temperate regions of the world. When comparing with the other crops groundnut has, the more advantage as it was reported to absorb metals from the contaminated soils through pods as well from the roots (22) and also a significant difference between the groundnut cultivars have been observed in the accumulation of metal (23). However, not much work was carried out on the consequences of Ni accumulation in groundnut.

Hence, it is of interest to study genotype variation in terms of the Physio-morphological and biochemical changes in local popular groundnut cultivars in response to different concentrations of Ni. The study was designed to investigate the impact of Ni on seedling growth, chlorophyll content and antioxidative enzymatic responses of the genotypes against different concentrations of Ni.

Materials and Methods

Pot experiment was conducted to accesses the genotypic variability of groundnut to Ni toxicity.

***Plant materials*:** Thirteen groundnut genotypes i.e., Abhaya, Anantha, Dharani, Greeshma (TCGS-888), ICGV-91114, JL-24, K-6, K-1375, Narayani, Prasuna (TCGS-341), Rohini (TCGS-913), TG-47 and TPT-4 were procured from the Regional Agricultural Research Station Tirupati 517502, Andhra Pradesh, India.

***Pot experiment*:** Pot experiment was conducted under greenhouse conditions at the Yogi Vemana University, Kadapa, India (Latitude 14°.47'N, Longitude 78°.71'E). Each pot was filled with 3kg of air dried soil and mixed with varied concentrations, 0.0 (Control), 100, 200, and 300 mg kg^{-1} soil) of Ni (*NiCl2.6H20).* The seeds of each and every genotype were surface-sterilized with 0.1% mercuric chloride and properly washed with distilled water and seedlings were raised in earthen pots in three replications. The treatment was continued for 30 days after sowing and measurements were taken on the 30th day across all genotypes and replications.

***Measurement of morphological characters*:** On the 30th day, all the plants were carefully harvested, gently washed to remove sand and other debris and spread on filter papers to remove surface moisture if any. All the phenotypic characters viz., root length (cm), shoot length (cm), total plant weight (g), total fresh root weight (g) and total fresh shoot weight (g) were measured immediately across all genotypes and treatments. Total root and shoot dry weight were recorded after exposing the samples to 60 °C for 72 h.

***SPAD (Soil Plant Analysis Development) Assay*:** SPAD Chlorophyll Meter Reading (SCMR) was recorded at 30 days after sowing by a Minolta handheld portable SCMR meter (SPAD-502, Konica Minolta, Japan) using four leaflets for a sample at 9.00-10.00 A.M across all groundnut genotypes and different Ni treatments.

***Proline assay*:** Total free proline was estimated following Bates *et al,* 1973 (24). Fresh leaves (100mg) were collected after 30 days and homogenized in 3ml of 3% sulfur-Salicylic acid and centrifuged at 12,000g at 4^0C for 15 min. Take 2 ml of supernatant and add 2 ml of acid Ninhydrine reagent (1.24 gm of Ninhydrin in a mixture of glacial acetic acid and 6M ortho-

phosphoric acid in 3:2 ratios) and glacial Acetic acid in 1:1:1ratio were added, the tubes were heated in a water bath at 100^0C for 1 hour and cooled on ice for 10 min. To the resulting mixture, 4 ml of toluene was added and incubated at room temperature for 30 min. The tubes were shaken for 15 Sec and allowed to stand for 10 min to separate the phases. The upper organic phase was separated and absorbance was measured at 520 nm using toluene as a blank.

Assay of antioxidant enzymes: Antioxidative enzymes were assayed by the method of Elavarthi and Martin, 2010 (25), a 200 mg of fresh leaf tissue was collected from heavy metal treated and control plants, ground to a fine powder in liquid nitrogen using a pre cooled mortar and pestle. The exact weight of each powdered sample was determined before it was thoroughly homogenized in 1.2 ml of 0.2M potassium phosphate buffer (pH 7.8 with 0.1mM EDTA) and samples were centrifuged at 15,000Xg for 20 min at 4ºC and the supernatant was removed and the pellet was resuspended in 0.8ml of the same buffer, and the suspension centrifuged for another 15 min at 15,000Xg. The combined supernatant was stored on ice and used to determine following antioxidative enzymes.

Catalase Assay (CAT): Catalase assay was determined according to Aebi and Lester, 1984 (26). The decomposition of H202 was followed as a decrease in absorbance at 240nm in a UV/Vis spectrophotometer. The 3 ml assay mixture contained 2 ml leaf extract (Diluted 200 times with 50 mM potassium phosphate buffer, pH 7.0) and 1 ml of 10 mM H202 .The extinction coefficient of H202 (40 mM^{-1} cm^{-1}at 240nm) was used to calculate the enzyme activity that was expressed in terms of milli moles of H202 per minute per gram fresh weight.

Ascorbate peroxidase Assay (APX): APX activity was assayed using a modified method of Nakano and Asada, 1981 (27). APX activity was determined from the decrease in absorbance at 290nm due to oxidation of Ascorbate in the reaction. The 1 ml assay mixture contained 50mM potassium phosphate buffer (pH 7.0), 0.5mM Ascorbate, 0.5 mM H2O2 and 10 µL of crude leaf extract. H2O2 was added last to initiate the reaction, and the decrease in absorbance was recorded every 30 seconds for 3 min. The extinction coefficient of 2.8 mM^{-1} cm^{-1} for reduced Ascorbate was used in calculating the enzyme activity that was expressed in terms of milli mole of Ascorbate per minute gram fresh weight.

Measurement of electrolyte leakage: Cell damage was assayed by measuring electrolyte leakage by the method of García-Marcos *et al,* 2013 (28). Twenty-five disks of 0.3 cm^2 were excised from upper leaf tissue using a cork borer. Disks were rinsed briefly with water and floated on 5 ml of double distilled water for 6 h at room temperature. The conductivity of the water was measured using a Crison conductivity meter. This represented the electrolyte leakage from the leaf discs (reading 1). Then, the samples were boiled for 20 min at 90°C. After the liquid cooled down, the conductivity of the water was measured again. This represented the total ions present in the leaf discs (reading 2). Electrolyte leakage was represented as the percentage of the total ions released [(reading 1/reading 2) × 100].

***Statistical analysis*:** The data were processed by analysis of variance (ANOVA) and Pearson correlation using the Software IBM SPSS Statistics v. 2.0.

Results and Discussion

Nickel chloride concentration showed significant effect on development of root and shoot across all groundnut genotypes (Fig. 1). Root length and weights (fresh and dry) were decreased with increased concentrations of Ni in all genotypes (Fig. 2 and 3). Genotypes significantly differed in their root morphology to Ni toxicity (Fig. 2). Greeshma had the highest root length (Fig. 2C) (22.3±1.52, 16.8±1.31, 10.6±1.15 and 5.3±0.57 cm respectively) and root fresh weight (Fig. 2A) (0.62±0.11, 0.53±0.03, 0.36±0.02 and 0.18±0.02 gm/plant) at all concentrations of Ni. In contrary, root dry weight (Fig. 2B) is more in the genotype Abhaya (0.44±0.05, 0.37±0.03,

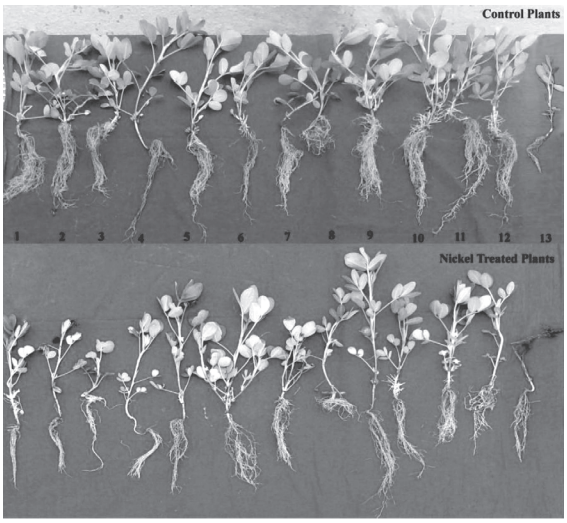

Fig. 1. Effect of Nickel (100 mg kg^{-1}) on groundnut genotypes 1) Abhaya 2) Anantha 3) Dharani 4) Greeshma (TCGS- 888) 5) ICGV-91114 6) JL-24 7) K-6 8) K-1375 9) Narayani 10) Prasuna (TCGS-341) 11) Rohini (TCGS-913) 12) TG-47 and 13) TPT-4.

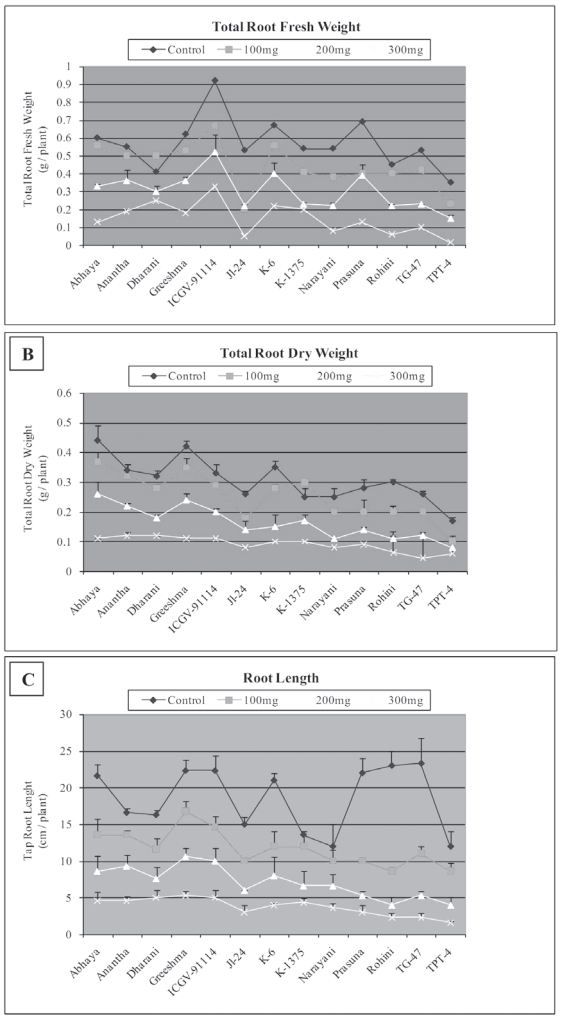

Fig. 2. Effect of Ni on root biomass and morphology of different groundnut genotypes grown in different concentrations (0.0, 100, 200 and 300 mg kg^{-1} soil) of Nickel ($NiCl_2.6H2O$). (A) Root fresh weight (g $Plant^1$), (B) Root dry weight (g $Plant^1$), and (C) Root length (Cm) (C). Error bars indicate ±SD.

0.26±0.04 and0. 11±0.00). Shoot length and biomass decreased with Ni concentration (Fig. 3). Greeshma genotype showed better shoot length (Fig. 2C) (32.6±2.08, 22.3±2.51, 17.5±2.5 and 12±0.2 cm); fresh weight (Fig. 3A) (3±0.01, 2.73±0.1, 1.99±0.03, and 1.19±0.03 gm/plant) when compared with the other genotypes. In overall genotypes Dharani, Abhya, Anantha and Greeshma showed better performance when compared with the other genotypes studied at elevated concentrations of Ni. It is generally observed that heavy metal stresses usually reduce the shoot and root Growth (29). Similar results were observed in onion (30); soybean seedlings (31) and *Zea mays* seedlings (32). The Inhibition of growth and morphogenesis might be due to the decreased plasticity of cell walls, which resulted from cell wall lignifications, and hindered mitosis (9). Variation in growth parameters among the genotypes against Ni stress showed clear genetic variability among the diverse genotypes of groundnut studied. A positive correlation was observed between the root: shoot ratio of the groundnut genotypes. However, Greeshma showed optimum root length and biomass, Abhaya performed well in terms of shoot parameters. There might be different tolerance mechanisms exist in these genotypes. The results obtained in this study are similar to the results of

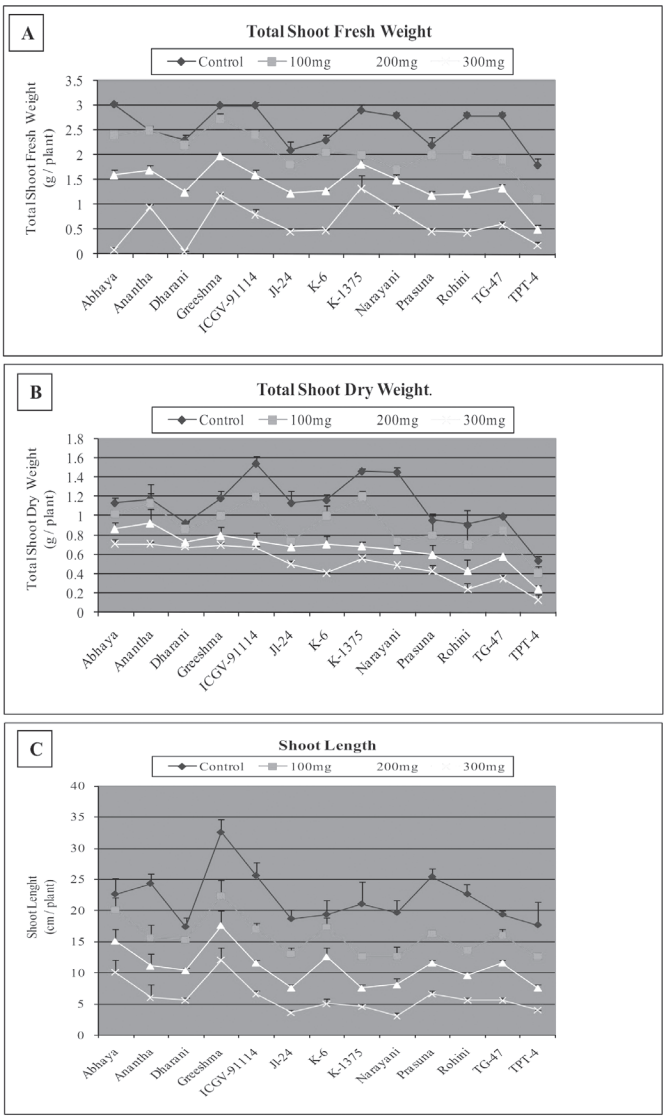

Fig. 3. Effect of Ni on shoot biomass and morphology of different groundnut genotypes grown in different concentrations (0.0, 100, 200 and 300 mg kg^{-1} soil) of Nickel ($NiCl_2$.6H2O). (A) Shoot fresh weight (g $Plant^{1}$), (B) shoot dry weight (g $Plant^{1}$), and (C) shoot length (Cm) (C). Error bars indicate ±SD.

different legume species showing intraspecific genetic variation in the tolerance to heavy metals, for instance Zn (33), Cu (34) Mn (35) and Cd (36).

SPAD values which are the indicators of chlorophyll content were determined on 30th DAS (Days After Sowing). Chlorophyll content is often measured in plants in order to assess the impact of environmental stress, as changes in pigment content are linked to visual symptoms of plant illness and photosynthetic productivity (37). Researchers have reported decreased Chlorophyll in several different plant species under the impact of heavy metals. There were significant differences among genotypes for SPAD values. The increase in Ni concentration led to a decrease in SPAD value (Fig. 4). A high SPAD value upon exposure of Ni was observed in ICGV-91114 followed by Dharani, while TPT-4 followed by Rohini had the lowest. The observed decrease of SPAD values in Ni stressed plants are accordance with the results of Dong *et al,* 2005 (38). It was reported that the decrease in chlorophyll content may potentially inhibit photosynthesis (15, 16, 17). But in our present results there was a positive correlation between the amount of chlorophyll retained by that of the biomass accumulation across the genotypes and treatments.

The effect of Ni on accumulation of proline content was shown in Fig. 5. Proline content was high in Ni treated plants. Decrease in proline content might be due to the decrease in mitochondrial electron transport activity and increase in proline *de novo* synthesis or decrease degradation under environmental stress conditions (39, 40). In addition, the comparison

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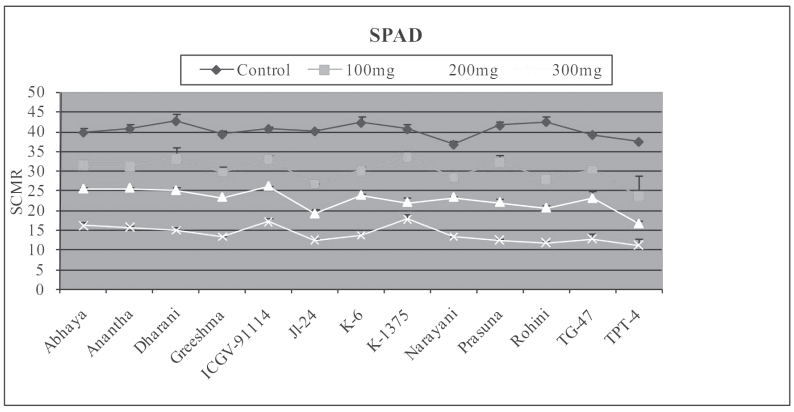

Fig. 4. Effect of Ni on SPAD values (Chlorophyll content) of different groundnut genotypes grown in different concentrations (0.0, 100, 200 and 300 mg kg^{-1} soil) of Nickel ($NiCl_2$.6H2O). Error bars indicate ±SD.

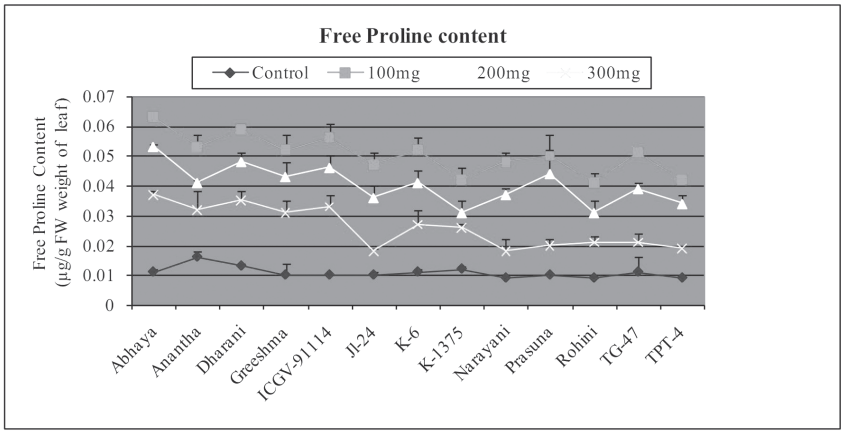

Fig. 5. Free Proline content in different groundnut genotypes grown in various concentrations (0.0, 100, 200 and 300 mg kg^{-1} soil) of Nickel ($NiCl_2$.6H2O). Error bars indicate ±SD.

of different genotypes revealed that maximum proline accumulation was observed in the genotypes Abhaya followed by Dharani. Similar results of increasing proline content by Cd^+ was also reported by Zengin, and Munzuroglu, 2006 (41) in sunflower. Similar results were observed in cabbage leaves when exposed Co, Ni and Cd Pandey and Sharma, 2002 (11) suggesting an association with the changed water status of the treated plants. Proline plays a major role in the heavy metal detoxification process and antioxidant defense mechanism during heavy metal stress (42, 43).

Like any abiotic stress Ni stress also induces the production of highly cytotoxic species of oxygen (ROS) which further leads to the peroxidation of membrane lipids and affect the functional and structural integrity of biological membranes (18). Membrane damage increases the permeability of leakage of potassium ions and other solutes (44). In this present study membrane injuries were measured by monitoring electrolyte leakage (Fig. 6). Electrolyte leakage was more in the Ni stressed plants compared with control plants. The optimum level of ion leakage (Fig. 7) which indicated the production of more

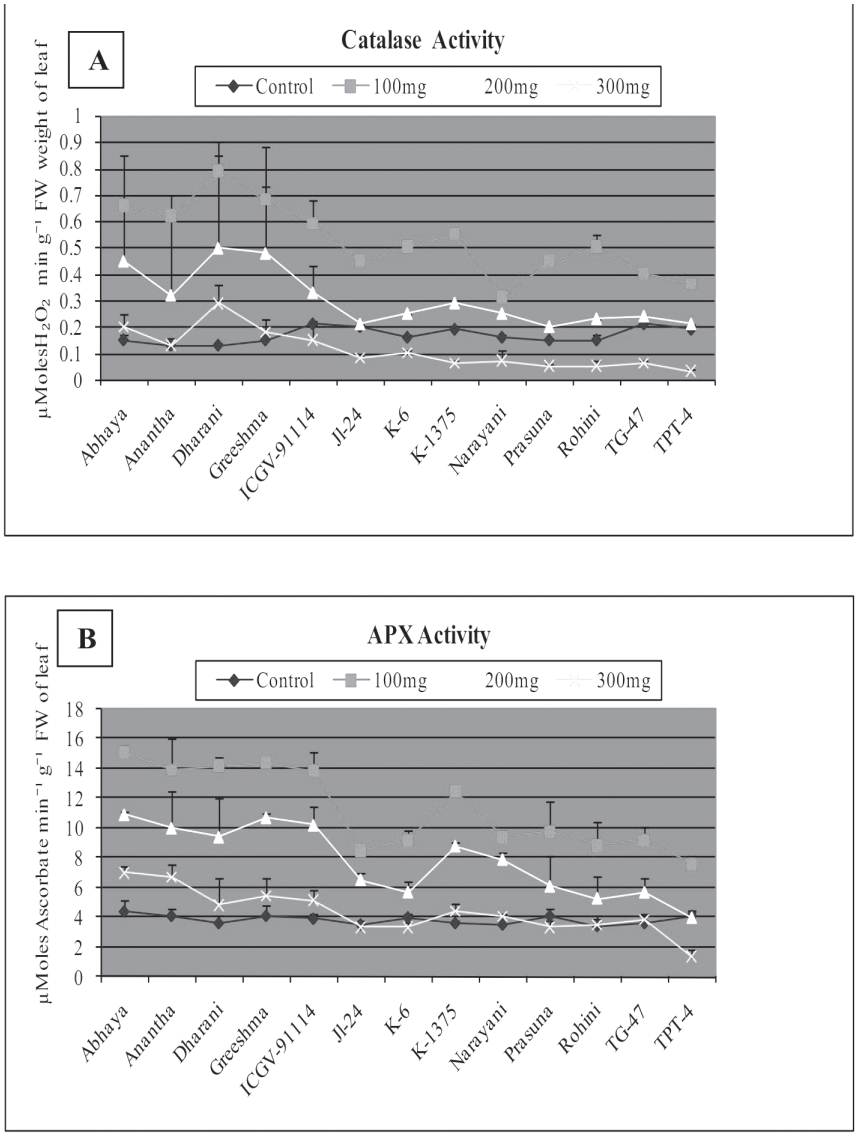

Fig. 6. Activities of antioxidants enzymes in different groundnut genotypes grown in various concentrations (0.0, 100, 200 and 300 mg kg^{-1} soil) of Nickel ($NiCl_2$.6H2O). (A) Catalase (CAT) (µMolesH, O, min g^{-1} FW weight of leaf) and (B) Ascorbate Peroxidase (APX) µMoles Ascorbate min^{-1} g^{-1} FW of leaf. Error bars indicate ±SD.

ROS was observed in the genotype TPT-4, whilst Abhaya observed with less ion leakage when compared with other genotypes. A similar trend was observed in soybean genotypes exposed to Cd stress (45).

The induction of a particular group of enzyme activities is considered to play an important role in the cellular defense strategy against oxidative stress caused by toxic metal concentrations (46). CAT and APX are among the major antioxidant enzymes involved in

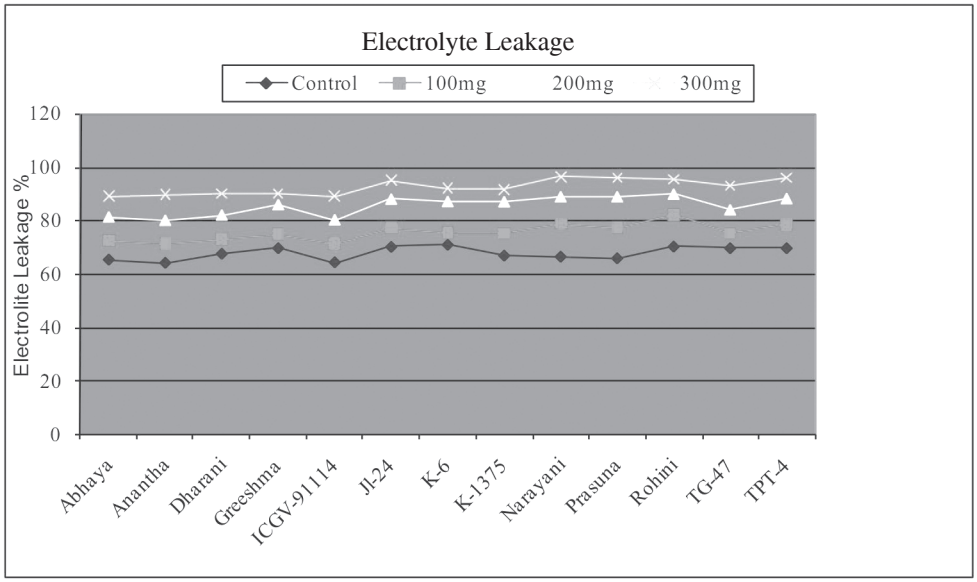

Fig. 7. Electrolyte Leakage (%) which indicates the cell membrane damage in groundnut genotypes grown in various concentrations (0.0, 100, 200 and 300 mg kg^{-1} soil) of Nickel ($NiCl_2$.6H2O). Error bars indicate ±SD.

scavenging ROS. There was a significant increase in the concentrations of antioxidative enzyme activities were observed in the groundnut genotypes treated with Ni. Effect of enzyme activities due to Ni stress can be seen in the Fig. 5. The enzyme catalase activity was increased in the Ni treated plants compared with control plants (Fig. 6A). The CAT enzyme activity was optimized in 100 mg kg^{-1} Ni treated plants. However, the activity was decreased in 200 mg kg^{-1}, 300 mg kg^{-1}. Earlier such decrease in CAT activity due to excess heavy metal was reported in cauliflower (47). The maximum activity was observed in the genotype Dharani followed by Greeshma. A pattern similar to CAT was also observed in the APX activity in different genotypes. APX activity significantly increased 100 mg kg^{-1} Ni treated plants (Fig. 6B). The optimum APX levels were observed in the genotype Abhaya, Anantha and Dharani. A similar trend was observed in a Ni hyperaccumulator plant Thlaspi (48); pea (19) and groundnut (49).

The antioxidative enzyme activities are in correlation with ion leakage and morphological parameters strongly suggest that antioxidative enzymes playing a major role tolerant genotype to cope up with Ni induced stress. Based on present study thirteen groundnut genotypes can be categorized as follows; Abhaya, Anantha, Greeshma, ICGV-91114 and Dharani as tolerant; K-1375, K-6 and TG-47 as moderately tolerant and genotypes JL-24, Narayani, Prasuna, Rohini and TPT-4 as sensitive genotypes for Ni induced stress.

Conclusion

In the present study, significant variation across genotypes of groundnut and across treatments with in genotype, in terms of Physio-morphological and biochemical variation when expose to elevated levels of Ni was found, suggesting diverse mechanisms contribute to the specific Ni tolerant levels across genotypes. There is a strong correlation between the accumulation of free proline, CAT, APX activity, electrolyte leakage and growth parameters. The varieties that were able to maintain elevated levels of chlorophyll, CAT and APX activity are more or less the ones which were able to accumulate higher

amounts of biomass (both shoot and root). The existence of genetic differences in groundnut genotypes in Ni tolerance indicates the possibility of developing the reasonable groundnut cultivars suitable for cultivation in Ni contaminated soils.

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KIR gene frequencies and haplotype in the selected population in Andhra Pradesh (India)

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Abstract

Under the phenomenon of allogenicity, a few sets of alleles that come under the domain of human immune function namely KIRs (Killer immunoglobulin-like receptor genes) and HLA-I were chosen to report from the population of Puttaparthi (India). The PCR protocols for the identified immune related genes viz.., KIR- 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 3DS1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 2DP1, 3DP1; HLA- C1 and HLA- C2 have been standardized. Online Statistical tool namely Fisher's exact test (in-silico.net/tools/statistics/chi2test) was employed to evaluate the association between pairs of KIR loci. Interestingly, majority of the activating KIR genes were least represented and varied from 5% to 32.5%. This observation is in compliance with the survival adaptation of the chosen population. The carrier gene frequencies of KIRs were compared with the other populations' viz., Chinese Mongolian, Chinese Han, Greek and Brazilian data. The expected heterozygosity of KIR alleles and their rank in gene diversity among the population of Puttaparthi were also discussed. The significant positive association between pairs of KIR loci and consequent linkage disequilibrium and haplotype analysis favored the presence of AB genotype with 12 genes in the chosen population from Puttaparthi (India).

Key words: KIR, Genotype, Haplotype, Heterozygosity

Introduction

Biological species are bestowed with a wide variety of salient features and inhabited myriad habitats in all the varied ecosystems. With the advent of molecular tools and diagnostic techniques, the approach in the evaluation of genetic polymorphism is focused on the individual species (1, 2). Particularly so with regard to humans due to their significance in ethnic relevance and exposed to a variety of environmental cues and stress (biological, emotional and physical) and cross migration across continents (3).

The KIR genes are localized on chromosome 19q13.4 and preferentially expressed by NK cells. KIRs constitute a large gene family comprising of a total of 16 (4, 5). Out of them, 7 genes encode KIR receptor transmembrane glycoproteins that are inhibitory in function and favor prevention of target cell lysis upon recognizing self HLA class I molecules on the target cells, 6 genes encode KIRs that are activation in function which upon detection of missing-self (HLA Class I) on the target cell execute the lytic function (6). The remaining two are pesudogenes. KIR 2DL4 does both inhibitory and activation functions. Extensive diversity has been reported in the distribution of inhibitory KIRs and their HLA C ligands (7). In a study involving 30 geographically distributed populations, Richard, et al., (1) demonstrated that there is a profound global diversity in the distribution of KIR

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genes and their ligands. Rajalingam (8) deduced through genotyping analysis that at least one inhibitory KIR is necessary for the survival of an individual.

The KIR gene family is one of the dense gene clusters spanning over 1 Mb within the leukocyte receptor complex (LRC) whose transcripts display characteristic Ig-like extra cellular transmembrane receptor domains. KIR loci spread over the span of 4 to 16 kb and represent 4 to 9 exons at least with one each inhibitory and activatory exons. KIR gene loci are distributed in both *A* and *B* group haplotypes, characteristically with non-variable and more variable exons respectively and the population is either designated as AA, BB or AB KIR genotypes. The two framework genes KIR3DL3 and KIR3DL2 are terminal alleles located at centromeric and telomeric ends at KIR loci within LRC respectively. The studies relating to the distribution of KIR genes and its haplotypes in the selected human population is of current interest in the context of immune surveillance. 23 dissimilar KIR gene patterns are reported in 21 Caucasoid families and a profound diversification is noticed among group *B* KIR haplotypes (9). Among northeast Asians and north Indians, groups *A* and *B* haplotypes distribution is noticed. Conversely, even the natives of Australia and America are reported to have the predominant distribution of *B* haplotypes (10).

While paying attention to the above salient features, it is envisaged to genotype a few immunogenetic genes which would be useful as markers to forecast any association between their presence/absence and the disease susceptibility of individuals of the population of Puttaparthi region situated in the District of Anantapur, Andhra Pradesh, India, primarily because of the reason that the population is highly conserved i.e., having marriages within the same community and most of them are agricultural workers.

Material and Methods

Authors sought the approval from the Institutional Ethics Committee for carrying out the work reported in the present article. The blood samples from healthy individuals (48 human subjects) were collected in Sri Sathya Sai General Hospital, Puttaparthi blotted on filter paper marked to the size of one rupee coin (IsoCode paper from Schleicher and Schuell) and soon after drying the same were mounted on sheets possessing the details of subjects. The present study was executed during the months April 2009 to March 2010 at the Department of Biosciences, Sri Sathya Sai Institute of Higher Learning, Prasanthi Nilayam and part of the experimental work was carried out at the Department of Immunogenetics, Karolinska Institute of Medical Sciences, Stockholm. The genomic DNA was extracted from the dried blood spots using Q1 A amp DNA Micro kit and obtained a good yield of about 9-10µg/100µl elution buffer. PCR reaction was performed using the sequence specific primers (Table 1) for all the KIR genes and the chosen HLA class I ligand genes. The PCR products were run through 1.5 % agarose gel electrophoresis with ethidium bromide as the stain and genes were visualized using gel doc (Vilber Lourmat). The data was analyzed to derive the values for observed and expected variations among KIR genes. Observed carrier frequencies (OF) of KIR genes were evaluated by simple empirical method *viz.*, subjects positive for the particular gene/total size of the sample population. Estimated gene frequencies (GF) of KIRs were calculated using the Berstein's formula *viz.*, GF= 1-("1-OF)$^{1/2}$ (11). Yet another test of variation adopted for the chosen population was the expected (Hardy–Weinberg) heterozygosity (H_E) = (2N/2N-1) (1-"OF^2) and the same was shown appropriate for small sample size (N<50) (12). 2 X 2 contingency table generated through Fisher's exact test as shown below is employed to calculate the association between pairs of KIR loci using online statistical tool: in-silico.net/tools/statistics/chi2test to evaluate the parameters of positive linkage disequilibrium and KIR haplotype.

Table 1: Primer sequences of KIR and HLA C1 and C2 genes employed in the present investigation.

Gene	Forward Primer		Reverse Primer		Amplicon size in base pairs (bp)
KIR	Name	Sequence 5'-3'	Name	Sequence 5'-3'	genes
2DL1	2DL1F	CCATCAGTCGCA TGACG	2DL1R1	CCACTCGTATGGAGAGTCAT	1,903
			2DL1R2	AATGTTCCGTTGACCTTGGT	1,818
2DL2	2DL2F2	AAACCTTCTCTCTCAGCCCA	2DL2R2	GCCCTGCAGAGAACCTACA	1877
2DL3	2DL3F3	CTTCATCGCTGGTGCTG	2DL3R1	CAGGAGACAACTTTGGATCA	816
2DL4	2DL4F1	CTGCATGCTGTGATTAGGTA	2DL4R1	CTGTTGAGGGTCTCTTGCT	695
2DL5	2DL5F	TGCCTCGAGGAGGACAT	2DL5R1	TCATAGGGTGAGTCATGGAG	1,151
3DL1	3DL1F1	AT(C/T)GGTCCCATGATGCT	3DL1R1	CTGAGAGAGAAGGTTTCTCATATG	1,661
3DL2	3DL2F1	TGCAGGAACCTACAGATGTTAT	3DL2R1	CTTGAGTTTGACCACACGC	1,882
3DL3	3DL3F1	CACTGTGGTGTCTGAAGGAC	3DL3R1	TCTCTGTGCAGAAGGAAGC	1,905
3DS1	3DS1F	GGCAGAATATTCCAGGAGG	3DS1R1	GGCACGCATCATGGA	1,847
2DS1	2DS1F1	2DS1F2CTCCATCAGTCGCA			1,922
		TGAGCTCCATCAGTCGCATGAA	2DS1R	AGGGCCCAGAGGAAAGTT	1,897
2DS2	2DS2F	TGCACAGAGAGGGGAAGTA	2DS2R1	CGCTCTCTCCTGCCAA	1,781
2DS3	2DS3F	TCACTCCCCCTATCAGTTT	2DS3R	GCATCTGTAGGTTCCTCCT	1,812
2DS4	2DS4F1	TCCTGCAATGTTGGTCG	2DS4R1	ACGGAAACAAGCAGTGGA	2,050
2DS4-full	2DS4F1	TCCTGCAATGTTGGTCG	2DS4 fullR1	CCCTCCCTGGATAGATGGTAC	1,956
2DS4 -del	2DS4F1	TCCTGCAATGTTGGTCG	2DS4delR	TTCCCTGGATAGATGGAGCTG	1,933
2DS5	2DS5F	AGAGAGGGGACGTTTAACC	2DS5R2		
			DS5RD	GGAAAGAGCCGAAGCATCCAGAG	1,952
				GGTCACTGGGC	180
2DP1	2DP1F	TCTGTTACTCACTCCCCCA	2DP1R	GGAAAGAGCCGAAGCATC	1,825
3DP1	3DP1F1	AGAGTATTCCGAAACACCG	3DP1R1	CTGACAACTGATAGGGGGAA	1,900
		KIR binding HLA-C allotypes			
HLA-C1	HLACN80f	CGA GTG AGC CTG CGG AAC	HLACr	GC CCA CTT CTG GAA GGT TCC	1,344
HLA-C2	HLACK80f	C CGA GTG AAC CTG CGG AAA	HLACr	GC CCA CTT CTG GAA GGT TCC	1,344
		Positive internal control (PIC)			
IC256	PIC-F	ATGATGTTGACCTTTCCAGGG	PIC-R	ATTGTGTAACTTTTCATCAGTTGC	256

Allele *j* \ Allele *i*	p	a
p	pp	pa
a	ap	aa

p: presence and a: absence, pp, pa, ap and aa: positive integers.

Results and Discussion

The presence and/or absence of KIR genes revealed through the PCR analysis and further through agarose gel electrophoresis (Plates 1, 2, 3 and 4) were noted down. The presence of framework genes viz., KIR 2DL4, 3DL2 and 3DL3 were first reviewed. Out of 48 subjects, 8 showed the absence of the same and hence these eight

subjects were not included in the final percentage analysis. Interestingly, 2DL2, 2DS5 and HLA C1 were reported to be absent in all the 40 samples (Table 2). The survey of literature revealed that the presence of KIR 3DS1 with its ligand has been shown to confer the highest risk in the development of cervical neoplasia (13). However, the involvement of a gradient has been postulated with activating genotypes conferring the maximum risk. In hepatitis C virus infection, the presence of KIR 2DL3 and its ligand HLA C1 conferred protection (14). A positive association of KIR 2DL2, 2DL5, 2DS2, 2DS3 and 3DS1 with Type 1 diabetes was shown (14-16). In the present study, except KIR 2DL2, the remaining inhibitory KIR genes were represented at higher percentage (Table 2) in the chosen population and the same possibly suggests the higher survival adaptation of the chosen population. Interestingly among the activating KIR genes, majority of them were least represented (Table 2) which also is in compliance with survival adaptation of the chosen population. A study based on rheumatoid arthritis also exhibited a unique characteristic feature of

Table 2: Observed carrier frequencies (OF), estimated gene frequencies (GF) and expected heterozygosity (H_E) of KIR genes derived from the chosen population of Puttaparthi.

Gene	No. of subjects having respective amplified genes out of a total of 40	OF	GF	H_E	Rank among KIR gene diversity
Inhibitory KIRs					
2DL1	23	0.575	0.349	0.678	5
2DL2	0	0	0	0	
2DL3	32	0.80	0.553	0.364	9
2DL4	40	1	1	0	
2DL5	24	0.600	0.368	0.647	6
3DL1	31	0.775	0.526	0.404	8
3DL2	40	1	1	0	
3DL3	40	1	1	0	
Activatory KIRs					
2DS1	9	0.225	0.120	0.961	3
2DS2	3	0.075	0.039	1.006	2
2DS3	2	0.050	0.026	1.009	1
2DS4	13	0.325	0.179	0.905	4
2DS5	0	0	0	0	
2DS5 RD	30	0.750	0.5	0.442	7
3DS1	13	0.325	0.179	0.905	4
KIR Ligand HLA					
-C1	0	0	0	0	
-C2	22	0.55	0.33	0.705	

the expression of KIR 2DS2 (17). Furthermore, KIR 2DS2 in the absence of its inhibitory counterpart 2DL2 was found to be associated with susceptibility to scleroderma (18, 19). 2DS1 and / or 2DS2 in the absence of HLA class I ligands was reported to be a major susceptibility factor in psoriatic arthritis (20, 21). Therefore, there is an enormous potential to unravel the association of KIR genes with the disease-states. In the present investigation, the inhibitory KIRs were shown to be present in their maximum percentage in the population while compared to activation KIRs (Table 2). Yet, another interesting observation is that HLA C1 which is a ligand for inhibitory KIRs was not represented at all in the subjects investigated (Table 2), whereas HLA C2 represented reasonably well. These findings suggest that the chosen subjects of Puttaparthi are unique with reference to the genotypes of KIR genes and their ligands and future studies associating the same with the endemic diseases will further pave the way to earmark them as genotype markers.

KIR gene frequencies and their comparison with other populations: Till date, 16 KIR genes and pseudogenes have been reported in various ethnic groups. Interestingly, the present PCR SSP data on KIR gene polymorphism is for the first time reported from the region of Puttaparthi (AP, India) and the calculated observed carrier frequencies (OF), estimated gene frequencies (GF) and expected (Hardy-Weinberg) heterozygosity (H_E) values are given in Table 2. The derived data on KIR genes revealed that KIR 2DL2 and 2DS5 are not represented within this population and the same inquisitively is not in compliance with the data of a few populations given in Table 3 wherein it is shown that the occurrence of the frequencies of these two genes are heterogeneous. The ligand for KIR 2DL2 receptor is HLA C1 antigen and that is also not represented in the present chosen population (Table 2). The OF of KIR 2DL3 and 3DS1 in the population of Puttaparthi were comparable with the other populations' *viz*., Chinese Mongolian (9), Chinese Han (22), Greek (23) and Brazilian (24) data as shown in Table-3. The remaining KIRs *viz*., 2DL1, 3DL1, 2DS1, 2DS2, 2DS3 and 2DS4 are all represented at lower frequencies in the present study on comparison with the aforementioned populations (Table 3). The gene frequencies of non-framework inhibitory KIRs ranged from 57% to 80% within the study population with an exception of 2DL2 having mixed representation, whereas the activation genes appeared at a lower range varying from 5.0% to 32.5% compared to inhibitory KIRs. The derived values of H_E and their corresponding rank among KIR gene diversity revealed that the two non-framework inhibitory KIRs namely 2DL3 and 3DL1 scored respectively the highest ranks (Table 2) upon comparison with others in the study population which possibly facilitates to recognize its ligands so as to ignore the self-corpuscles. In addition, frequencies of these two inhibitory KIRs are more profoundly represented in all the four populations (Table 3), which are considered for comparison in the present study. The positive association between the pairs of KIR loci and linkage disequilibrium analysis (Fig.1) favored the prevalence of KIR AB genotype with 9 genes (Table 4) in the chosen population from Puttaparthi (India) representing both by predominant inhibitory and activator alleles. Immunological attribute of KIRs envisages that they bind to specific motifs on the self HLA class 1 molecules and further inhibit the lysis of host's healthy corpuscles and thus prevent host's cells from lysis (6). Whereas, bacterial pathogens do not have HLA molecules and malignant cells in their metastasis stage do have missed-self molecules and they are the targets of NK cells provided that NK cells are endowed with full complement of KIRs and hence the evaluation of polymorphism among KIR genes is important to diagnose the vulnerability of a population to a set of diseases (6). Thus, the study of KIR gene polymorphism in the representative groups of Indian population, their inheritance patterns and their possible association with diseases would give us an insight for the development of prophylactic strategies and to enrich Indian ethnical gene resources.

Table 3: Observed carrier frequencies of KIR genes of different populations compared with the present experimental data.

Gene	Observed carrier frequencies (OF) of KIR genes represented in %				
	Puttaparthi (Present study)	Chinese Mongolian Population (9)	Chinese Han Population(20)	Greek Population(21)	Brazilian Population(22)
Inhibitory KIRs					
2DL1	57.0	84.0	99.0	89.0	97.4
2DL2	0	16.0	17.3	50.0	59.4
2DL3	80.0	89.0	99.0	88.0	84.6
2DL4	100.0	100.0	100.0	100.0	100.0
2DL5	60.0	48.0	(A)30.8	NR	51.9
3DL1	77.5	93.0	94.2	90.0	92.7
3DL2	100.0	100.0	100.0	100.0	100.0
3DL3	100.0	100.0	100.0	NR	100.0
Activatory KIRs					
2DS1	22.5	30.0	33.7	43.0	42.1
2DS2	7.5	20.0	17.3	54.0	59.2
2DS3	5.0	11.0	12.5	37.0	32.5
2DS4	32.5	49.0	80.7	88.0	93.6
2DS5	0	40.0	23	21.0	31.9
2DS5 RD	75.0	NR	NR	NR	NR
3DS1	32.5	48.0	32.8	46.0	37.3

NR: not reported

KIR haplotype and KIR genotype: Pairs of alleles (2DL5-2DS5RD; 2DL5-3DS1; 2DS1-3DS1 and 2DS4-3DS1) showing significant positive associations (Table 4) inevitably represent *B*-group KIR haplotype, another pair (2DL1-2DS4) represents *A*-group and yet another pair (2DL3-2DS3) represents the presence of both *AB* group. The framework genes 3DL3, 2DL4 and 3DL2 are uniformly represented in all individual samples genotyped. The significant positive association among 7 pairs of KIR alleles (Table 4) revealed that other than the allele 2DS4, none of the other pairs of non-framework alleles repeated in both haplotypes. To confirm the presence of *A*-group haplotype in the chosen population, there is a significant positive association between alleles 2DL1-2DS4. Whereas, the other 4-pairs of alleles *viz*., 2DL5-2DS5RD; 2DL5-3DS1; 2DS1-3DS1 and 2DS4-3DS1 with significant positive association confirmed the presence of *B*-group haplotype. Ultimately, the positive association between 2DL3 and 2DS3 confirms the presence of both KIR groups *AB* in the population of Puttaparthi. Uniquely, the present data reveal the absence of 2DL2 and 2DS5 alleles. Thus, the population of Puttaparthi is reported to be heterozygous for KIR haplotypes and represent AB- genotype with 12 genes including 3 framework genes (3DL3, 2DL4 and 3DL2). The reported *AB* hetero-haplotype is endowed with 5

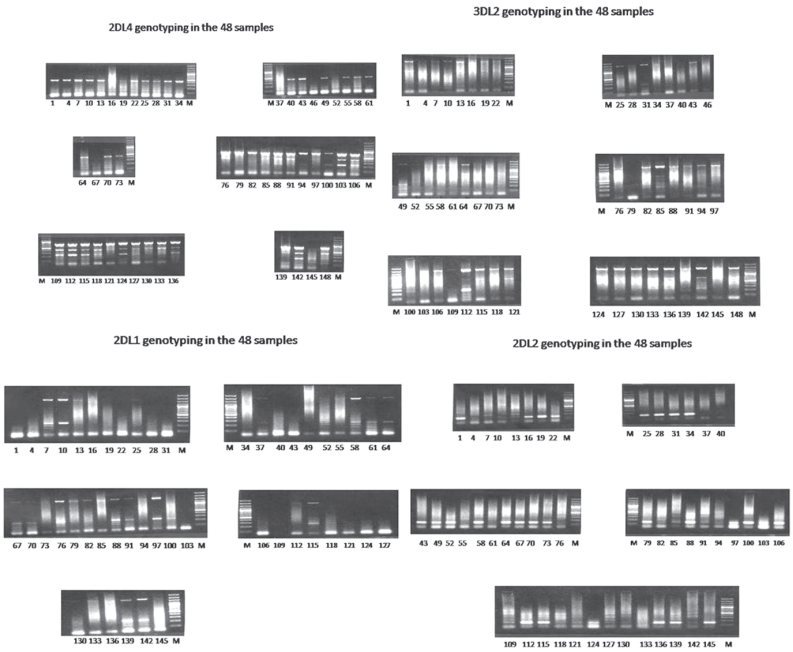

Plate-1: PCR amplification and genotyping of KIR 2DL4, 3DL2, 2DL1 and 2DL2 genes are shown in 48 subjects of the mixed ethnic groups.

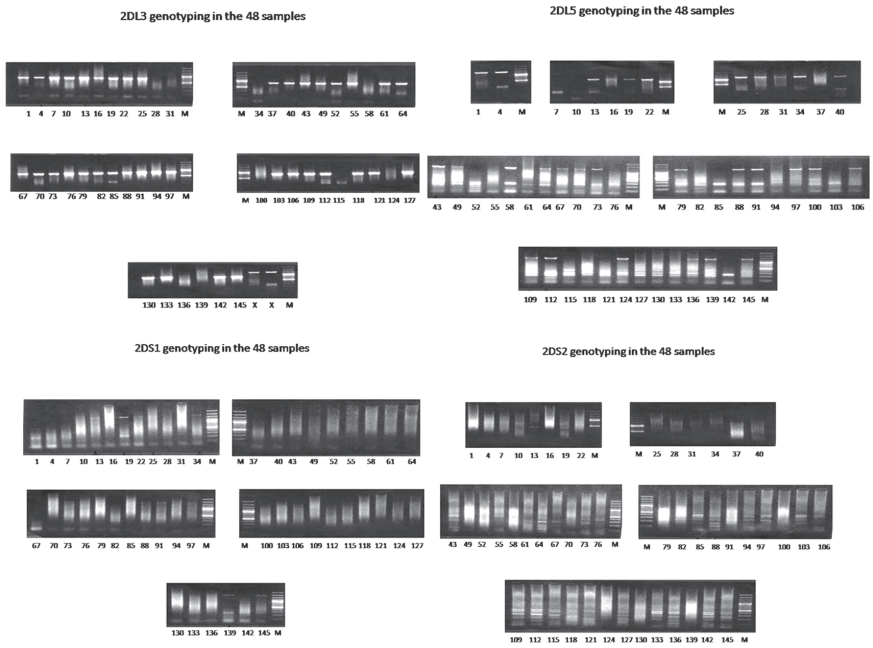

Plate-2: PCR amplification and genotyping of KIR 2DL3, 2DL5, 2DS1 and 2DS2 genes are shown in 48 subjects of the mixed ethnic groups.

KIR genotyping

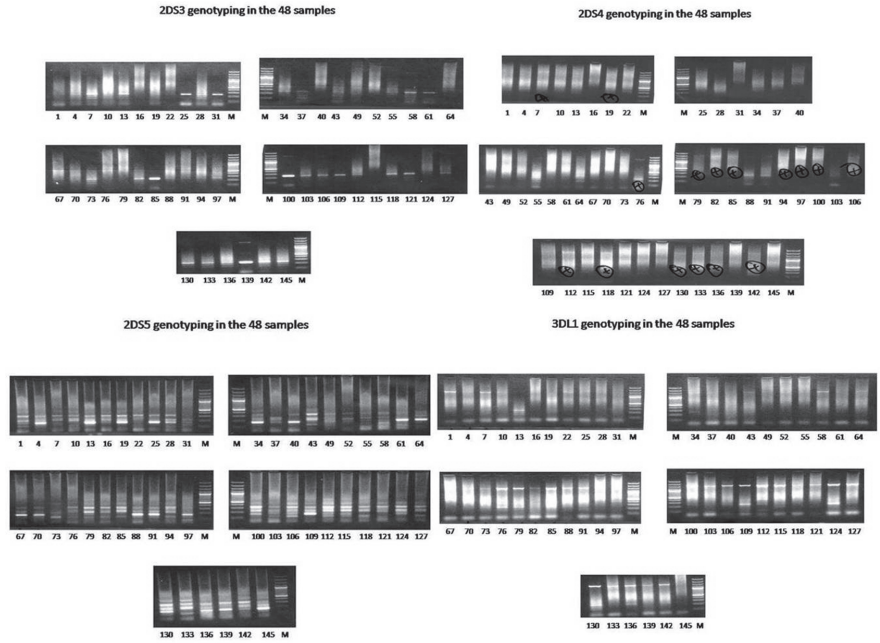

Plate-3: PCR amplification and genotyping of KIR 2DS3, 2DS4, 2DS5 and 3DL1 genes are shown in 48 subjects of the mixed ethnic groups.

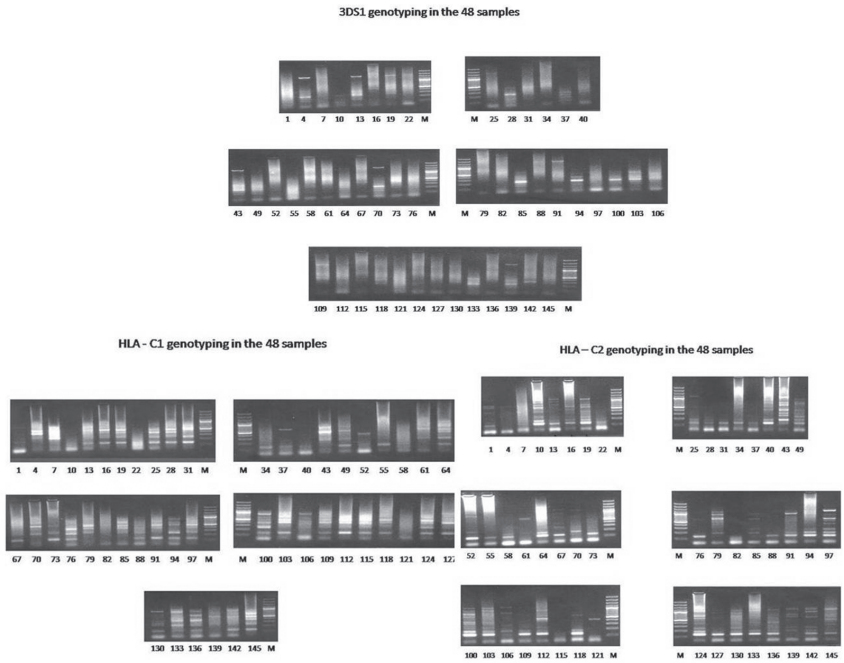

Plate-4: PCR amplification and genotyping of KIR 3DS1, HLA-C1 and HLA-C2 genes are shown in 48 subjects of the mixed ethnic groups.

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Table-4: Pairs of KIR loci showing significant positive association in the population of Puttaparthi derived from Figure-1. These pairs of significant association favor the prevalence of AB genotype with 12 genes.

2DL1 - 2DS4
2DL3 – 2DS3
2DL5 – 2DS1
2DL5 - 2DS5RD
2DL5 - 3DS1
2DS1 - 3DS1
2DS4 - 3DS1

3DL3----2DL3----------2DL1---2DL4----------2DS4-3DL2- *A hap*

-3DL3----2DL5--2DS3----2DL1---2DL4--3DS1-2DL5-2DS5RD---2DS1-2DS4-3DL2-B *B hap*

KIR : AB genotype with 12 genes along with framework genes(3DL3, 2DL4 and 3DL2) excluding pseudogenes (2DP1 and 3DP1).

	2DL1	2DL3	2DL5	3DL1	2DS1	2DS2	2DS3	2DS4	2DS5RD	3DS1
2DL1	X	0.7024 NS	0.1042 NS	0.4560 NS	1.000 NS	0.1216 NS	0.2122 NS	**0.0204** NS	0.7166 NS	0.7756 NS
2DL3		X	0.0759 NS	0.8498 NS	0.8498 NS	0.3679 NS	**0.0037** NS	0.1769 NS	0.0679 NS	0.2634 NS
2DL5			X	0.2162 NS	**0.0054** NS	0.8064 NS	0.2361 NS	0.0088 NS	**0.0026S** NS	**0.0053** S
3DL1				X	0.9999 NS	0.545 NS	0.4343 NS	0.226 NS	0.1903 NS	0.4371 NS
2DS1					X	0.1215 NS	0.4038 NS	0.2266 NS	01903 NS	**0.0001** S
2DS2						X	0.6795 NS	0.9999 NS	0.7289 NS	0.2317 NS
2DS3							X	0.3140 NS	0.4022 NS	0.9999 NS
2DS4								X	0.1238 NS	**0.0336** S
2DS5 RD									X	0.1238 NS
3DS1										X

Fig. 1: Pairs of KIR loci association calculated using the online statistical tool (in-silico.net/tools/statistics/chi2test).

The calculated values of 'p' given for each of the two KIR loci indicate statistical significant (S) and non-significant (NS) association.

alleles having inhibitory and 4 alleles with activation function which suggests a reasonably high immune profile to combat autoimmune disorders. It has been observed that groups-*A* and *B* KIR haplotypes are having equal distribution among Africans and Europeans and it is also shown that group *B*-haplotypes are common in the natives of India, whereas the

predominance of group-*A* haplotype is observed in northeast Asians (10). In another study (25) showing the evidence of co-evolution of KIR and HLA I revealed that the genetic positive association between these two groups of gene clusters reduce the disease risk. Incidentally, the estimated receptor and ligand association calculated through Fisher's exact test between KIR2DL1 and HLA C2 alleles revealed an insignificant association (p=0.1308) in the present data.

The most significant positive KIR associations within Greek population are seen among the pairs of loci 2DL2-2DS2; 2DL2-2DS3; 3DL1-2DS4 and 2DS1-3DS1 (23) and reported the most prevalent AA1 genotype. Furthermore, the high association across all loci is seen between the pairs KIR alleles viz., 3DS1-2DS1 and 3DS1:2DS5 (25). In consonance, in the present study a significant positive association is also noticed among 3DS1-2DS1 (Table 4).

The KIR distribution in group AB genotype reported in the present study needs to be understood in the context of the possible conditioned natural killer cell responses to infections and autoimmune risks primarily due to the presence of both variable stimulatory KIR genes in group-*B* and non-variable inhibitory genes in group-*A* haplotypes. Further, the studies relating to KIR gene diversity and KIR haplotype composition in the native population of India possibly enhance the prophylactic measures toward the unforeseen risks.

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Evaluation of *in vitro* Free Radical Scavenging Potential and Inhibition of Glutathione-S-transferase activity in Tasar Waste Products

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Abstract

Investigations were conducted on free radical scavenging potential and inhibition of glutathione-S-transferase (GST) activity of methanolic extracts of tasar silk waste such as fresh pupae, boiled pupae, cut cocoons and litters of tasar silkworm (*Antheraea mylitta* D.). Tests used to measure the free radical scavenging potential of silk waste extracts included 2,2-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide scavenging, hydroxyl scavenging and inhibition of lipid peroxidation (LPX) levels. The total phenolic and tannic acid contents were quantified in methanolic extracts of silk waste. Antioxidant activities determined were compared with synthetic antioxidants such as butylated hydroxytoluene (BHT) and ascorbic acid (ASA). The antioxidant activities in different samples showed the following order: fresh pupae > boiled pupae > cut cocoons > litter. The phenolic and tannic acid contents were more in litter as compared to other samples. Further, litter extract has higher glutathione-s-transferase inhibition capacity. The results suggest that the extracts of tasar waste products contain various natural antioxidants which may be useful for curing diseases associated with oxidative damages.

Key words: *Antheraea mylitta*; Antioxidant activity; Total phenolics; Glutathione -S-transferase

Introduction

Reactive oxygen species (ROS) is a generic name given to a variety of molecules derived from molecular oxygen. Addition of one electron to molecular oxygen produces the superoxide anion ($O_2^{\cdot-}$) while the addition of two electrons yields the peroxide ion (OO^{-}). These peroxides when protonated in the biological systems give rise to hydrogen peroxide (H_2O_2). Further reaction of hydrogen peroxide with superoxide anion in the presence of ferric or cupric ions in the Harber-Weiss reaction, produces the highly reactive hydroxyl radical ($^{\cdot}OH$) (1). Number of biochemical pathways occurring in the cytosol and other cell organelles like mitochondria, endoplasmic reticulum and micro bodies gives rise to ROS production (2, 3). Exogenous sources such as smoking, environmental pollutants, drugs, UV light, exposure to radiation are also responsible to produce ROS in cells (1).

The imbalance between ROS and antioxidant defence leads to oxidative modification in cellular membrane or intracellular molecules (4). This results in various diseases such as aging, cancer, inflammation, rheumatoid arthritis, hypertension, and atherosclerosis (5). Human body has multiple mechanisms especially enzymatic and non-enzymatic antioxidant systems to protect the cellular molecules against ROS induced damage (1). However the innate

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antioxidants may not be enough for severe or continued oxidative stress. Recently, interest has increased considerably in finding naturally occurring antioxidants in food or medicinal flora to replace synthetic antioxidants which are being restricted, due to their adverse side effects and toxic properties (6, 7).

A pupa is the stage in the life cycle of silkworm in which maximum storage nutrients are available. Approximately 10-12 thousand tonnes of tasar pupae after reeling are available in India. This protein rich tasar pupa is simply discarded as waste after reeling and used as animal feed. Recently, interest has increased considerably in finding naturally occurring bioactive molecules for use as food or medicinal supplement to replace synthetic one to avoid side effects of carcinogenicity (6). Earlier reports indicated that silkworm pupae (*Bombayx mori)* are rich in proteins and vitamins (8), antijuvenoid (9), antioxidant (10) and estrogenic effects (11). Presence of proteins, lipids, total mineral, phosphorous, glycoprotein and amino acids have been observed in tasar waste of *A. mylitta* (larval and pupal cuticle) (12-13). The tasar silkworm, *A.mylitta* D is an important silk producing insect. During rearing, grainage and reeling process, large quantity of waste accumulates in the form of litter, flimsy or cut cocoons, pupae and left over unreelable silk, which could be utilized as value added products using suitable technology. Studies on the antioxidant potential of tasar silk waste are very limited. The present observations were therefore undertaken to: (i) evaluate the free radical potential; (ii) quantify total phenolic and tannic acid contents present in the tested samples; (iii) to confirm that phenolic and tannic constituents are responsible for inhibition of GST activity of the tasar silk waste.

Materials and Methods

Sample preparation: A 10% (w/v) tissue extracts (fresh pupae, boiled pupae, flimsy/cut cocoon shell and litter) were prepared in methanol (95%) and filtered through Whatman paper No 1. The extracts were concentrated through Soxhlet extraction apparatus (hot plate type), Jain scientific glass works. The condensed methanol extract containing 400 mg / ml of total dissolved solid (after blotted dry), was preserved at -20°C until further use. All the experiments were conducted in triplicate.

Total phenols: Samples were extracted with 95% methanol and were estimated by using Folin-Ciocalteau reagent (FCR) (14). The reaction mixture contains 0.1ml of extracted samples and its volume was made up to 3ml with double distilled water. In the test tube containing test sample, 0.5ml of FCR was added. Then after 2 min, 1ml of 20% Na_2CO_3 were added and mixed thoroughly. Then the mixture was kept in a boiling water bath for about 5min and cooled in running tap water. The absorbance was taken against blank at 650 nm. The total phenolic content was expressed in mg/gm using a standard curve prepared from catechol.

Estimation of tannin: Tannin was estimated by using Folin-Dennis reagent (14). The reaction mixture contains 0.1 ml of extracted sample, 2.9 ml of double distilled water, 0.5 ml Folin Denis reagent and 1ml of 20% Na_2CO_3. The blue colour appeared was measured at 700 nm. Tannic content was expressed in mg/gm dry weight by using a standard curve prepared from tannic acid.

Free radical scavenging assay: Free radical scavenging potential was measured by 2, 2-diphenyl-1-picry-hydrazil (DPPH) by the method of Blois (15). The reaction mixture containing 0.9 ml of DPPH solution (0.1 mM in methanol) and extract (0.04 - 0.1 ml) was adjusted to a total volume of 1 ml by adding methanol. The absorbance was measured at 0 min and after 5 min at 517 nm. Butlylated hydroxytoluene (BHT) was used as the control. Scavenging effect (%) was calculated as below and expressed as percent

$$[A_o - A_1 / A_o] \times 100$$

A_o was absorbance at 0 minutes

A_1 was absorbance at 5 minutes

Hydrogen peroxide scavenging assay: The H_2O_2 scavenging power of extracts was estimated

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using the method of (16) with some variations. A solution of H_2O_2 (20 mM) was prepared in phosphate buffer (pH 7.4). Reaction mixture containing 2.9 ml of H_2O_2 solution and varying concentration of test samples (0.04-0.1 ml) was adjusted to 3 ml by adding phosphate buffer solution (0.1 M, pH 7.4). The absorbance was measured at 0 min and after 60 min at 240nm. The percentage of scavenging effect (%) was calculated as

$[A_o - A_1 / A_o] \times 100$

A_o was absorbance at 0 minutes

A_1 was absorbance at 60 minutes

Hydroxyl scavenging assay: Hydroxyl radical scavenging ability was measured according to procedure by (17), with few modifications. The degradation of deoxyribose by OH^- was measured colorimetricaly in presence and absence of test samples. The reaction mixture contained 100 mM of $FeSO_4$, 2 mM of H_2O_2, 3 mM of deoxyribose and varying concentration of test sample. The test tubes were then incubated for 30 min at 37°C. After incubation, TCA (0.5 ml, 5%) and TBA (0.5 ml, 1%) were added to reaction mixture and kept in boiling water bath for 45 minutes. The absorbance was measured at 532 nm. The scavenging effect (%) was calculated as

$[1- (A_o - A_1 / A_2)] \times 100$

Where, A_o was the absorbance in the presence of extract,

A_1 - absorbance without FeSO4, and H_2O_2

and A_2 - absorbance of the control (without extract)

Preparation of sheep liver fractions: Sheep liver was obtained from slaughter house (Piska Nagri, Jharkhand), washed with ice cold KCl (1.15%) and homogenized (10% w/v) with Teflon Potter-Elvejhem homogenizer. Homogenate was filtrated through cheese cloth and centrifuged at 10000 rpm for 10 min, at 4°C. Supernatant was used for LPX assay and GST activities.

Lipid peroxidation assay : Peroxidation of liver homogenate was induced by FeSO4 solution. Liver homogenate was incubated with 100 mM of $FeSO_4$ for 30 minutes at 37° C, the formation of thiobarbituric acid reactive substances(TBARS) in the incubation mixture was measured at 532nm (18).

The percentage inhibitory effect was calculated as

$[1- (A_o - A_1 / A_2)] \times 100$

Where A_o was the absorbance in the presence of extract,

A_1 - absorbance without sheep liver homogenate

and A_2 - absorbance of the control (without extract)

Glutathione-S-transferase activity: GST activities were determined spectrophotometrically by monitoring the thioester formation at 340 nm using 1-chro-2, 4-dinitrobenzene (CDNB) as the substrate (19). Sheep liver cytosolic fractions were prepared and used as the enzyme source to measure GST activity. The enzyme activity was calculated by taking the extinction coefficient of CDNB 9.6 mM^{-1} cm^{-1}. The percentage of GST inhibition was calculated by following expressions:

Percentage of inhibition = $[(A_o - A_1) / A_o] \times 100$

Where, A_o is activity of control and A_1 is activity of sample.

Statistical analysis: All experiments were conducted with three replicate and results were expressed as mean ± standard deviation (SD). Difference among the means samples were analyzed by ANOVA. Differences were considered statistically significant when $P < 0.05$.

Result and Discussion

Total phenolic and tannic acid content: Antioxidant is known as beneficial health substances and hence its exploration worldwide has recently been increased (20). Most of the natural antioxidants are phenolic compounds which are commonly found in both edible and inedible plants (21, 22).Total phenolic and tannic acid content of the methanol extract was investigated by using the Folin-Ciocalteu and Folin-Denis reagent assay, respectively. In the present study, higher level of total phenolic contents and tannic acid contents were observed in litter, boiled pupae, cocoon shell and fresh pupae respectively (Fig.1A&B). Recently, there

has been an upsurge of interest in the therapeutic potentials of plants, as antioxidants in reducing free radical induced tissue injury. This is because synthetic antioxidants that are commercially available are quite unsafe, their toxicity being the major problem of concern (6, 7). The strong restrictions have been therefore placed on their application. Natural antioxidants especially phenolics and flavonoids from tea, wine, fruits, vegetables and spices are exploited commercially either as antioxidant additives or as nutritional supplements (23).

Free radical scavenging activity: DPPH assay is well recommended for the study of free radical scavenging potential of samples. Assay is based on the reduction of DPPH. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm. After reaction with antioxidants DPPH becomes paired off in the presence of a hydrogen donor and is reduced to the DPHH and as consequence the absorbance's decreased from the DPPH (15). DPPH scavenging potential of the extract (from tasar waste) and standard depicted in Fig.1 (C) reveals that increasing extract concentrations significantly enhanced the free radical scavenging potential. This suggest that scavenging potential of extract is directly proportional to extract concentration ($P<0.05$). Among the different samples analyzed, fresh pupae showed higher DPPH scavenging potential compared to other silk waste (Fig. 1C). Similar observations on free radical scavenging activities of the silkworm pupae oils are also reported by Jung *et al*. (24).

Hydrogen peroxide scavenging assay: ROS and hydrogen peroxide (H_2O_2) derived from molecular oxygen, are not a free radicals but these are precursors of free radicals. These molecules react with metals such as Fe^{2+} or Cu^{2+} as well as superoxide anions in the Fenton reaction, to produce highly reactive hydroxyl radicals (1). As shown in Fig. 1D, the H_2O_2 scavenging activity of all extracts indicated a dose dependent response, while standard (ascorbic acid) showed more effective capacity to scavenge H_2O_2. It has been reported that electron donor may accelerate the conversion of H_2O_2 to H_2O which could possibly scavenge H_2O_2. A significant correlation observed between reducing power and H_2O_2 scavenging action (Fig.1D, $P<0.05$) could support this statement. Earlier report on the H_2O_2 scavenging potential of sericin (protein) isolation on *A.mylitta* indicated that this could provide protective effect on fibroblast by acting as

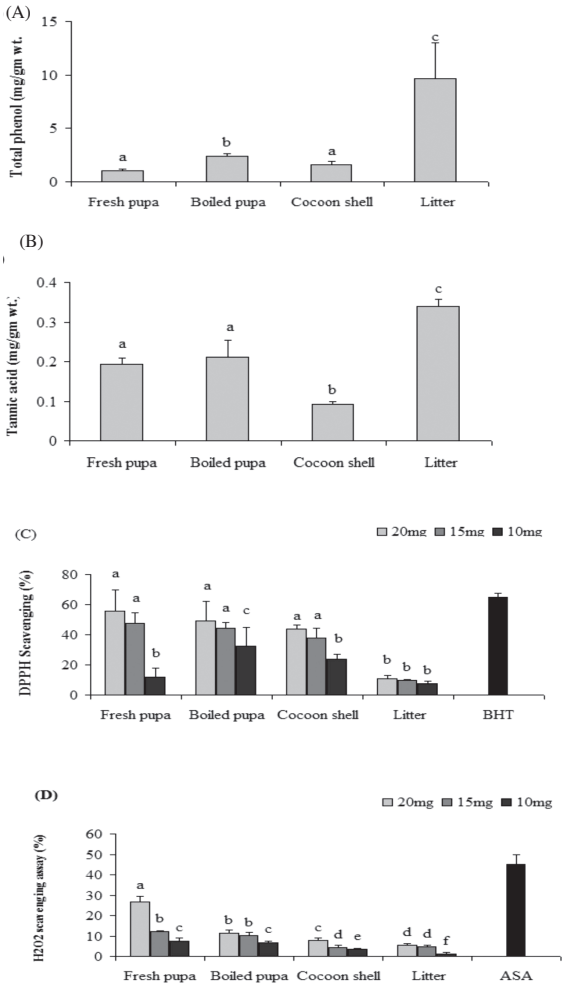

Fig 1. (A) Total Phenolic content, (B) Tannic acid content, (C) DPPH scavenging potential and (D) H_2O_2 scavenging potential of silkworm extracts. Values are mean of triplicate determination ± SD (n=3). Superscripts different letters are significantly different from each other at $P<0.05$.

antioxidant as well as by promoting endogenous antioxidant enzymes *in vitro* (25).

Hydroxyl Radical scavenging activity: Among the oxygen radicals, hydroxyl radical is most reactive with a wide range of biomolecules in living cells, such as carbohydrates, proteins, lipids and nucleotides. The resultant effect is lipid peroxidation (26), protein oxidation (27), DNA damage (28), and other cellular damages. Such consequences can either be barred and/or reversed by increasing the cellular antioxidants. Therefore, it is vital to explore for $OH^{\cdot}$ radical scavengers in food materials to avoid several dreadful diseases associated with this oxyradical. In living systems formation of $OH^{\cdot}$ radicals mainly occurs through Fenton and Haber-Weiss reaction (1). In the present study, the scavenging potential was measured by Fenton reaction. Our results indicate that methanolic extract of silk waste product has scavenging potential of $OH^{\cdot}$ radicals (Fig.2A), which could be due to scavenging capacity for H_2O_2 or metals. Similarly the significant correlation between $OH^{\cdot}$ radicals v/s H_2O_2 ($r = 0.81$ for fresh pupae; $r = 0.73$ for boiled pupae and $r = 0.7$ for litter, $P < 0.05$, Chart-1) further strengthens our views on scavenging potential of $OH^{\cdot}$ radicals by silkworm extract.

Lipid peroxidation: Among other biomolecules, lipids are more vulnerable to oxidative damage in physiological process (29). Oxidation of lipids generates various degradation products, among which malondialdehyde (MDA) forms a major cause of cell damage (30). Inhibition of LPX in cells and food materials is therefore of significant importance in preventing several diseases. *Invitro* induction of lipid peroxidation by Ferrous ions is an ideal tool for measuring antioxidant potential of extracts. Ferrous ions stimulate lipid peroxidation and supports decomposition of lipid peroxides, generating highly reactive intermediates such as hydroxyl radicals, perferryl and ferryl species (31). The present study indicates that, methanolic extracts of silk waste significantly inhibited lipid peroxidation (Fig-2B, $P < 0.05$). This implies that LPX was inhibited by the extract of fresh pupae. These results compared with other extracts probably suggest the scavenging action of free radicals and /or the reducing potential of the extract. Further, in fresh pupae a significant correlation between $OH^{\cdot}$ radical scavenging capacity v/s LPX ($r = 0.73$ $P < 0.05$, Chart-1), H_2O_2 scavenging action vs LPX ($r = 0.76$ $P < 0.05$, Chart-1) and DPPH scavenging potential v/s LPX ($r = 0.8$, $P < 0.05$, Chart-1.),

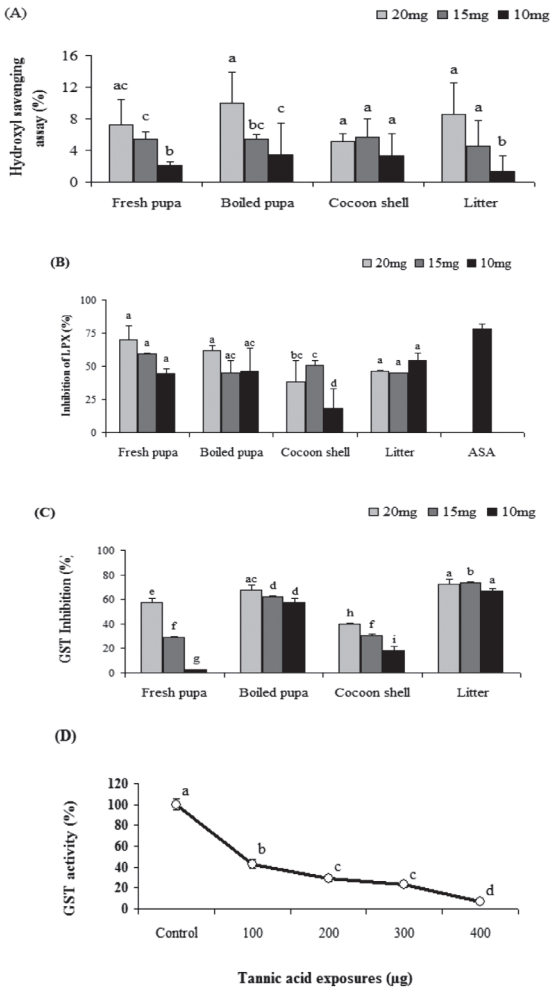

Fig. 2. (A) Hydroxyl radical scavenging, (B) Inhibition of LPX (C) Inhibition of GST activity of silkworm extracts and (D) inhibition of GST activity through the exposure of tannic acid. Values are mean of triplicate determination ± SD (n=3). Superscripts different letters are significantly different from each other at P<0.05.

		DPPH	H_2O_2	LPX	GST	$OH^•$	DPPH	H_2O_2	LPX	GST	$OH^•$	
Fresh Pupae	DPPH	1.00					1.00					Cocoon Shell
	H_2O_2	**0.77**	1.00				**0.64**	1.00				
	LPX	**0.80**	**0.76**	1.00					1.00			
	GST	**0.86**	**0.95**	**0.86**	1.00		**0.86**	**0.84**		1.00		
	$OH^•$	**0.81**	**0.81**	**0.73**	**0.91**	1.00					1.00	
Boiled Pupae	DPPH	1.00					1.00					Litter
	H_2O_2	**0.67**	1.00					1.00				
	LPX			1.00				**-0.83**	1.00			
	GST		**0.73**		1.00					1.00		
	$OH^•$		**0.73**		**0.71**	1.00		**0.70**			1.00	

Significant Non significant

Chart 1. Correlation matrix of biochemical indices in fresh pupal extract, boiled pupal extract, cocoon shell extract and litter extract. Black colours are significant at $P < 0.05$.

which indicates the higher potential of fresh pupae to scavenges the free radicals.

GST inhibition assay: GST catalyzes the conjugation of reduced GSH to nucleophilic xenobiotics, thus resulting in more polar compounds to be excreted or further metabolized (1). In the present study, inhibition of GST activity was observed on exposure of sheep liver post mitochondrial fraction (PMF) to silk waste extract. Higher amount of GST inhibition was detected in litter extract as compared to other extracts (Fig.2C). Also more phenolic and tannic acid contents were detected in litter samples as compared to other silk extracts (Fig.1A&B). Earlier studies suggest the inhibitory effects of plant phenolics such as tannic acid, ellagic acid, ferrulic acid, caffeic acid, silybin, quercetin, curcumin, and chlorogenic acid against GST activity (32, 33). In the present study, inhibition of GST activity was observed by *invitro* exposure of tanninc acid (Fig.2D). Similar to this findings, phenolic compounds such as tannic acid have the potential to bind proteins including GST enzymes through hydrogen bond formation causing steric hindrance and hence enzyme inactivation (34). The search for novel antioxidants with GST inhibitory capacity has become an important issue because of their role in tumour cells (35, 36).

The present study determines that the methanol extract of *A. mylitta* D waste showed strong antioxidant activity against DPPH scavenging activity, lipid peroxidation and hydrogen peroxide. In addition to these, extracts having strong GST inhibition activity, which needs to be evaluated at molecular level. Based on the results of the present studies it is suggested the need to initiate further works for identification of the antioxidant components in *A.mylitta* D for its industrial and pharmaceutical application.

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3D QSAR Study and Designing of Novel Prolinenitriles Derivatives as Dipeptidyl Peptidase IV Inhibitor

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Abstract

The QSAR study was performed on 21 derivatives of 4-substituted prolyl prolinenitriles using kNN-MFA method. The stepwise, genetic algorithm and simulated annealing was used for building the QSAR models. The most significant values of model generated were internal predictivity 73% (q^2) and external predictivity 95 % (pred_r^2) by simulated annealing method, steric interactions play important role in determining DPP IV inhibitory activity. On the basis of contour plots provide by the QSAR analysis, ten novel compounds were designed, showed excellent DPP IV inhibitors predicted activity.

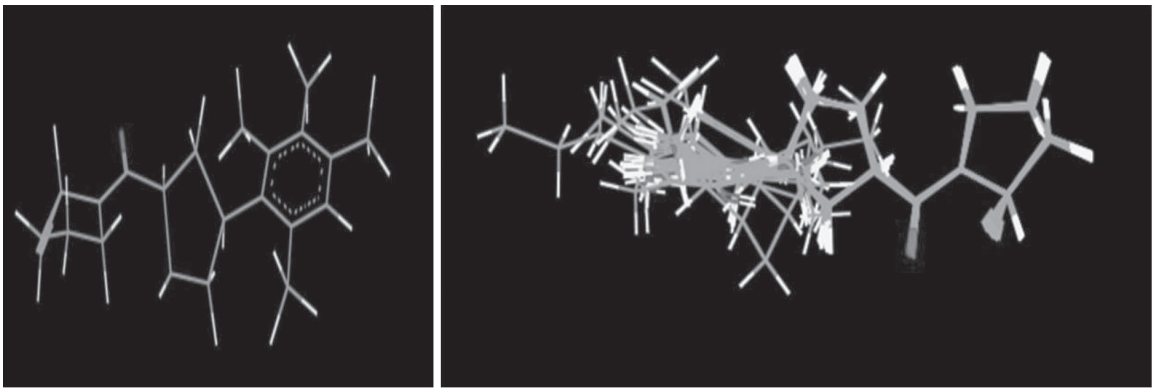
Keyword: Quantitative structure activity, dipeptidyl peptidase IV, kNN-MFA, simulated annealing, prolyl prolinenitriles

Introduction

Diabetes mellitus is a global widespread disease approximately 4-7% will be affected all over the world by 2025 (1) and situation more aggressive in future (2). It causes death in developed as well as developing countries (3,4). Type-2 diabetes mellitus (T2DM) is characterized by hyperglycemia, damage of â pancreatic cell of islet of Langerhans, insulin resistance, progressive metabolic disorder of carbohydrate and lipid metabolism (5). It creates complications such as peripheral vascular insufficiencies, neuropathy and retinopathy end stage renal disease. Patients with T2DM often suffer from dyslipidemia produced from high triglycerides, cholesterol and phospholipids (6). It was found that sudden enhance in cholesterol level range during atherosclerosis is a common feature, involvement of arterial damage, lead to ischemic heart disease, myocardial infarction, and cerebro-vascular accidents are responsible for one-third of deaths in industrialized nations (7,8). In addition to lifestyle interference, treatment of T2DM consists of oral antihyperglycaemic drugs and insulin (9). Suppress the several possible targets, the expansion of development of dipeptidyl peptidase IV inhibitors can prevent the degradation of the incretin hormones appears to be one of the most attractive, rational agents for the treatment of T2DM (10). Dipeptidyl peptidase IV also known as T-cell antigen CD26 (11,12) is a member of serine protease class family that selectively cleaves dipeptide from polypeptides, including proline or alanine at the N-terminal penultimate position (13,14).

Materials and Methods

In a QSAR analysis data set of 4-substituted prolyl prolinenitriles derivatives (21 molecules) (15) has been taken from the published literature, dataset showed in Tab. 1. The reported IC_{50} values (nM), have been transformed to pIC_{50}, for QSAR analysis. The QSAR analysis performed using the software Molecular Design Suite (MDS) 4.1.19092011 (16). The dataset aligned by using most active molecule (P09), through template based alignment method and reference molecule (1) as shown in Fig. 1. The alignment of all the molecules on the template is



1 2

Fig. 1. Template structure (1) and reference aligned Molecule structure (2)

presented in Fig. 1 as an aligned molecule (2). After alignment, a molecular descriptors field is calculated on a grid space provide near by the molecule. The fields are steric, electrostatic and hydrophobic, computed at the each lattice points of the grid using a CH_3 probe of charge +1.

To predict the QSAR analysis externally and internally, dataset was divided into training and test set using sphere exclusion methods. In the present study sphere exclusion algorithm was selected for development of training and test sets (17). As the dissimilarity level increases, the lower the training set is and the higher the test set is and vice versa. Different training and test set of 4-substituted prolyl prolinenitriles derivatives were constructed using sphere exclusion with dissimilarity level 4 to 6. Training and test set were selected and calculated Unicolumn statistics (18-20). The electrostatic and the steric fields were selected in model generation, dielectric constant is 0.2, charge type is gasteiger marsili, cut off value are 30 kcal/mol and 30kcal/mol respectively. Data generated by k nearest neighbor molecular field analysis (kNN-MFA) in conjunction with Stepwise forward-backward variable selection method (SW-kNN MFA), Genetic algorithm k-NN QSAR Algorithm (GA-kNN MFA) and Simulated Annealing k-NN QSAR Algorithm (SA-kNN MFA) applied to generate the most significant models which were helpful in finding a lead molecule with better activity.

Interpretation of Models: In QSAR analysis, validation is an important step. Models were generally validated internally with training set and externally with test set. In internal validation, a data set is segregated in the training set and its biological activity is predicted on the basis of the kNN principle.

Model 1: Dissimilarity value 4 is selected for model in which out of 21 compound set, 3 compounds are under test set as P05, P15, P22 and rest are in the training set, SW-kNN MFA q^2 0.44 $Predr^2$ 0.78, GA-kNN MFA q^2 0.60 $Predr^2$ 0.92, SA-kNN MFA q^2 0.55, $Predr^2$ 0.90. The model 1 is considered as less significant model which contains the q^2 and predicted r^2 value in the limited range.

Model 2: Dissimilarity value 4.1 is selected for model in which out of 21 compound set, 4 compounds are under test set as P05, P08, P15, P22 and rest are in the training set, SW-kNN MFA q^2 0.60 $Predr^2$ 0.81, GA-kNN MFA q^2 0.67 $Predr^2$ 0.70, SA-kNN MFA q^2 0.73, $Predr^2$ 0.95. The model 2 is considered as significant model which contain the q^2 and predicted r^2 value in the better range as compared to model 1. The most

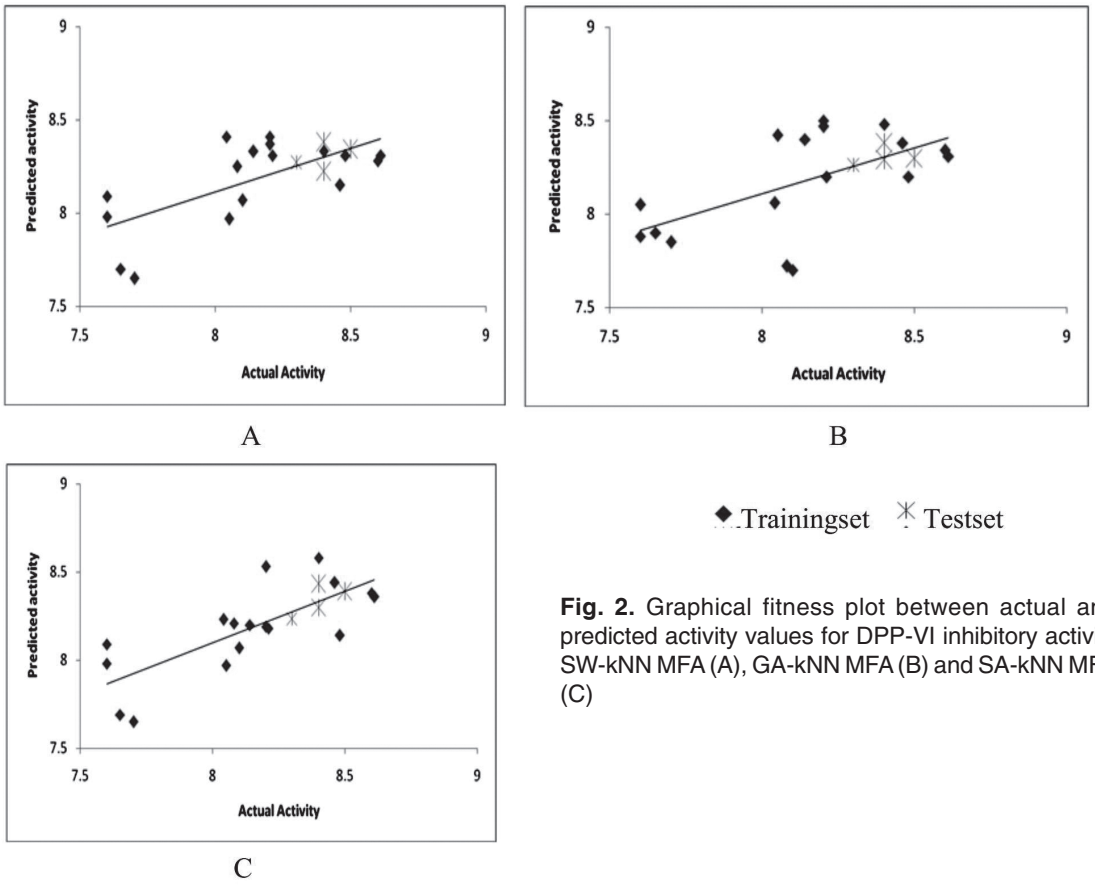


Fig. 2. Graphical fitness plot between actual and predicted activity values for DPP-VI inhibitory activity SW-kNN MFA (A), GA-kNN MFA (B) and SA-kNN MFA (C)

significant values of model 2 generated with internal predictivity 73% (q2 =0.73) and external predictivity 95 % (pred_r^2 = 0.95) by GA-kNN MFA methods.

Results and Discussion

The several training and test set of 4-substituted prolyl prolinenitriles derivatives were built through using sphere exclusion (dissimilarity level 4 to 6) and better result obtained at dissimilarity value of 4.1. The selection of the training and test set depends on the unicolumn statistics value shown in Tab. 2. The model 2 showed q^2 73% (q^2 =0.73) and pred_r^2 95 % (0.95). The most noteworthy model 2 of SA-kNN MFA methodology showed that steric (S_515, 588) interactions i.e. bulky groups play important role in recognizing DPP IV inhibitory activity.

This result shows that the test is interpolative i.e., derived from the min-max range of training set. The mean and standard deviation of the training and test set provides insight to the relative difference of mean and point density distribution of the two sets. k-Nearest neighbor molecular field analysis (kNN-MFA) was applied using stepwise (SW), genetic algorithm (GA) and simulated annealing (SA) approaches for building QSAR models. Results of models developed by SW-kNN MFA, GA-kNN and MFA SA-kNN MFA using sphere exclusion methods. Significant QSAR model generated is shown in Tab-3.

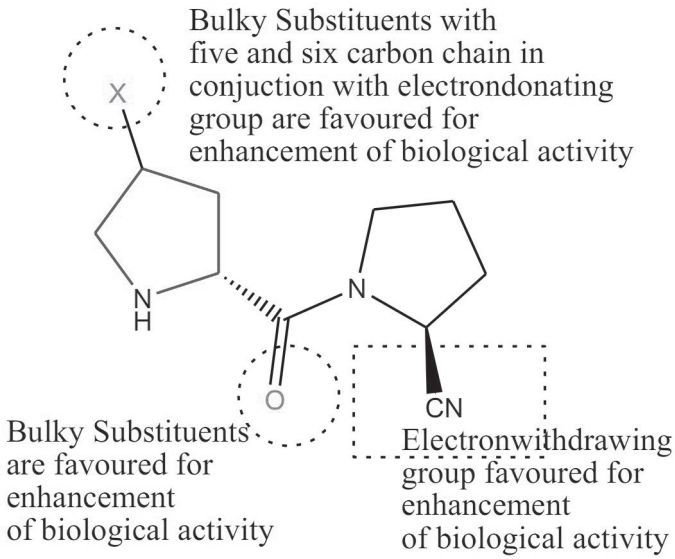


Fig. 3. Training set (A) and Test set (B) biological activity is predicted graph by SA-kNN MFA method

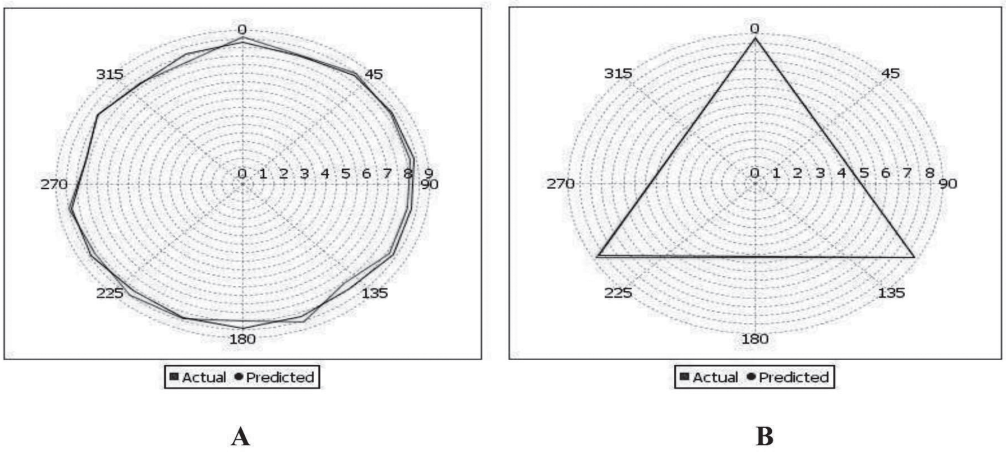


Fig. 4. Showing 3D-Allignment of molecules and descriptor of model by wire frame model by SA-kNN MFA method

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Statistical measures used are shown in Tab-4 to correlate biological activity and molecular descriptors. Data fitness plot for model is shown in Fig. 2. Result of the observed and predicted biological activity for the training and test compounds for the Model is shown in Tab. 4. The plot of observed vs. predicted activity of training and test sets for model is shown in Fig 3. From the plot it can be seen that kNN-MFA model is able to predict the activity of training set quite well (all points are close to regression line) as well as external. Sphere exclusion (SE) algorithm and random selection methods were used for constructing training and test sets. kNN-MFA methodology with stepwise (SW), genetic algorithm (GA) and simulated annealing (SA) was used for building the QSAR models and alignment molecule with descriptor shown in Fig. 4. The kNN-MFA contour plot (Fig. 5) provided further understanding of the relationship between structural features of 4-substituted prolyl prolinenitriles derivatives and their activities which should be applicable to design newer potential as dipeptidyl peptidase IV (DPP IV) inhibitors. The positive value of steric parameter near the keto group is required for the biological activity and can be replaced with the similar isosteric group for increasing the biological activity. Near the R_1 and R_2 the steric value is negative so it shows that bulky group is not contributing much for the biological activity.

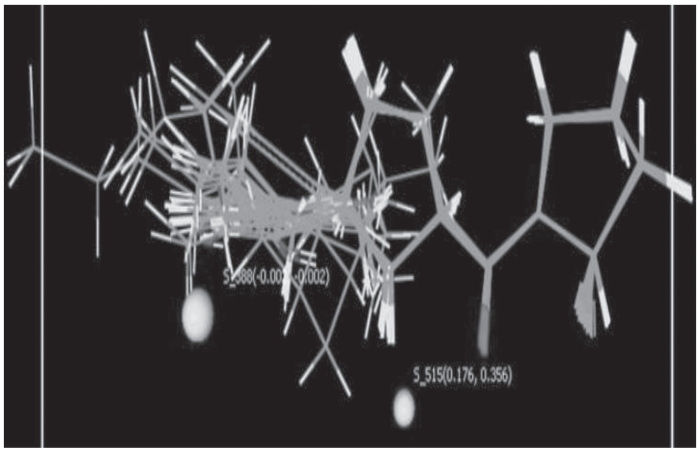

Fig. 5. The kNN-MFA contour plots shows structural features of derivatives and their activities by SA-kNN MFA

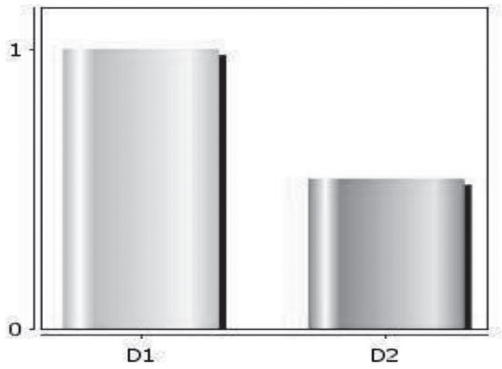

Fig. 6. The structure activity relationship revealed by QSAR analysis.

Structural Activity Relationship and Designing: The QSAR analysis supplied enough information regarding the structural necessities for better antidiabetic activity. The suggestion from QSAR analysis promotes us to propose and design some novel antidiabetic compounds. The QSAR contours contribution maps guide us to optimize the existing scaffold. Based on the approach suggestion in aspect, more bulky and electron-donating groups at the X position enhance activity; bulky and electron-withdrawing substituent are favored near C=O group; and minor, no bulky substituents and electron-withdrawing substituent enhance potency at on replacement to CN through QSAR approach. Furthermore QSAR advise that unsaturated and aliphatic side chain showed intermediate contribution. The chain or rings containing five and six carbon with electron-donating were essential for binding to the inhibitors site. The structure-activity relationship explored by this study is presented in Fig. 6. Based on the studies, a series of new DPP-IV inhibitors were designed. These designed molecules were aligned by template based method, and their pIC_{50} values were predicted by the formerly established QSAR models showed in Tab 5 and Fig. 7. The compound SP-4 showed better predicted activity comparison to other designed molecules.

Conclusion

Significant models were generated in sphere exclusion data selection method. Model developed to predict the structural features of 4-substituted prolyl-prolinenitriles derivatives to inhibit dipeptidyl peptidase IV reveals useful information about the structural features requirement for the molecule. The sophisticated result obtained from the various kNN-MFA models show that positive contribution range in steric descriptors indicates bulky substituents are preferred in that region. The positive contributing value of steric parameter near the keto group shows that keto group is required for the biological activity and can be replaced with the similar isosteric group and bulky substituents for

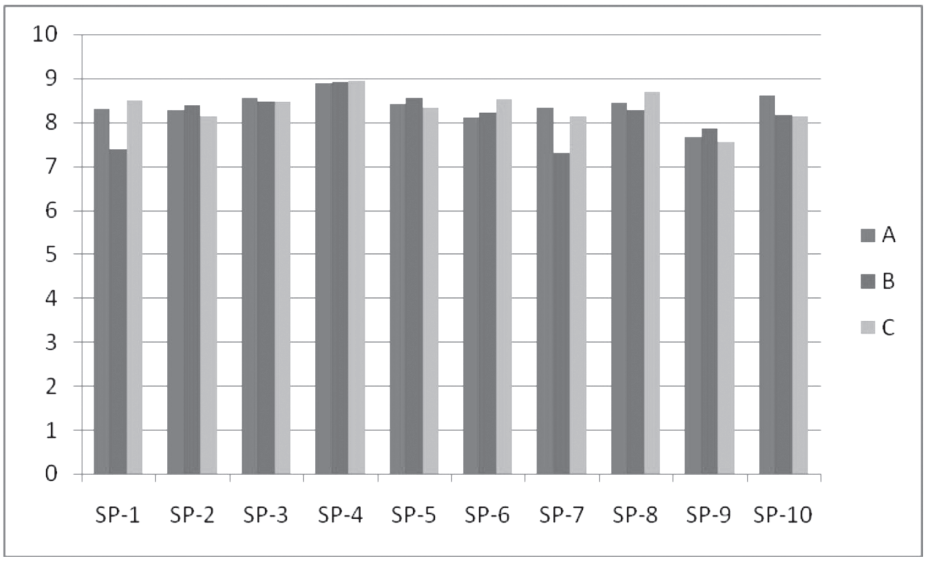

Fig. 7. Graph of predicted biological activity of designed novel compounds for SW-kNN MFA (A), SA-kNN MFA (B) and GA-kNN MFA (C)

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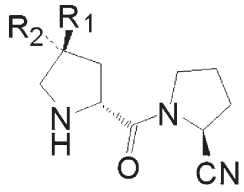
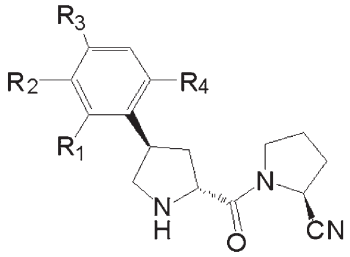
Table 1. General structure of the compounds of 4-substituted prolyl prolinenitriles derivatives and their biological activities (data set of 21 molecules)



Compound	R1	R2	IC50 (nM)	pIC50
P04	H	H	20	7.6
P05	Allyl	H	3.5	8.4
P06	Propyl	H	3.4	8.46
P07	Methallyl	H	5.7	8.2
P08	Isobutyl	H	2.9	8.5
P09	c-hexyl	H	2.4	8.61
P10	2-adamantyl	H	7.8	8.1
P11	Ph	H	3.5	8.4
P12	H	Ph	16	7.7
P13	2,6-di Me-Ph	H	6.1	8.21



Compound	R1	R2	R3	R4	IC50 (nM)	pIC50
P14	OH	H	H	H	9.1	8.04
P15	H	OH	H	H	3.9	8.4
P16	H	H	OH	H	2.5	8.6
P17	Me	H	OH	Me	3.3	8.48
P18	OMe	H	OH	Me	8.3	8.08
P19	OMe	H	OH	OMe	23	7.6
P20	Me	H	OH	Et	8.9	8.05
P21	OEt	H	OH	OEt	22	7.65
P22	Me	OH	H	Me	4.9	8.3
P23	OMe	OH	H	Me	7.1	8.14
P24	Me	OH	Me	Me	5.3	8.2

Table 2. Uni-Column Statistics for Model 2 for training and test set activity.

Column Name	Average	Max	Min	Std Dev	Sum
Training set	8.1456	8.6100	7.6000	0.33431	46.6200
Test set	8.3667	8.4000	8.3000	0.0577	25.1000

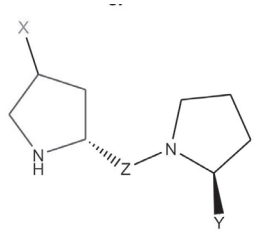
Table 3. Result of kNN-MFA study using sphere exclusion selection method

S. No	Dissimilarity value	Test set	SW-kNN MFA		GA-kNN MFA		SA-kNN MFA	
			q^2	$Predr^2$	q^2	$Predr^2$	q^2	$Predr^2$
1	4	p05, p15, p22	0.44	0.78	0.60	0.92	0.55	0.90
2	4.1	p05, p08, p15, p22	0.60	0.81	0.67	0.70	0.73	0.95

Table 4. Actual and predicted biological activity for Training set and test set (Model 2).

Compound	Actual	Predicted			Residual Value		
		SW-KNN MFA	GA-KNN MFA	SA-KNN MFA	SW-KNN MFA	GA-KNN MFA	SA-KNN MFA
P04	7.60	8.09	7.88	8.09	-0.49	-0.28	-0.49
P05	8.40	8.22	8.29	8.43	0.18	0.11	-0.03
P06	8.46	8.15	8.38	8.44	0.31	0.08	0.02
P07	8.20	8.41	8.50	8.53	-0.21	-0.3	-0.33
P08	8.50	8.34	8.30	8.39	0.16	0.2	0.11
P09	8.61	8.31	8.31	8.36	0.3	0.3	0.25
P10	8.10	8.07	7.70	8.07	0.03	0.4	0.03
P11	8.40	8.33	8.48	8.58	0.07	-0.08	-0.18
P12	7.70	7.65	7.85	7.65	0.05	-0.15	0.05
P13	8.21	8.31	8.20	8.18	-0.1	0.01	0.03
P14	8.04	8.41	8.06	8.23	-0.37	-0.02	-0.19
P15	8.40	8.38	8.38	8.30	0.02	0.02	0.1
P16	8.60	8.28	8.34	8.38	0.32	0.26	0.22
P17	8.48	8.31	8.20	8.14	0.17	0.28	0.34
P18	8.08	8.25	7.72	8.21	-0.17	0.36	-0.13
P19	7.60	7.98	8.05	7.98	-0.38	-0.45	-0.38
P20	8.05	7.97	8.42	7.97	0.08	-0.37	0.08
P21	7.65	7.70	7.90	7.69	-0.05	-0.25	-0.04
P22	8.30	8.27	8.26	8.23	0.03	0.04	0.07
P23	8.14	8.33	8.40	8.20	-0.19	-0.26	-0.06
P24	8.20	8.37	8.47	8.19	-0.17	-0.27	0.01

Table 5. Predicted biological activity of designed novel compounds by SW-kNN MFA (A), GA-kNN MFA (B) and SA-kNN MFA methodology



Code	X	Y	Z	SW-kNN MFA	GA-kNN MFA	SA-kNN MFA
SP-1	-C6H4-4-OH	-CN	CH-NH2	8.29	7.38	8.49
SP-2	-C6H4-4-OMe	-F	C=O	8.26	8.39	8.13
SP-3	-C6H4-4-Me	-NO2	CH-CN	8.55	8.48	8.47
SP-4	-C6H4-3-Me	-C6H4-4-CN	C=O	8.89	8.90	8.93
SP-5	-C6H4-3-OMe	-C6H4-4-NO2	C=O	8.42	8.56	8.32
SP-6	-C6H4-3-OH	-C6H4-4-F	C=O	8.11	8.21	8.52
SP-7	-C6H4-4-OEt	-C6H4-4-Cl	C=O	8.34	7.31	8.12
SP-8	-C6H4-4-Et	-Cl	C=O	8.43	8.28	8.68
SP-9	-C6H4-3-OEt	-C6H4-2-CN	C=O	7.65	7.85	7.55
SP-10	-C6H4-3-NH2	-C6H4-2-NO2	C=O	8.61	8.15	8.13

increasing the biological activity. On the basis of steric and electrostatic potential contributions to the developed model in this work is useful in describing QSAR of 4-substituted prolyl prolinenitriles derivatives, on the basis of analysis, ten novel derivatives were designed with potent inhibitory activity.

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Variation in Antioxidative Potential of Processed and Unprocessed Honey samples from Central India. A plausible role of Quinolin and Gallic acid as antioxidants.

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Abstract

Honey is a natural product having high economic importance since ages. Traditional medicine has recognised it as a potent medicine for a variety of ailments. However, only recently modern medicine has recognised its medicinal importance. Many reports have shown that honey exhibits antioxidant, free radical scavenging, anti bacterial and other bioactive properties and these properties depend mostly on the differences attributed to varied regional as well as floral origins of honey samples. The present investigation was carried out to access bioactive properties of different honey samples (processed and unprocessed) belonging to different regions of central India. A comparison was made with flowers of *Rosa hybrid* (Rose) and *Calendula officinalis* (Marigold), which were commonly employed by honey bees as a source of nector. The results demonstrated that flowers exhibit maximum antioxidant activity (Marigold-80%, Rose-76.7%) and total phenolic content (Marigold-1105.7mgGAEQ/kg, Rose-1741.3 mgGAEQ/kg) as compared to honey samples. Processed honey samples exhibited a lower antioxidant activity as compared to unprocessed honey samples. Sample G and H, which were unprocessed honey sample showed maximum antioxidant activity 64.5 and 71.8%, and phenolic content-(765.3 and 814.4mgGAEQ/kg). MS analysis showed that Sample G-H contained Quinolin and Gallic acid, which might be responsible for the observed property. It appears that during industrial processing of honey these constituents are lost, which leads significant reduction in antioxidant property.

Keywords: Honey; Antioxidant; DPPH; Mass Spectroscopy; Anti bacterial

Introduction

There are number of flowers, which synthesize metabolites having antioxidant activity that can be employed as a natural source of free radical scavenging compounds (1). Most of these flowers are used by the bees to collect nectar for the synthesis of honey (2) and consequently, these metabolites from flowers are transferred to honey. Honey is a natural product with high economical importance worldwide, mainly synthesised by honeybees (*Apis mellifera*) (3) which has highly variable composition due to diverse origin of plants from which it is harvested (4). Honey is considered as a natural source of sugars, as well as an important ingredient in traditional medicine; having health-protective, therapeutic impacts, anti-inflammatory properties (5). Moreover, honey has been shown to be effective in the healing of wounds and burns, as an antimicrobial agent as well as having an important antioxidant activity (6). Although, honey has not been considered as a therapeutic agent in modern medicine due to lack studies on its medicinal properties (5). It has been reported that different honey samples have varying phenolic content and consequently different antioxidant activity. Processing, handling and storage of honey also influence its composition and

antioxidant property (7). It is also been proven to be effective against deteriorative oxidation reactions in foods, such as lipid oxidation (8) and enzymatic browning of fruits and vegetables. Honey contains diverse components such as pollen grains, bee wax, and small parts of the bees and many other substances such as enzymes, vitamins, amino acids, pigments, phenolic acids, and flavonoids(9). Therefore, presences of these phytochemical constituents are considered to be very important parameters in the assessment of honey flavour quality. (10)

It has been shown that oxidative stress plays a significant role in the onset of numerous pathological conditions including coronary heart disease (8). Free radicals and reactive oxygen species (ROS) also cause damage to the tissues and contribute to the process of aging. Therefore ingestion of food and supplements having high antioxidant content is considered as a suitable alternative for protection against these damaging entities (11). The present investigation focuses on elucidation of chemical components that contribute to the antioxidant activity of Indian honeys. Current literature indicates that components such as phenolic acids, and flavonoid have floral origin, show high antioxidant properties. In this study, we have compared the phenolic content, radical scavenging activity and antibacterial activity of selected flowers with commercial honey and unprocessed honey samples.

Materials and Methods

Chemicals: DPPH, Ascorbic acid, Gallic acid, Catechin from Sigma Aldrich USA, Folin-Ciocalteu reagent from Fischer, India and dextrose, peptone, agar from Himedia, India were purchased. All chemicals used in this study were of analytical grade.

Honey and flower samples: All commercial honey samples were obtained from local market of Bilaspur and each sample having different areas of origin. Unprocessed honeys were collected from different region of Chhattisgarh state located in Central India. Eight honey samples were employed in this study, among them six were commercial available honeys (Sample A-F) having heterofloral origin and two unprocessed honey (Sample G-H) having heterofloral origin from different regions of Chhattisgarh state belonging to central India (Table 1). Two flower samples were collected from local flower source of Rose (*Rosa hybrida)* and Marigold (*Calendula offcinalis*) flower.

Table 1. Honey Sample and their geographical origin

Sample Name	Geographical Origin	Manufactu ring Date
Sample A	Delhi	August,2011
Sample B	Haridwar	September,2011
Sample C	Delhi	December,2010
Sample D	Gwalior	February,2011
Sample E	Bareli	December,2010
Sample F	Firozabad	October,2010
Sample G	Achanakmar	December,2011
Sample H	Jashpur	February,2011

Preparation of flower extracts: Flower extracts were prepared by drying fresh flower at 60°C, dried flowers (10g) were extracted in soxhlet apparatus with 80% ethanol and water (100ml) at room temperature for 4h (12). This experiment was used for both the flower in the solvent. Then extract was centrifuged for 10 min at 10000 rpm at 4°C, supernatant was used for further experiments.

Characterization of antioxidant activity

Total phenolic content: Diluted honey sample (0.2g/ml) was mixed with 1ml of Folin Ciocalteu reagent. After 3min, 1mL of 10% sodium carbonate solution was added to the mixture and adjusted to 10ml with distilled water. Same experiment was repeated with flower extract; in this case powder form of extract was used. The reaction was kept in the dark for 90min, after which the absorbance was read at 725nm by UV/

VIS spectrophotometer. Gallic acid was used standard.

Total flavonoid content: Total flavonoid contents (TF) of honey were determined according to the colorimetric assay (13). 1mL of properly diluted honey (0.2g/mL was mixed with 4ml of distilled water. At zero time, 0.3mL of (5%w/v) $NaNO_2$ was added. After 5min, 0.3ml of (10%w/v) $AlCl_3$ was added. After 6min, 2ml of 1M solution of NaOH were added. After that, the volume was made up to 10ml, immediately by the addition of 2.4ml of distilled water. The mixture was shaken vigorously and the absorbance of the mixture was read at 510nm. Same experiment was repeated with dried flower extract. A calibration curve was prepared using catechin.

DPPH free radical scavenging activity: The scavenging activity against 1,1-diphenyl-1-picrylhydrazyl hydrate (DPPH) was evaluated (14) with minor modification. 6.5X10-5mol/l DPPH in methanol was freshly prepared before measurement 3ml of DPPH solution was added with 50µl of honey. The absorbance of the reaction mixture was determined after 16min at 515nm. Blank sample contained the same amount of methanol and DPPH. The measurements were performed in triplicate. Same experiment was repeated with flower extract. The radical scavenging activity was calculated by the formula:
I = ((AB-AA)/AB) x 100
Where, I = DPPH inhibition %; AB = absorption of a blank sample (t = 0min); AA = absorption of honey solution at the end of the reaction (t = 16min). 1mM Ascorbic acid was used as positive control and distilled water as negative control.

DPPH free radical scavenging assay for IC50 determination: Honey and flower samples of 0.75mL with varying concentration ranging from 10 to 100mg/L were mixed with 1.5mL of methanol-dissolved DPPH (0.1mg/mL) and allowed to stand for 15min before measuring the absorbance at 517nm. The blank was honey at the same concentration in methanol without the DPPH radical (15).

Color intensity (ABS 450): Honey samples were prepared as follows: diluted to 50% (w/v) with warm water (45–50°C), sonicated for 5min and filtered with 0.2µm pore size filter to eliminate large particles. Colour intensity was defined as the difference between spectrophotometric absorbance at 450 and 720nm (16).

Chemical profiling of Commercial Honey, Unprocessed Honey and Flower extract: Chemical profiling of commercial honey, unprocessed honey and flower extract were carried out by determining the pH, reducing sugar and total carbohydrate of sample. pH of all samples were determined by pH meter. Reducing sugar was analysed by DNS reagent (7) and total carbohydrate was analyzed by anthrone reagent (17).

Antibacterial activity: The agar well diffusion method was employed to test the antimicrobial activity of honey. Petri plates were prepared with nutrient agar medium. 0.1ml of the *Bacillus subtilis* (MTCC 441) culture suspension was spread on each plate. Wells of 5mm diameter (approximately) were cut with sterile cork borer in the inoculated agar. The wells were filled with 40ìl test sample of honey (50v/v) with the help of micropipette. The plates were incubated for 24 hours at 37°C. Chloramphenicol was used as positive control and distilled water was used as negative control and 80% ethanol is used for ethanolic flower extract. (12)

Mass Spectrometery

Sample preparation: Honey samples were membrane filtered with 0.2µm pore size filter, then the samples were prepared in 100ppm (parts per million) concentration using methanol and distilled water of HPLC grade as the solvent (both taken in equal ratios of 1:1). In 2mL of solvent (1mL methanol and 1mL of distilled water) 0.1mL of filtered honey were added and mixed properly.

Mass spectrometer analysis: MS analyses were performed on an API2000 triple stage quadrupole mass spectrometer equipped with a turbo-ion

spray interface. The electrospray capillary voltage was 5500V. MS detection was performed in positive mode using Multiple Reaction Monitoring.

Result and Discussion

Characterization of antioxidant activity

Total phenolic content: The total polyphenol content of all flower and honey samples were determined by using modified Folin-Ciocalteu assay which is sensitive to phenol and polyphenol entities and other electron donating antioxidants (Ascorbic acid, Vitamin E). The results showed that different honey types and flower sample had varying levels of phenolic content (Fig. 1). Flower extracts exhibited highest phenolic content as compared to honey samples. Also it was observed that ethanolic extracts exhibited higher phenolic content as compared to water extracts. It was observed that commercial honey samples had a lower phenolic content when compared with unprocessed samples. Phenolic content of commercial samples was in the range of 80.5 to 147.5 mgGAE/kg. Sample G and H showed the highest content of 765.3 and 814mgGAE/kg. Similar to our observation, Orange honey of Southern Rainforest also contained 834mgGAE/kg phenolic concentration and Acacea honey contained 43.6 (mgGAE/kg) (2), Manuka honey of New Zealand contained 1133 (mgGAE/kg) (18) and Malecian honey contain 876.56 (mgGAE/kg) (19). Tualang honey had the highest total phenolic content 839.6 mg/kg, followed by gelam (721.6mg/kg), Indian forest (456.3mg/kg), and pineapple honeys (277.5 mg/kg) (20). Rose ethanolic extract (Ret) showed maximum phenolic concentration 1173 mgGAE/kg. Sample G and H (unprocessed) had higher phenolic content as compared to processed honey samples, these differences might be attributed to the effect of processing which removes most of the phenolic content in honey samples. Also, it might also be due to differences between other honey samples might be attributed to natural variations in composition arising due to different location and floral sources (15). General observation can be made that dark honeys were characterised by considerably higher phenolic content than the other samples (4).

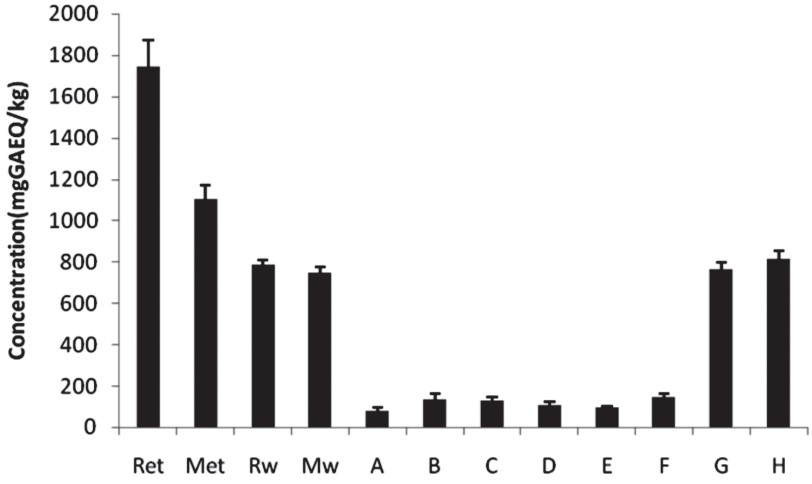

Fig. 1. Total Phenolic content of flower extract and honey samples. Ret-Ethanolic extract of Rose flower, Met- Ethanolic extract of Marigold, Rw- Water extract of Rose, Mw- Water extract of Marigold, Sample A-F, Processed honey samples, Sample G-H, unprocessed honey samples.

Total flavonoid content: In this study, we used a spectrophotometric quantification of flavonoids with aluminium chloride. The flavonoid quantification method is based on the complexation of flavones and flavonols to aluminium cations. Total flavonoid content of honey samples (mg CEQ/kg) using the standard curve generated by catechin. Flavonoid content of different honey samples are summarized in (Fig. 2). It was observed that flower extracts exhibited higher flavonoid content as compared to honey samples. Among all honey sample, H show maximum amount of flavonoid 229mgCEQ/kg followed by sample G, which contained 209.5 mgGAE/kg and commercial honeys samples contained lowest flavonoid content. Flower sample Met contain highest amount of flavonoid 896mg/kg. Similarly, Malecian honey showed 227.57mg/kg, (19), Manuka honey-10.8 mg/kg (18). Flavonoid content in Lithuanian honey was reported as 281.5mg/kg (3). Our study reported that 15-155mgCEQ/kg flavonoids are present in Indian commercially available honey. This difference might be due to the effect of processing. The real content of total flavonoids must be the sum of flavonoid contents determined by the aluminium chloride method which is specific for flavones and flavonols (21). Polyphenols in honey are mainly flavonoids (chrysin, quercetin, luteolin, kaempferol, apigenin, and galangin), phenolic acids and phenolic acid derivatives (18). Flavonoid content was lower than phenolic content.

DPPH free radical scavenging activity: DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples. Among all the honey samples H exhibits the strongest scavenging activity having 71.8%, followed by sample G, which had 64.5 %. (Fig. 3). Also, flower extracts exhibited higher radical scavenging activity as compared to processed and unprocessed honey samples. In a similar study, Borneo tropical honey had 26.79% inhibition while the highest activity was found in Tualang honey 81.64% (19). Further, Buckwheat honey exhibited the strongest scavenging activity, of the honey products followed by mixed-breed honey, honey (Japanese bee), royal jelly, and pure honey (23). It has been reported that an increase in the concentration of honey sample resulted in an increase in radical scavenging activity (16)

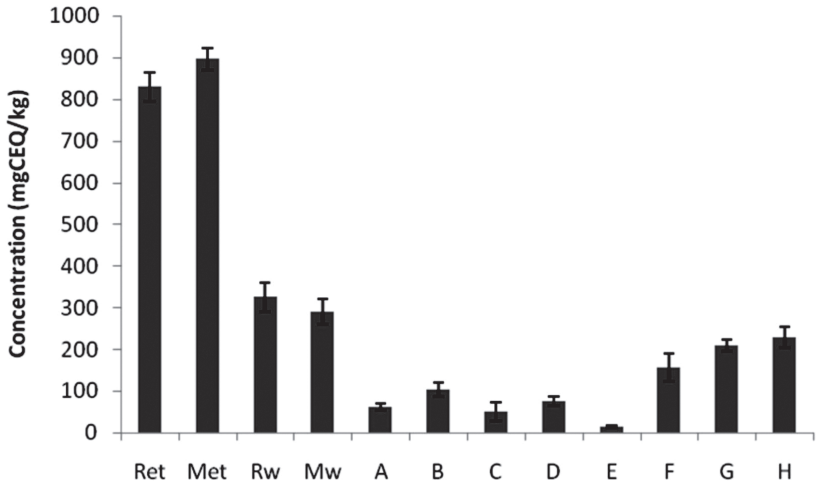

Fig. 2. Total Flavanoid content of flower extract and honey samples. Ret-Ethanolic extract of Rose flower, Met- Ethanolic extract of Marigold, Rw- Water extract of Rose, Mw- Water extract of Marigold, Sample A-F, Processed honey samples, Sample G-H, unprocessed honey samples.

Antioxidant Potential of Honey samples

suggested that antioxidant activity of honey varies widely, depending on the floral source possibly due to the differences in the content of plant secondary metabolites and enzyme activity. Also, reports have confirmed that the radical scavenging activity of a sample cannot be predicted only on the basis of its total phenolic content (9). It appears that antioxidant capacity of honey is the result of the combined activity of a wide range of compounds including phenolic, peptides, organic acids, enzymes, maillard reaction products and possibly other minor components (18).

Colour intensity (ABS 450): Many reports have shown that the colour of honey samples reflects its antioxidant activity. It is believed that dark coloured honey contains more radical scavenging activity than light honey. The results of colour intensity of different honey sample are summarized in (Fig 4). In the present study an interesting observation was observed. Three processed honey samples showed higher color intensity, which was comparable to unprocessed honey samples. However, these samples showed a low level of total phenolic and flavonoid content. This result indicated that there is a possibility that in processed honey samples, extra color is added to mimic the color of unprocessed honey samples and to increase its market value. Therefore, we feel that ABS450 parameter cannot be employed as a reliable index of the presence of antioxidant activity. However, in few studies a direct correlation was obtained with color intensity and antioxidant property(16, 19, 15).

DPPH free radical scavenging assay for IC50 determination: IC50 (50% Inhibitory concentration) is the concentration required to scavenge 50% of the DPPH radicals. In this study, IC50 value of flower extracts was lowest, which renged fron 11-19 mg/ml. IC50 value of processed honey samples ranges from 52-102 mg/ml (Fig 5). The higher the IC50 values, the lower the ability to scavenge the free radicals. Among all honey samples, unprocessed honey samples (G and H)

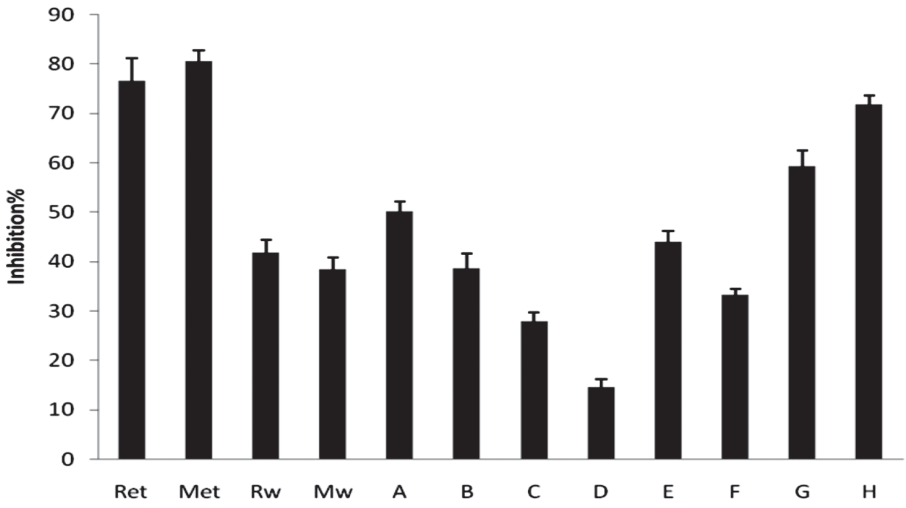

Fig. 3. Percentage DPPH inhibition of honey and flower sample. Ret-Ethanolic extract of Rose flower, Met- Ethanolic extract of Marigold, Rw- Water extract of Rose, Mw- Water extract of Marigold, Sample A-F, Processed honey samples, Sample G-H, unprocessed honey samples.

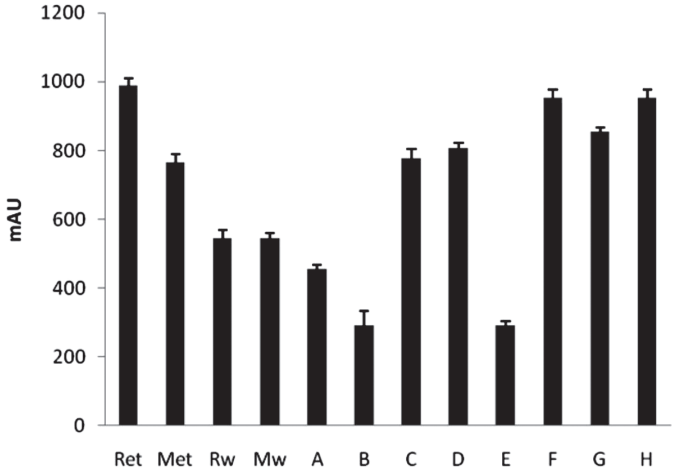

Fig. 4. Colour intensity of honey and flower sample. Ret-Ethanolic extract of Rose flower, Met-Ethanolic extract of Marigold, Rw- Water extract of Rose, Mw- Water extract of Marigold, Sample A-F, Processed honey samples, Sample G-H, unprocessed honey samples.

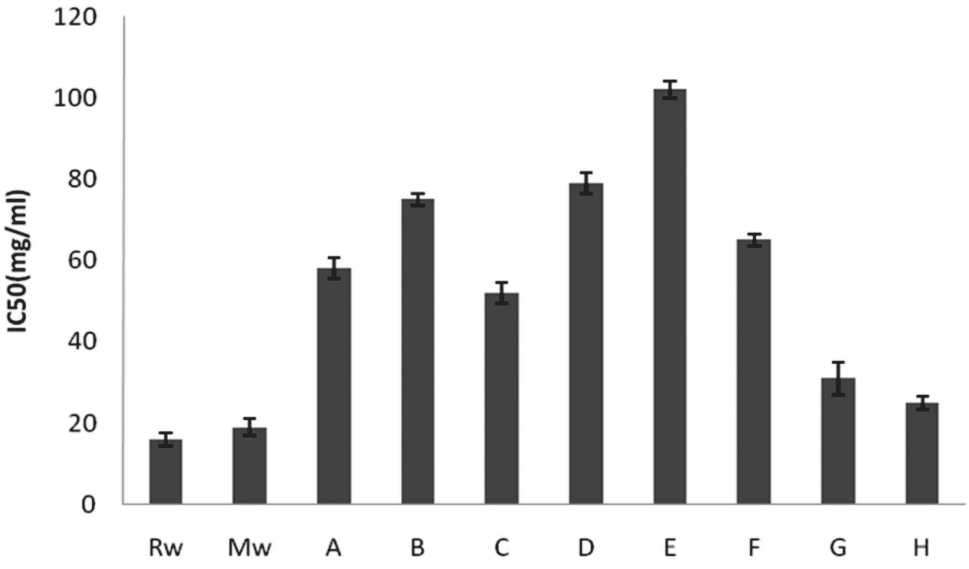

Fig. 5. IC50 value of Honey and flower sample. Ret-Ethanolic extract of Rose flower, Met- Ethanolic extract of Marigold, Rw- Water extract of Rose, Mw- Water extract of Marigold, Sample A-F, Processed honey samples, Sample G-H, unprocessed honey samples.

Antioxidant Potential of Honey samples

exhibited a lower value. Similar to our study, value of 168.94, 130.49 and 106.67mg/ml were reported on light, amber and dark honey from Portugal respectively (3). In a similar report monofloral honey exhibited IC50 value from 10.19 mg/ml to 404.9mg/ml and Acacia honey exhibited 111.05mg/ml (2). Indian commercial honey showed 22.40mg/ml of IC50 value (24). The most significant IC50 value was reported on strawberry tree honey 1.63 mg/mL (16).

Chemical profiling of Commercial Honey, Unprocessed Honey and Flower extract: Honey contain many compounds such as minerals, vitamins, sugar; all of them contributing on their chemical properties. In this study sugar concentration as well as total carbohydrate of honey were also determined, result were described in (Table 2). Flower contains more sugar than honey sample because they are the natural source of sugar and polysaccharide. Commercial honey showed the lower level of sugar than unprocessed honey. The average sugar content was 81.16 % and the range was 77.60- 83.80 % reported in Italian honey (17). pH of honey also plays a significant role in honey antiradical and antibacterial property. The pH values varied according to sample to sample because floral and geographic origins can cause great variations in honey pH values, as the nectar pH and soil conditions can greatly influence honey physicochemical characteristics. The low pH was responsible for all non-cationic bactericidal activity of MGO (Methylglyoxal) neutralized manuka honey against E. coli (25).

Antibacterial activity: Antibacterial properties of honey have been related to the level of hydrogen peroxide determined by its relative levels of glucose oxidase, catalase and other nonperoxide factors such as lysozyme, phenolic acids and flavonoids (21). Bactericidal activity was tested against each sample of honey (Table 3). Un processed honey sample G and H showed better antibacterial effects than other samples. Sample G and H were found to be effective antimicribial agent against *bacilius subtilis,* with a zone of inhibition 1.7 cm and 2.0 cm respectively. Interestingly, Samples A, B, D, F, G did not show any clear zone which indicated that they do not possess antibacterial activity. Honey has a low pH, mainly because of the conversion of glucose into hydrogen peroxide and gluconic acid by glucose oxidase. The high sugar concentration, MGO (Methylglyoxal), low pH contributed to the bactericidal activity of manuka honey (25). Clover honey made 1.6mm clear zone on bacterial plate (21). It has been reported that, Northeast Portugal honey, was effective against is *Staphylococcus aureus, Bacillus subtilis,* due to its phenolic compounds extracts (26). Taulang honey was effective against Gram positive bacteria (27).

Table 2. pH, sugar and total carbohydrate of honey samples

S.N.	Sample Name	Ph	Sugar Concentration (mg/ml)	Total Carbohydrate Concentration (mg/ml)
1	A	5.5	0.72±0.043	1.1±0.085
2	B	5.1	1.32±0.11	3.775±0.99
3	C	4.8	1.34±0.08	1.07±0.22
4	D	3.4	0.34±0.02	1.77±0.09
5	E	5.5	1.31±0.092	2.57±0.034
5	F	5.2	0.33±0.044	2.520.075
7	G	4.1	1.85±0.032	5.07±0.12
8	H	5.0	1.65±0.12	4.05±0.88

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Mass spectrum analysis: Honey samples were analyzed by mass spectrometer and several peaks were obtained in graph. A few peaks and their molecular mass were recognized and compounds were identified. Compounds were listed in (Table 4).

The results of MS suggest that, Sample A contain hydrotropic acid and geranyl nitrile, it was reported on Greek cottan honey. Sample B contain hydroxyl benzoic acid, ferulic acid, gallic acid, myricetin, benzene propanol. Benzene propanol was also reported on Greek cottan honey. Hydroxyl benzoic acid, ferulic acid, gallic acid, elenolic acid, acetoin, protectol PP are identify in sample C and cetoin has been mentioned as a eucalyptus honey marker. Acetoin, phenyl acetaldehyde, pinocembrin are

Table 3. Antibacterial activity of honey samples on *Bacillus cereus* (MTCC 441).

S.N.	Sample Name	Zone of Inhibition (Diameter in cm)
1	A	-
2	B	-
3	C	1.3±0.14
4	D	-
5	E	1.3±0.09
6	F	-
7	G	1.7±0.08
8	H	2±0.11
9	Positive control (chloramphenicol)	4±0.34

Table 4. Compounds present in honey samples as identified by MS analysis.

COMPOUND	A	B	C	D	E	F	G	H
Quinolin	-	-	-	-	-	-	+	+
Chloramphenicol	-	-	-	-	+	-	-	+
Acetoin	-	-	+	+	-	-	-	+
Gallic acid	-	+	+	-	-	-	+	+
Geranyl nitrile	+	-	+	-	-	-	-	+
Ferulic acid	-	+	+	-	+	+	+	-
Scopoletin	-	-	-	-	-	-	+	-
Phenyl ethanol	-	-	-	-	-	-	-	-
Syringic acid	-	-	-	-	-	+	-	-
Catechol	-	-	-	-	+	+	-	-
Phenyl acetaldehyde	-	-	-	+	-	-	-	-
Hydroxy benzoic acid	-	+	-	-	+	-	-	-
Nonanol	-	-	-	-	+	-	-	-
Pinocembrin	-	-	-	+	+	-	-	-
Ferulic acid	-	-	-	-	-	-	-	-
Elenolic acid	-	-	+	-	-	-	-	-
Myricetin	-	+	-	-	-	-	-	-
Benzene propanol	-	+	-	-	-	-	-	-
Hydrotropic acid	+	-	-	-	-		-	-
Coumaric acid	-	-	-	-	-	-	-	-
Chlorogenic acid	-	-	-	-	-	-	-	-
Kaempferol	-	-	-	-	-	-	-	-

Sample A-F, Commercial honey samples, Sample G-H, unprocessed honey samples.

present in Sample D. Sample E contain hydroxy benzoic acid, nanonal, ferulic acid, chloramphenicol, catechol, pinocembrin. Scopoletin, quinolin, Ferulic acid, gallic acid were identified in Sample G. Sample H contain chloramphenicol, quinolin, acetoin, gallic acid, geranyl nitrile. Similar to our study, Eucalyptus honey contained acetoin, aldimethyldisulphide, dimethyltrisulphidek, alkane and nonane. Methyl anthranilate lilac aldehyde, limonene diol, hotrienol and 1-p-menthen-al are characteritic compound of citrus honey that are absent in our all sample. 1-Penten-3-ol, phenylacetaldehyde, Isophoron was reported on Heather honey (28). Hesperetin, ellagic acid, and quercetin, ferulic acid, kaempferol, gallic acid, chlorogenic acid, myricetin, elenolic acid were identified in Malecian honey (29). 3-pyrrolidinyl-kynurenicacid (3-PKA), kynurenic acid were identified in chestnut honey (18). Homovanillyl alcohol, 2-methyl-*p*-phthalaldehyde, coniferaldehyde, hexyl-cinnamaldehyde, syringic acid hydrazide, scopoletin, scoparone were also reported on Greek cottan honey (22). Geranyl nitrile and acetoin are present in many sample but marker of citrus honey are absent as well as 3-PKA, kynurenic acid are also absent. Compounds of Greek cotton honey were mostly present in unprocessed and commercial honey sample. Heather honey component are also identified in our honey sample. All this data showing that heterofloral origin honey contain great number of phenolic and other volatile chemicals.

Conclusions

Six commercial and two unprocessed honey samples were evaluated for their total antioxidant capacities, chemical profiling and antibacterial activity. A comparison was also made with flowers belonging to *Rosa hybrid* (Rose) and *Calendula officinalis* (Marigold). Which were used as a source of nector by honey bees. It was observed that flower extracts and unprocessed honey samples exhibited high antioxidant capacity as compared to processed honey samples. In addition, an amount of total phenolic compounds and total flavenoids was also high in flower samples and unprocessed honey samples. MS analysis indicated presence of Quinolin and Gallic acid in these samples, which were absent in processed honey samples. In addition as we recognize that honey is the floral originated compound and physiochemical synthesized by plant were transferred to honey, thus these comment satisfied that flower extracts had higher phenolic content and antioxidant activity. Commercial honey might loose many essential phenolic compounds during the processing and storage. The process of repeated filtration and heating, reduces some important enzymatic and non enzymatic antioxidant compounds, therefore unprocessed sample exhibit the higher phenolic content, similarly antioxidant activity, thus it is essential and required to modify processing and storing method.

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Vacuolar Targeting of Cry1Ac and its Effects on Expression and Stability in Tobacco

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Abstract

Increasing heterologous expression of delta endotoxins of *Bacillus thuringiensis* in transgenic plants is being actively pursued as a means to increase their efficacy and to delay insect resistance. To examine if vacuoles could be used as alternate localization sites of delta endotoxins we developed binary vectors with a chimeric vacuole targeting signals and verified its localization efficiency by creating GFP fusions of Cry1Ac. Transgenic tobacco plants expressing Cry1Ac localized either to cytosol and vacuoles were generated and confirmed by PCR, QPCR and ELISA. Comparative protein expression analysis by quantitative ELISA showed that maximum, percentage total soluble protein of Cry1Ac was 0.64 and 1% in cytosol and vacuole targeted plants, respectively. However, detailed protein expression analysis showed that there are no significant differences in expression of Cry1Ac between cytosol and vacuole targeted plants. These results were further corroborated by immunoblot analysis as well as insect bioassays. Nevertheless, our study demonstrated that delta endotoxins could be targeted to vacuoles and expressed successfully which is of importance when gene stacking is being pursed where alternate localization sites are employed for different genes.

Key words: Cry1Ac, delta endotoxin, Vacuole targeting, *Helicoverpa armigera*

Introduction

The economic and environmental costs of insect control in agriculture and the losses incurred in spite of different measures are very high. As an alternative strategy, generation of exogenous *Bt* gene expressing insecticidal proteins in the transgenic plants has shown promise for increasing plant resistance to insects and reducing the use of traditional chemical insecticides (1). The delta endotoxin arsenal of *Bacillus thuringiensis* (*Bt*) possesses a wide array of insecticidal proteins that are active against different groups of insects. *Bt* proteins are specific to certain species of insects and are non-toxic to beneficial insects on crops, birds or mammals, including humans (2). This biotechnological approach of generation of *Bt* transgenic insect resistant plants is a major breakthrough in protecting crops from damaging infestation (1, 3).

Bacillus thuringiensis is a gram-positive spore forming bacterium that forms parasporal crystals during its sporulation. These parasporal crystals consist of one or more delta-endotoxins or crystal (Cry) proteins that makes *Bt* an effective insect pathogen. Insect midgut having an alkaline environment causes the crystal to dissolve and release the protoxins. Subsequently protoxin converts to 65-70 kDa N-terminal truncated form, the activated toxin through the action of midgut proteases (4). Sequential binding events of activated toxin with the diferrent insect gut

proteins leads to membrane insertion through specific binding to insect midgut epithelial cell receptors (4,5) and forms lytic pores in the microvilli of the apical membrane that kill the epithelial cells and eventually the insect (4, 6-9). Initial efforts in generation of viable transgenic crops carrying *Bt* genes resulted in failure due to the low expression of the transgene due to the prokaryotic nature of the gene and protein degradation. Innovations by using codon optimized genes (modifying coding sequences), deleting the mRNA destabilizing sequences, truncated genes (via deletion of C-terminal domain) have significantly increased the transgene expression levels in plants (10-14) providing viable insect resistant plants. However, with the wide spread use of different *Bt* crops in the field, the field evolution of insect resistance to delta endotoxin is an area of huge concern (15, 16). Insect resistance to *Bt* genes are reported to occur through different mechanisms that include reduced binding of Bt proteins to the midgut receptors, reduced activation of protoxins by midgut proteases, increased immune response and detoxification of Bt toxins and enhanced esterase removal (15, 17-20). However, different approaches have been proposed to curtail/delay this insect resistance. These include use of refugia, high protein dosage and gene stacking (21, 22). Of these, the use of refugia is carried out in the field. It is necessary to increase the expression of *Bt* genes to the maximum level possible without affecting the normal physiological state of the plant.

Targeting of the protein to different sub-cellular organelles has been envisaged to enhance the stability of the foreign proteins in the cells there by increasing the quantity of the heterologous proteins. This was proved to be right for a wide array of proteins (23-26). Similar strategy was adapted to Bt proteins but only the chloroplasts were tested (27-31). Vacuole acts as an alternate site for the storage of proteins in large quantities and is a promising candidate for the storage of heterologous proteins. The protein storage vacuoles are endoplasmic reticulum-derived cisterns that are specialized in stable protein storage and accumulation. They are characterized by absence of amino peptidases and since these organelles do not undergo membrane fusion with lytic vacuoles protein degradation are avoided. The vacuoles provide an attractive system for targeting heterologous proteins and an excellent sub-cellular location for long-term protein storage (32, 33). This approach has been successfully applied for different proteins such as chloramphenicol acetyl-transferase (34), human insulin (35), barley lectin (36), human lysozyme (37) to name a few. In addition, when phaseolin was expressed in transgenic tobacco under a constitutive promoter, it was sorted to the vacuole in leaf cells and was stored as protein body-like aggregates (38, 39). Therefore, we used codon optimized, truncated version of Cry1Ac and expressed it in cytosol as well as targeted it to vacuoles, to verify if expression of Bt proteins in plants could be increased by targeting them to vacuoles.

Materials and Methods

Construction of vector*: cry1Ac* gene was excised from pBinBt3 (40) using *BamH*I and *Sal*I restriction enzymes and cloned in corresponding sites present between CaMV35S promoter and OCS terminator of pBinAR (41) to obtain *pBinAc* and was used for cytosol targeting of the Cry1Ac protein. For targeting of the protein to the vacuole a 69 bp N-terminus peptide targeting sequence from soybean vegetative storage protein (VSPαS) (42) was amplified from total DNA isolated from soybean, using forward primer (5'-GGGGTACCATGAAAATGAAGG-3') and reverse primer (5'-CGGGATCCTCCATGGCATT-3') tagged with *Kpn*I and *BamH*I restriction sites respectively along with breathing nucleotide sequences to facilitate restriction digestion. This fragment was then cloned into *Kpn*I-*BamH*I sites of pBinAR binary vector containing *cry1Ac* sequence. 27 bp C-terminal vacuolar targeting sequence of tobacco chitinase A sequence (43) was added to the end of *cry1Ac* by extension PCR using a single forward primer (5'-GGGG TACCATGAAAATGAAGG-3') tagged with *Kpn*I

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restriction site along with four sets of reverse primers, RP-I (5'-TCCATTTCCTT CAGCCTC GAGTGTTGC-3'), RP-II (5'-GACT AAAAGT CCAT TTCCTTCAGC CTC-3'), RP-III (5'-CATAGTATCG ACTAAAAGTCCATT TCC-3') and RP-IV (5'-ACGCGTCGACTC ACATAGTAT CGACTAA -3') that are having overlapping sequences of the targeting signal. Fragment was amplified first with forward primer containing *Kpn*I site with breathing nucleotide and 'I' reverse primer. Amplified product obtained from the 'I' primer were again amplified with forward primer and 'II' reverse primer, product obtained from 'II' reverse primer has been PCR amplified with forward primer and 'III' reverse primer. Product obtained from the 'III' reverse primer were further amplified with forward primer and IV reverse primer containing *Sal*I restriction site with breathing nucleotides to obtain the final product containing C-terminal 27 bp tobacco chitinase A sequence. After sequencing confirmation of the product this fragment was cloned in *Kpn*I and *Sal*I restriction sites of the pBinAR binary vector to generate *pBinARVac-Ac* vector. To confirm the targeting of Cry1Ac to the sub-cellular compartments, *cry1Ac-GFP* fusions with respective targeting signals were constructed by cloning the *GFP* gene at the 3' end of the *cry1Ac* gene along with or without targeting signals using a similar strategy (Fig. 1).

Agroinfiltration, transient expression and microscopy: The cytosolic and vacuole targeted *cry1Ac-GFP* fusion constructs were mobilized into *Agrobacterium tumefaciens* EHA105 by freeze thaw method. *Agrobacterium* harboring the vector was inoculated in YEM media with appropriate concentrations of rifampicin and kanamycin. An overnight culture of *Agrobacterium* was harvested at 0.6 to 0.8, OD_{600} and sedimented at 4,000 ×g for 20 minutes, and washed in 25 ml of infiltration medium. Finally, *Agrobacterium* cells were suspended in 100 ml of infiltration medium supplemented with 10 mM $MgCl_2$, 25 mM MES-KOH (pH 5.6), and 200 μM acetosyringone. The bacterial solution was incubated at room temperature for four hours with gentle shaking in dark before infiltration. *Nicotiana tabacum var.* Petit Havana plants were grown for 4-5 weeks in the soil. *Agrobacterium* infiltration medium was applied using blunt-tipped plastic syringe and was forced into abaxial epidermis of fully expanded leaves of tobacco (44). After 72 hr of incubation, the leaves were washed twice with water and cut

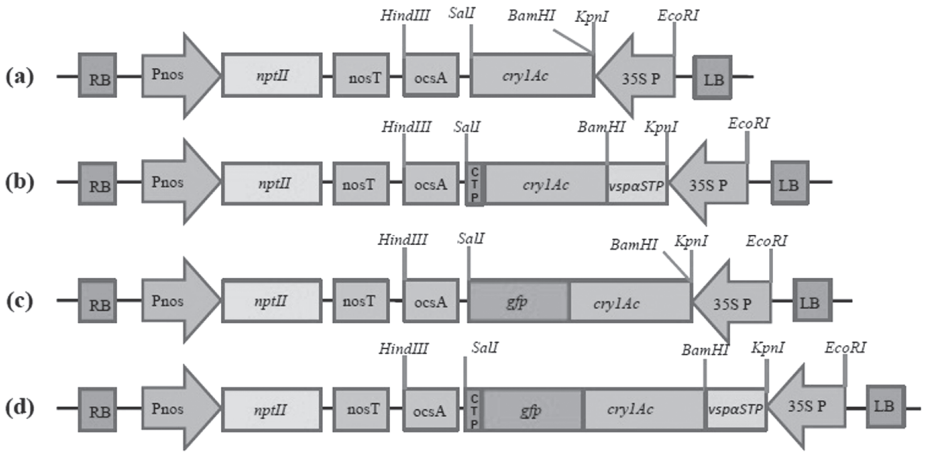

Fig. 1. T-DNA map of binary vectors (a) pBinAc (b) pBinVacAc (c) pBinAc-gfp (d) pBinVacAc-gfp. In all four vectors *cry1Ac* gene was driven by CamV35S promoter and selectable marker gene (*nptII*) was driven by nos promoter (Pnos). vspαSTP is N-terminal vegetative storage protein signal transit peptide from soyabean and CTP is C-terminal transit peptide from Chitinase A of tobacco

into small sections with a surgical blade. The cut sections were observed under confocal microscope from Leica, TCS SP6 (Germany).

A. tumefaciens-mediated transformation: Fully expanded tobacco leaves were excised from axenically grown wild-type plants and cut into 1-2 cm leaf discs. The explants were placed for 10 min in a culture of *A. tumefaciens* carrying the plasmid of interest, and then blotted on to sterile filter paper to remove excess *Agrobacterium* culture. Leaf discs were incubated at 25 °C in 16 h of light on shoot-inducing medium (MS salts containing 3 % sucrose, 2 mg/l 6-benzylamino purine and 0.1 mg/l α-naphthalene acetic acid). After 2 days, the leaf discs were transferred to MS agar containing 2 mg/l 6-benzylaminopurine, 0.1 mg/l α-naphthalene acetic acid, 300 mg/l kanamycin and 500mg/l cefotaxime for the selection of transgenic shoot growth. Elongated shoots were excised from calli and transferred on to 3 % sucrose MS agar, 300 mg/l kanamycin and 250 mg/l Cefotaxime. Transformed plants were grown at 25 °C for 16 hr in light in axenic conditions in the presence of kanamycin and were sub-cultured every 5-6 weeks.

ELISA: A double-antibody sandwich enzyme-linked immunosorbant assay (ELISA) was used to detect the presence of the Cry1Ac protein expressed in the leaves of transgenic plants. Experiments were performed with double sandwich quantitative Cry1Ac/Ab ELISA plates from Envirologix, Portland, USA. Proteins from leaf samples of targeted, untargeted and wild type tobacco plants were extracted using the protein extraction buffer provided in the kit. Leaf extract was diluted to fit in the linear range of the provided Cry1Ac standards and steps were performed essentially according to manufacturer's instructions. Halo MPR-96 microplate reader (Dynamica, Ottawa, Canada), was used to read the plate at 450 nm.

Western blotting: Sample proteins from young leafs of transgenic and wild type plants, was extracted using a protein extraction buffer (pH 9.5, 50 mM Na_2CO_3, 150 mM NaCl, 1 mM PMSF, β-mercaptoethanol). The total extracted crude proteins were quantified using Bradford reagent (Bio-Rad, Hercules, USA), and protein concentrations were determined against a standard of bovine serum albumin. Crude protein samples were then run on 12 % SDS-PAGE gel according to the method described by Laemmli (45) with a Dual Mini Slab Chamber (Bio-Rad). This protein gel was transferred to Immobilon-P membrane (Millipore, Billerica, USA) using a Mini Trans-Blot electrophoretic cell (Bio-Rad) by applying 40 V for 3 h and transferred to a blocking solution containing 5 % skimmed milk powder in phosphate buffered saline (PBS). This was followed by incubation in primary antibody (mouse anti-Cry1Ac antibody, Envirologix) at 1:5000 dilutions, and secondary antibody (goat anti-mouse IgG Alkaline phosphatase conjugate, GeNei, Bengaluru, India) at 1:5000 dilutions for 1 h each at room temperature. The signal was detected using BCIP/NBT substrate (Sigma-Aldrich, St Louis, USA) after developing for 5-10 min.

QRT PCR: Total RNA, from the transgenic and wild type plant leaves was isolated with the use of Plant RNA Isolation Kit (Sigma-Aldrich) following the manufacturer's instructions. It was visualized in 0.8 % agarose gel to confirm its quality and quantified using nanodrop (Thermo scientific). cDNA was synthesized with 1ìg of total RNA using oligodT (18mer) primers using SuperScript-III cDNA Synthesis System (Invitrogen, Carlsbad, USA). Real-time PCR was performed in a 96-well reaction plate by using Stratagene Mx3005P unit, using VeriQuest SYBR Green qPCR Master Mix (Affymetrix, Santa Clara, USA). For specific amplification of *cry1Ac*, oligonucleotides 5'-TTCTTGACGGAA CAGAGT TCG CCT-3', and 5'-TGGGTGGC ACATTGTTG TTCTGTG-3' were used as forward and reverse primers. Results were analyzed and calculated by comparative threshold cycle method according to the manufacturers' instructions for data normalization. L25 ribosomal protein from tobacco was used as a reference gene (46).

Northern blotting: Thirty micrograms of the total RNA extracted from the leaf sample of the tobacco plant was separated on a 1.5 % formaldehyde agarose gel in 1X MOPS buffer and transferred to a positively charged microporous (0.45-μm pores) nylon immobilon membrane (Millipore, India) by capillary method and fixed through ultraviolet cross-linking. The membrane was probed with 1 kb *cry1Ac* probe labeled with αP^{32}-CTP using Mega Prime DNA labelling kit (GE Healthcare, Buckinghamshire, UK), and hybridized in UltraHyb buffer (Ambion, Austin, USA) at 55°C. After hybridization, the membranes were washed with different stringency buffers and developed after 2 days.

Insect bioassay: Cotton bollworm (*Helicoverpa armigera*) insects were obtained from the Division of Entomology, IARI, New Delhi; and were reared on a semi-synthetic diet (47). Hatched eggs were maintained up to second instar larvae. Five healthy neonate larvae of cotton bollworm were released onto leaf discs placed on a moist filter paper. Leaf discs from non-transformed plants were used as controls. Each bioassay was repeated twice. These cups were stored at 27±1°C and larval mortality data was recorded at 24, 48, 72 and 96 hours post release of the larvae.

Results

Construction of vectors: Different vectors were constructed to target Cry1Ac protein either to cytosol or vacuole and also *Cry1Ac-GFP* fused vectors to verify the same (Fig. 1). The green fluorescent protein (GFP) coding region was fused in frame to the C-terminus of synthetic *cry1Ac* under the control of the 35S promoter, generating the vector *pBinCry1Ac-GFP* which is used as control in form of cytosol localization. The N-terminal leader sequence of soybean vegetative storage protein (VSPαS) that is responsible for targeting the protein to endoplasmic reticulum (42) and C-terminal signal sequence (GNGLLVDTM) from tobacco chitinase A that is responsible for targeting the protein to vacuole (43) were fused with *Cry1Ac-GFP* towards N-terminus and C-terminus respectively to target the protein into the plant vacuole; generating the *pBinARCry1Ac-GFPVac* (Fig. 1). Binary vectors that were devoid of GFP were used for stable transformation of tobacco plants and compared.

Agrobacterium mediated transient expression and localization of Cry1Ac-GFP fusion protein: Laboratory grown four to five week old tobacco plants were used for transient expressions studies. An infiltration medium containing the fusion construct *pBinCry1Ac-GFP* and *pBinARCry1Ac-GFPVac* was applied to abaxial epidermis of full-expanded leaves of the tobacco plant using blunt-tipped plastic syringe. Three days after the infiltration, the infiltrated portions of the leaves were examined for the GFP expression. Portions of leaves infiltrated with vector devoid of targeting signals showed a homogenous green fluorescence throughout the cells demonstrating that the GFP fused Cry1Ac is spread throughout the cytoplasm. Whereas the portions infiltrated with *Agrobacterium* harboring *Cry1Ac-GFPVac* vector showed localized green fluorescence in form of concentric rings confirming the vacuolar targeting GFP fused Cry1Ac and the ability of the targeting signals to localize the Cry1Ac protein to the vacuoles (Fig. 2).

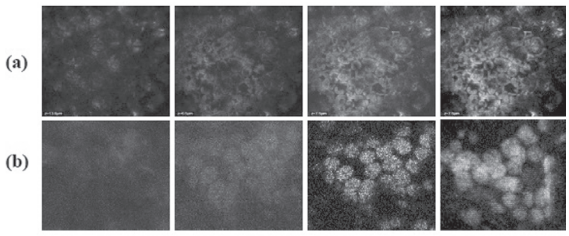

Fig. 2. Merged images of Ac-GFP fusions transient expression in tobacco leaf. (a) Cytosol localization (b) Vacuole localization

Agrobacterium mediated stable transformation of tobacco: Transformation of tobacco leaf discs was carried using *Agrobacterium* harboring *pBinAc* and *pBinVac-Ac* to obtain putative transgenic plants with Cry1Ac expressed

in cytosol and localized in vacuoles respectively. Initially, 15 independent plants regenerated from *Agrobacterium* mediated stable transformation of tobacco leaf discs with these two constructs were confirmed with PCR analysis for gene integration, Cry protein ImmunoStrip and qualitative ELISA for protein expression. Of these a total of 5 and 8 morphologically similar independent transgenic lines obtained with *pBinAc* (untargeted) and *pBinVac-Ac* (vacuole targeted) were selected and analyzed comparatively to determine the efficiency of vacuole localization to attain higher protein expression. The untargeted putative transgenic plants were confirmed by *cry1Ac* gene specific primers and the putative vacuole targeted *cry1Ac* plants were confirmed by PCR with a forward *cry1Ac* specific primer and a reverse primer specific to the C-terminal vacuole targeting signal (Fig. 3). Transgenic plants confirmed with PCR analysis were subjected to protein analysis using ImmunoStrips specific for *cry1Ab*/*Ac* gene products. Two bands appeared on each ImmunoStrip that are immersed into the extracts from transgenic plants as compared to the control where a single band appeared (Fig. 3). Also, expression analysis of mRNA was carried out with qRT-PCR for different transgenic lines to confirm the successful expression of the transgene. The

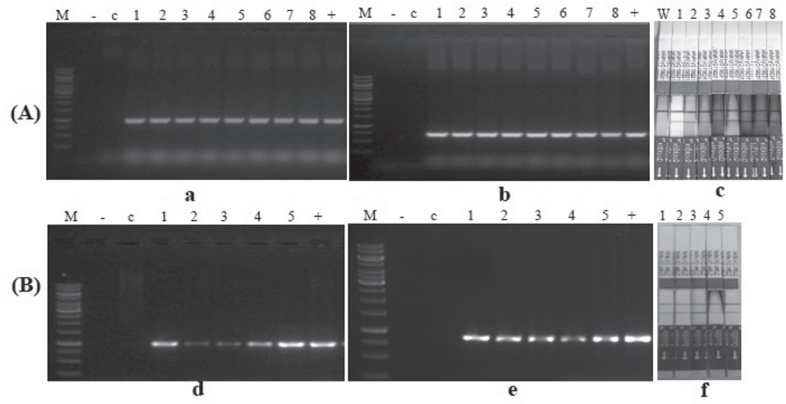

Fig. 3. Confirmation of putative transgenic plants. Panel A: confirmation of cytosol targeted tobacco transgenic plants. Panel B: confirmation of vacuole targeted tobacco transgenic plants. a and d: PCR analysis with *cry1Ac* specific primers. b and e : PCR analysis with *nptII* specific primers. C and f: Immunostrip assay

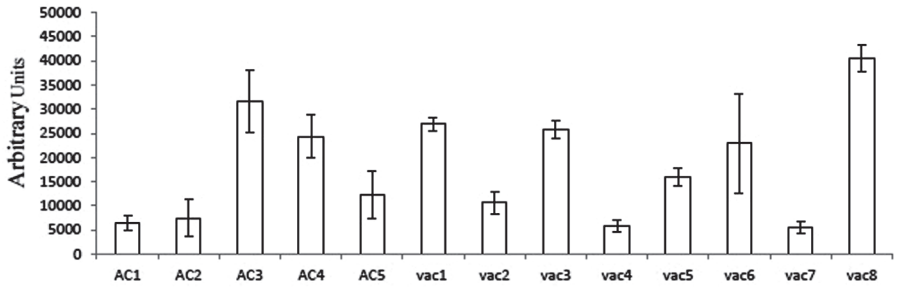

Fig. 4. qRT-PCR analysis. Ac1-Ac5 are cytosol targeted samples and vac1-vac8 are vacuole targeted samples

results showed that the lines that were selected were expressing the transgene and the expression levels were varied (Fig. 4).

Cry1Ac protein expression analysis: Quantitative ELISA was performed to determine the protein concentrations in independent transgenic lines belonging to both the groups. The average expression levels in terms of percentage of total soluble protein (% TSP) of Cry1Ac protein in the untargeted group of transgenic lines was 0.453 whereas for the vacuole targeted transgenic lines it was 0.458. Minimum and maximum levels of protein expression found in this study were 0.24 and 0.64 % TSP for the untargeted group and 0.2 and 1 % TSP for the vacuole targeted group respectively (Fig. 5). Immunoblot analysis of total soluble protein from the transgenic tobacco leaves was also performed to confirm the results of ELISA. The pattern of expression detected in the immunoblot analysis was similar to the results obtained with ELISA. The results obtained from ELISA experiments showed that there were no significant differences between the untargeted and vacuole targeted groups in terms of expression levels of Cry1Ac protein.

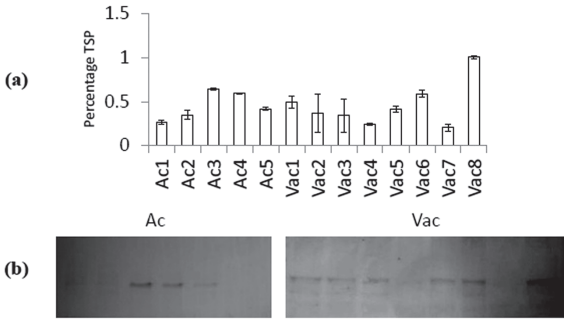

Fig. 5. Protein expression profile of different targeted groups. (a) quantitative ELISA (b) Western analysis. Ac, and Vac represents different lines of untargeted group and vacuole targeted samples

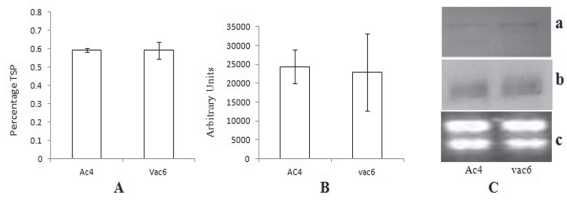

Fig. 6. Comparative expression profiling. A: ELISA, B: QRT-PCR, C: Blot analysis. a, b and c are Western Blot, Northern blot and loading control for the northern blots respectively

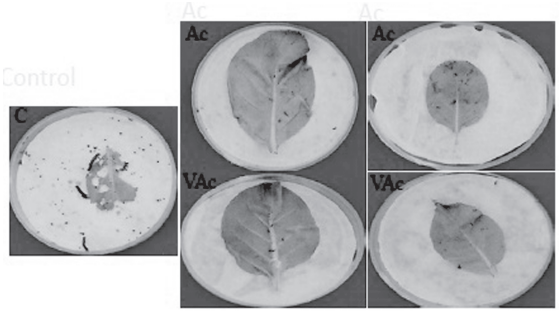

Fig 7. Leaf disk bioassay of wild type cytosolic *cry1Ac* and vacuole targeted *cry1Ac* transgenic lines against *H. armigera*

Western analysis and quantitative ELISA demonstrated that approximately equal levels of protein is expressed in the two different lines (Fig. 6).

However, to further validate these results we selected a single independent transgenic line from each group showing approximately equal levels of *cry1Ac* mRNA as assessed by qRT-PCR results (Ac4 and vacAc6) and performed comparative mRNA expression profiling by qRT-PCR and Northern analysis and also protein expression profiling by quantitative ELISA and Western analysis. QRT-PCR analysis and Northern analysis of the samples indicated approximately equal expression of *cry1Ac* mRNA, while Western analysis and quantitative ELISA demonstrated that approximately equal levels of protein is expressed in the two different lines (Fig. 6).

Insect bioassay: Transgenic lines expressing Bt-toxin Cry1Ac in the cytosol, as well as vacuole were also evaluated for entomocidal activity by insect feeding bioassays performed with second instar larvae of *H. armigera* to study if there are any differences in the biological activity of the expressed Cry1Ac protein among the two groups. Leaves from the young tobacco plants were fed to the larvae and mortality was monitored. Insects fed on the leaves of the transgenic lines showed retarded growth and also the leaves were less damaged when compared to the control non-transgenic leaves. A 40-60 % mortality rate was

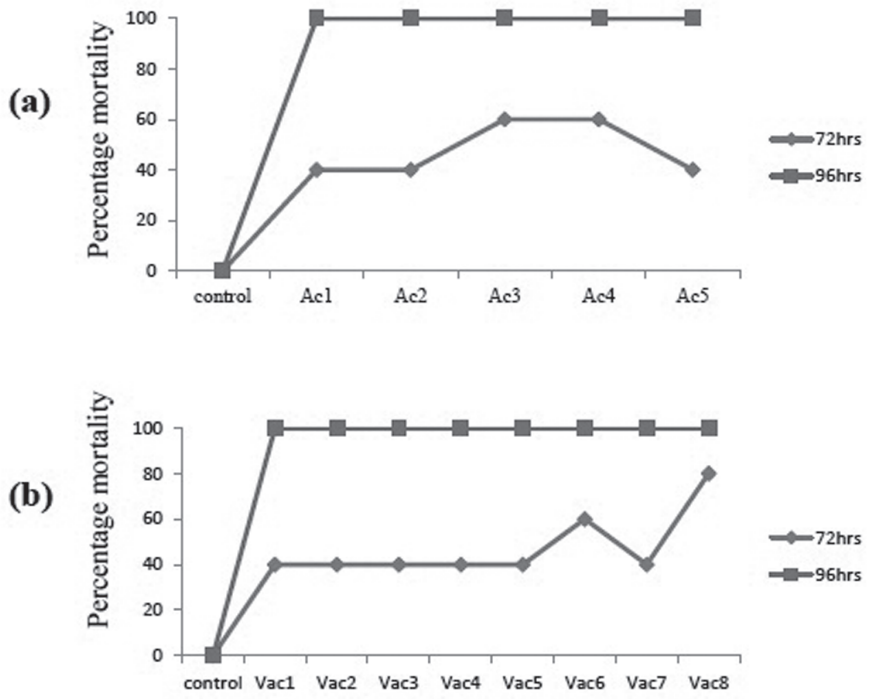

Fig. 8. Percentage mortality rate of *H. armigera* challenged with the leaf of different *cry1Ac* targeted lines of tobacco. (a) cytosol targeted *cry1Ac* transgenic lines (b) vacuole targeted *cry1Ac* transgenic lines. 72 and 96 hrs are the time intervals for mortality observation

observed in different transgenic lines of both the groups at 72hr. Also, the mortality rate corresponded to the Cry1Ac protein levels in both groups. At 96hrs 100 % mortality was observed in nearly all the lines (Fig. 7, Fig. 8).

Discussion

Different approaches were followed in increasing the transgene expression especially of *Bt* genes that lead to the development of viable transgenic events and thereby their commercialization. However, to sustain the success novel approaches to increase the transgene expression as well as stability are to be developed. Targeting of proteins to different cellular compartments of the plant cells has become an important and effective method to increase the production and stability of recombinant proteins. Targeting peptide sequences obtained from either plant or other organism have been utilized to target the protein in the various specialized organelle compartments such as apoplast, ER lumen, and vacuole, leading to a higher level of protein accumulation (48). In the present study, we comparatively analyzed the efficacy of delta endotoxin targeting to tobacco vacuole to find whether this is an ideal localization site for not only obtaining higher protein expression but also to increase biological efficacy.

In this study, we constructed a vacuole targeting vector with a chimeric targeting signal having an N-terminal ER targeting signal from Soybean vegetative storage protein (VSPáS) (42) and a C-terminus vacuole targeting signal from tobacco chitinase A (43) and tested the localization efficiency using GFP localization. Transgenic plants were developed with both the

untargeted and vacuole targeted vectors and the integration and expression of the transgene was confirmed with PCR, qRT-PCR and ELISA. We observed that there were no differences among the groups with regard to Cry1Ac expression. The average expression of Cry1Ac among the targeted and untargeted groups was approximately 0.45 %. Also, comparative analysis of lines showing similar levels of mRNA also supported that there were no differences in the expression of the transgene. Although, different reports demonstrated that higher expression of heterologous proteins was possible when targeted to vacuoles (34-39), present study shows that no added advantage can be obtained by targeting Bt proteins or at least truncated Cry1Ac to the vacuoles. The reasons behind the inability to increase the levels of Bt proteins could be multifold like the natural resistance of Bt proteins to different proteases, one of the main reason for increased heterologous protein expression when targeted to vacuoles, the acidic nature of the vacuole environment as against the alkaline environment required to solubilize the Bt proteins. The cytosol is having a neutral environment, which is also not ideal for the Bt proteins. Since the same constraints are present for the expressed Cry1Ac protein in the cytosol and vacuole, it may be the probable reason behind no added advantage of targeting Bt proteins to the vacuole. However, our present study shows that the vacuole localization can still be used for expressing of Bt proteins on par with the cytosol and this could be especially useful when adopting the gene pyramiding strategy.

Conclusion

In summation, we have created vectors that can efficiently target Cry proteins to the vacuole and generated transgenic tobacco plants with Cry1Ac localized to cytosol and vacuoles. Detailed protein expression analysis showed that there are no significant differences in the expression of Cry1Ac between cytosol and vacuole targeted plants. This result was further corroborated by immunoblot analysis as well as insect bioassays. However, our study demonstrated that delta endotoxins could be targeted to vacuoles and expressed successfully albeit with no added advantage over cytosol expression.

Acknowledgments

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In vitro rooting efficiency in *Morus indica* cultivars (S34, S54, M5 and Mysore-local) from *in vitro* shoot cultures

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Abstract

Development of plant tissue culture has contributed greatly to our understanding of the factors responsible for the growth and differentiation of plant cells which resulted in regenerating whole plants from the cultured material. Mulberry plant is usually heterozygous due to its cross pollination habit. Use of micropropagation as an integral component of tree improvement program has lagged behind for some important forest tree crops because of the failure to develop protocols for successful root induction. An attempt has been made to describe an efficient root induction protocol from *in vitro* regenerated shoots of all the mulberry cultivars (cvs. S34, S54, M5 and Mysore-local) under study. Efficient rooting 92% response was observed on MS media containing IBA with 1.0mg/l and 61% of rooting response noted on media containing1.0mg/l IAA with mulberry cultivars (cvs. S34). The rooting ability was found to be superior in cvs. S34 when compared to other cultivars of mulberry.

Keywords: Murashige and Skoog, Indole-3-Butyric acid, Indole acetic acid, Cultivar (cv.).

Introduction

One of the essential requirements for the successful application of plant propagation technology to agriculture is the capacity to regenerate elite plantlets. The successful outcome to micropropagate plantlets through tissue culture essentially depend on induction of rooting. Mulberry leaves show the synthesis of pharmaceutical and commercial products such as the sericin protein consumed by *Bombyx mori* yields cocoons. The leaf biomass produces energy rich substances like vitamins A and D, also helps in prevention of certain gastric disorders (1). Any means of micropropagation leads to success only on establishing efficient root induction protocols in tissue culture (2,3). Micropropagation as an important component of crop improvement systems has lagged behind for some significant tree crops because of the failure to develop techniques for successful root initiation (4,5). Problems encountered during rooting of *in vitro* regenerated shoots are mainly related to the phenomenon of callus induction from the cut ends of the shoots, when high auxin concentration is usually applied for root initiation (6). Here we describe efficient root induction (7) and development protocol from *in vitro* regenerated shoots (8,9) of all the mulberry cultivars. Our study demonstrates the rooting ability to be superior in cv. S34 (Plate -I Fig. a-d) compared with other cultivars (M5, Mysore-local and S54) under study.

Materials and Methods

Nodal explants of four mulberry cvs. namely M5, S34,S54 and Mysore-local were collected from 3-4 months old field grown mature plants (10). Explants were surface disinfected with 0.1 % HgCl2 for 3-5 minutes and followed by 4-5 rinses in sterile distilled water. The explants were

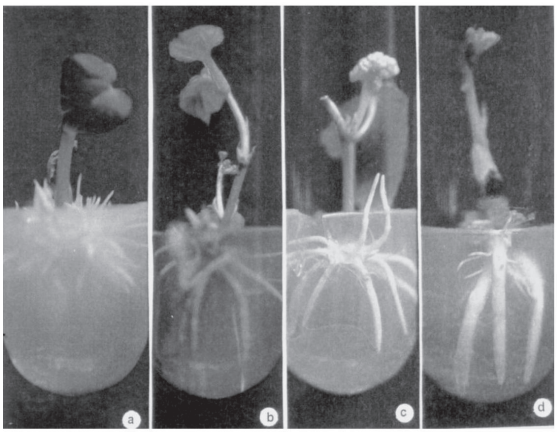

Fig. 1(a-d). Efficient rooting performance recorded from *in vitro* regenerated shoots in mulberry cv.S34 on MS + 1 mg/l IBA.

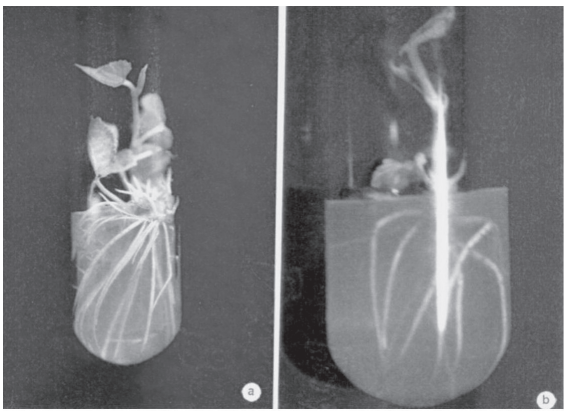

Fig. 2. (a). Root initiation from *in vitro* regenerated shoots on MS + 1 mg/l IBA in mulberry cv. S34. **(b).** Root induction on MS + 1 mg/l IBA from *in vitro* grown shoots in mulberry cv.M5

cultured on MS medium supplemented with different concentrations (0.5-2 mg/lit) of BA or KN and combinations of BA (1.0 mg/lit) and KN (0.5-2 mg/lit). The pH was adjusted to 5.7 and solidified with 0.8% difco bacto agar. Single explants were inoculated in each culture tube and incubated at 25 ± 2 °C for 2-3 weeks under 16 hours photoperiod.

In vitro regenerated shoots were rooted on full strength MS medium (11,12) supplemented with auxins like IAA or IBA (0.5 - 2.0 mg/l) after 3-4 weeks of culture (13). Percentage of rooted plants, average number of roots per shoot and root length was recorded in all the cultivars (S34, M5, Mysore-local and S54) after 20-25 days in culture.

Results and Discussion

***Effect of IBA*:** Effect of IBA on *in vitro* root development and the percentage of response, average number of roots and average root length in all the mulberry cultivars were studied. Regenerated shoots (14, 15, 16) from all the cultivars responded with a high rooting efficiency at 1 mg/l IBA in cv. S34 (92%) (Fig-2(a)) followed by cvs.M5 (80%) (Fig-2(b)), Mysore-local (72%) (Fig-3(b)) and S54 (60%) (Fig-3(a)). However at 0.5 mg/l IBA there was no significant response when compared with 1 mg/l IBA. The average number of roots produced at 0.5 mg/l IBA was less when compared to higher concentrations (1.0 – 1.5 mg/l) of IBA.

In vitro regenerated shoots of cvs. M5, S34, S54 and Mysore-local were cultured on MS medium supplemented with different concentrations of IBA (0.5 – 2.0 mg/l). From the above concentrations 1 mg/l IBA was reported to be optimum for root induction in all the cultivars studied.

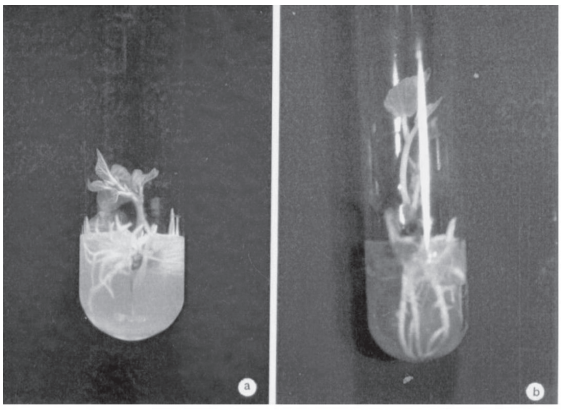

Fig. 3. (a). Root initiation from *in vitro* regenerated shoots on MS + 1 mg/l IBA in mulberry cv. S54. **(b).** Root induction on MS + 1 mg/l IBA from *in vitro* grown shoots in mulberry cv.Mysore-local.

The average number of roots were found to be maximum in cv. S34 (19.2 ± 0.3) (Table-1,) followed by cvs. M5 (13.6 ± 0.2) (Table-2), Mysore local (9.6 ± 0.2) (Table-3) and S54 (8.2 ± 0.2) (Table- 4) at 1 mg/l IBA. There was a gradual decrease in the average number of roots with increase in concentrations (1.5 – 2.0 mg/l) of IBA. Maximum mean length of the root was recorded in cv. S34 (6.5 ± 0.6) followed by cvs. M5 (4.5 ± 0.4), Mysore-local (3.2 ± 0.4) and S54 (2.2 ± 0.2) at 1 mg/l IBA. At higher concentrations of IBA (1.5 – 2.0 mg/l) showed a gradual decrease in the mean length of the root in all the cultivars studied. Moreover, 0.5 mg/l IBA was reported to be least favorable for root induction.

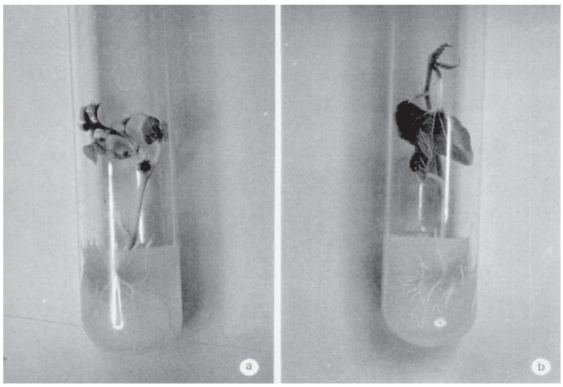

Fig. 4. (a). Root initiation from *in vitro* regenerated shoots on MS + 1 mg/l IAA in mulberry cv. S34. **(b).** Root induction on MS + 1 mg/l IAA from *in vitro* grown shoots in mulberry cv.M5.

***Effect of IAA*:** Effect of IAA on *in vitro* root development, percentage of response, average number of roots and average root length was also studied. Regenerated shoots cultured on rooting medium showed highest percentage of rooting in cv. S34 (61%) (Fig-4 (a)) followed by cvs. M5 (48%) (Fig-4 (b)), Mysore-local (40%) (Fig-5(b)) and S54 (32%) (Fig-5(a)) at 1mg/l IAA. Percentage of response was poor at 0.5 mg/l IAA and was less significant when compared to higher concentrations of IAA (1.5 and 2.0 mg/l).

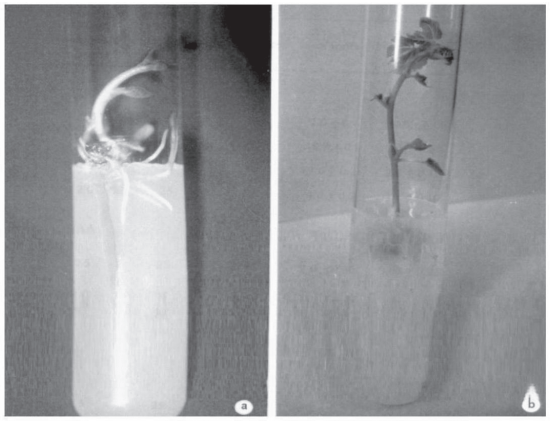

Fig. 5. (a). Root initiation from *in vitro* regenerated shoots on MS + 1 mg/l IAA in mulberry cv. S54. **(b).** Root induction on MS + 1 mg/l IAA from *in vitro* grown shoots in mulberry cv. Mysore-local.

The average number of roots grown were found to be high in cv. S34 (9.6 ± 0.3) (Table-1) followed by cvs. M5 (9.2 ± 0.2) (Table-2), Mysore local (7.8 ± 0.2) (Table-3) and S54 (6.0 ± 0.2) (Table-4) at 1 mg/l IAA. There was a considerable decrease in the average number of roots produced at higher concentrations (1.5 and 2.0 mg/l).

The mean length of the root was maximum at 1.0 mg/l IAA in all the mulberry cultivars studied. It was found to the maximum in the cv. S34 (5.7 ± 0.6) at followed by cvs M5 (3.0 ± 0.4), Mysore – local (2.2 ± 0.1) and S54 (1.4 ± 0.1) at 1 mg/l IAA. There is a gradual decrease in the mean length of the root at higher concentrations (1.5 & 2.0 mg/l).

The study is conducted to mark the rooting efficiency and also to determine the effect of auxins on rooting performance of *in vitro* raised mulberry plants. It was found that 1 mg/l IBA was more favorable in inducing *in vitro* root development in mulberry. The size and length of the root were variable in different mulberry cultivars. Root length is maximum in S34 and M5 followed by Mysore local and S54.

The thickness of the root in S34 and M5 were more when compared with Mysore-local and S54. At 0.5 mg/l IBA thickness was found to be less, but it increased when higher concentrations (1.5 and 2.0 mg/l IBA) were used. Roots showed

positively phototropism at the early stages of development. Root induction was extensive in terms of length, thickness and size at 1.0 and 1.5 mg/l IBA and was not much significant at 0.5 mg/l IBA. The highest root number was recorded in S34 followed by M5, Mysore local and S54. Effect of IAA is poor when compared to IBA in the root initiation from *in vitro* regenerated shoots of

Table 1. Effect of different concentrations of IBA and IAA individually on rooting performance of *in vitro* grown shoots in mulberry cv. S34.

Growth regulators (mg/l)	No. of Cultures Maintained	% of response	Avg.no. of roots	Avg. root Length
IBA				
0.5	25	15	2.6 ± 0.1	0.82 ± 0.09
1.0	**25**	**92**	**19.2 ± 0.3**	**6.5 ± 0.60**
1.5	25	55	7.6 ± 0.2	2.1 ± 0.10
2.0	25	28	4.0 ± 0.2	1.1 ± 0.10
1AA				
0.5	25	9	2.0 ± 0.06	0.68 ± 0.08
1.0	**25**	**61**	**9.6 ± 0.3**	**5.7 ± 0.60**
1.5	25	46	6.8 ± 0.2	1.6 ± 0.10
2.0	25	17	3.2 ± 0.09	0.96 ± 0.09

Values are mean of 25 explants ± S.E

Table 2. Effect of different concentrations of IBA and IAA individually on rooting performance of *in vitro* grown shoots in mulberry cv. M5.

Growth regulators (mg/l)	No. of Cultures Maintained	% of response	Avg.no. of roots	Avg. root Length
IBA				
0.5	25	12	2.1 ± 0.1	0.74 ± 0.08
1.0	**25**	**80**	**13.6 ± 0.2**	**4.5 ± 0.40**
1.5	25	48	6.4 ± 0.2	1.9 ± 0.10
2.0	25	22	3.6 ± 0.2	0.98 ± 0.10
1AA				
0.5	25	6	1.6 ± 0.04	0.46 ± 0.06
1.0	**25**	**48**	**9.2 ± 0.2**	**3.0 ± 0.4**
1.5	25	36	5.6 ± 0.1	1.4 ± 0.1
2.0	25	12	3.0 ± 0.09	• 0.08

Values are mean of 25 explants ± S.E

Table 3. Effect of different concentrations of IBA and IAA individually on rooting performance of *in vitro* grown shoots in mulberry cv. Mysore-local.

Growth regulators (mg/l)	No. of Cultures Maintained	% of response	Avg.no. of roots	Avg. root Length
IBA				
0.5	25	9	1.85 ± 0.09	0.52 ± 0.07
1.0	**25**	**72**	**9.6 ± 0.20**	**3.2 ± 0.40**
1.5	25	36	5.0 -± 0.20	1.5 ± 0.10
2.0	25	19	3.0 ± 0.20	0.9 ± 0.10
1AA				
0.5	25	4	1.2 ± 0.04	0.36 ± 0.06
1.0	**25**	**40**	**7.8 ± 0.20**	**2.20 ± 0.10**
1.5	25	28	4.2 ± 0.10	0.98 ± 0.10
2.0	25	8	1.5 ± 0.06	0.42 ± 0.08

Values are mean of 25 explants ± SE

Table 4. Effect of different concentrations of IBA and IAA individually on rooting performance of *in vitro* grown shoots in mulberry cv. S54.

Growth regulators (mg/l)	No. of Cultures Maintained	% of response	Avg.no. of roots	Avg. root Length
IBA				
0.5	25	4	1.24 ± 0.03	0.38 ± 0.06
1.0	**25**	**60**	**8.2 ± 0.20**	**2.2 ± 0.20**
1.5	25	30	4.2 ± 0.20	1.2 ± 0.10
2.0	25	16	2.8 ± 0.20	0.85 ± 0.09
1AA				
0.5	25	2	1.0 ± 0.02	0.18 ± 0.02
1.0	**25**	**32**	**6.0 ± 0.20**	**1.4 ± 0.10**
1.5	25	16	3.6 ± 0.09	0.64 ± 0.09
2.0	25	4	1.21 ± 0.06	0.21 ± 0.02

Values are mean of 25 explants ± S.E

mulberry. Studies on the use of IBA in rooting ripe mulberry cuttings is a well established fact. Efficient rooting 92% response was observed on MS media containing IBA with 1.0mg/l with mulberry cultivars cvs. S34 (Fig-1(a-d)).

Conclusion

Among the different mulberry cultivars studied cv S34 showed better response in rooting efficiency when compared to other cultivars. Efficient root induction results in better nutrient

absorption which in turn helps in enhancing the leaf biomass for commercial production of silk and improving other qualitative and quantitative traits.

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Mutative Strain Improvement of Bacterial Soil Isolates for Enhanced Lipase Activity

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Abstract

Bacterial lipase isolates, B1, B3 & B4 were isolated and selected through various screening procedures for best lipase isolates. These best lipase producing isolates were subjected to the strain improvement techniques by UV radiation, microwave radiation and ethidium bromide treatment for further screening and isolation of best wild and mutant lipase producing isolates. In the results, the UV irradiation method produced the maximum number of mutant strains yielding to different exposure time intervals compared to other strain improvement techniques. However, the UV irradiation at an exposure of 30s produced the best mutant lipase producing isolate named as B3UVS30 from 11.49% survived colonies of B3, producing 24mm of clear lipolytic zone and 14.5U/ml of lipase activity subjected to agar well diffusion method and titrimetric method respectively. Similarly, microwave irradiation at an exposure of 30s produced the second best mutant lipase isolate named as B1MWS30 from 57.26% survived colonies of B1, recording 16mm lipolytic zone and 12 U/ml lipase activity subjected to agar well diffusion method and titrimetric method respectively. Conversely, ethidium bromide treatment did not produce any positive mutants and further its effect resulted in the loss of lipase activity and left distorted morphology in the cultures of B1, B3 & B4. In case of isolate B4, only UV irradiation treatment method produced 10 better lipase producing mutants, but they were unstable in the subsequent generations resulting in either reduced or lost lipase activity. So the lipase isolate B3 was subjected to 16S rDNA sequencing and subsequently identified and named this parent isolate B3 as *Pseudomonas* sp. SPSU B3 and its mutant B3UVS30 as *Pseudomonas* sp. SPSU B3UVS30. Hence this bacterial isolate and its mutant were stored and the novel isolate *Pseudomonas* sp. SPSU B3 is made available at national agriculturally important culture collection repository (Acc. No.: NAIMCC-B-01768) for further studies in the frontiers of biotechnology as a potential bacterial lipase producing strain and for its use in commercial and academic institutions.

Keywords: Mutants, UV irradiation, Microwave, Ethidium bromide, Bacterial lipases, *Pseudomonas*.

Introduction

The microbial derived lipases (1-8) are of great demand for their known reputation in the bioprocess industries (9, 10). This reputation of microbial lipases especially in biotechnological processes and systems could be attributed to their broad spectrum of biochemical activities in microbial organisms and the amicability of microbial systems for genetic manipulation using the latest developments in technology for better enzyme production, purification and characterization (11-15). Various kinetic models for enzyme functioning, activation and deactivation with the reaction parameters of substrate, activator, modulator, inhibitor, enzyme concentration, pH, redox potential, temperature etc. are extensively used for design and

development of enzyme linked bioprocesses (16-18). However for higher enzyme production, some of the important improvement techniques employed at microbial level by many researchers are UV-irradiated mutation, point mutation, chemical mutation, microwave irradiated mutation etc. leading to the selection of improved mutant microbial strains (19-24). In this report, selective strain improvement studies were carried out on the soil bacterial lipase isolates, using different mutative techniques, to screen and isolate the better lipase producing bacterial isolate(s).

Materials and Methods

***Screening of lipolytic bacteria*:** The lipolytic bacteria were obtained from soil samples collected from Guntur, Nagpur and Udaipur by serial dilution method & pour plate method using trybutyrin agar media (25). The bacterial isolates were further screened using Rhodamine-B agar plates (26), agar well diffusion method (27-28) and lipase titrimetric method (29-32). The obtained bacterial lipase isolates were further subjected to optimization studies and the best lipase producing bacterial isolates were named as B1, B3 & B4 (33-34). These isolates were sub-cultured once in fortnight and preserved them in agar slants at 4^{O}C in the refrigerator for the use of regular experiments.

***Preparation of cell suspensions*:** A loopful culture of bacterial isolates B1, B3 & B4 were transferred from slants into the respective 50 ml nutrient broth aseptically and incubated them at 30^{O}C for 24 h at 150 rpm in an orbital shaker incubator. Broth cultures of 1.5ml from the culture flasks of B1, B3 & B4 were withdrawn aseptically in vials and centrifuged at 10000g and 4^{o}C for 10 min. The supernatant of each vial was drained and the biomass pellet was dissolved in 1 ml of sterile saline water under aseptic conditions and these cell suspensions were adjusted to a concentration of 10^{5}cfu/ml separately for subsequent experiments (35).

***Mutagenesis by UV irradiation method and selection of mutant strains*:** The prepared cell suspensions from B1, B3 & B4 cultures were distributed (0.5ml) to their respective sterile glass petri plates and exposed to UV irradiation (254nm) for variable time intervals (19, 36-38) ranging from 5 seconds to 60 minutes, keeping the vertical distance of UV source fixed at 45cm. Sterilized tributyrin agar media were poured aseptically in to the UV irradiated culture petri plates by pour plate method and placed them in dark for 3hrs; subsequently incubated then at 30^{O}C for 48 h. Emerged colonies in the respective plates were counted along with the control plate without UV irradiation. The positive mutated bacterial colonies with larger clear zones of hydrolyzed tributyrin as compared to the respective wild strains of B1, B3 & B4 were transferred aseptically to nutrient agar slants and named as shown in Table-1. These isolates were subcultured on nutrient agar slants regularly and stored at 4^{O}C in the refrigerator until use.

***Mutagenesis by microwave treatment and selection of mutant strains*:** The prepared cell suspensions of B1, B3 & B4 cultures were (5ml) placed aseptically in sterile test tubes and kept them in cold conditions and placed them in microwave oven (800W). These culture suspensions were subjected to microwave irradiation (39) for periods of 30s, 60s, 90s, 2min., 3min., 4min., 5min. and 10min. separately. These irradiated cultures were taken (1ml) separately in sterile petri plates and sterilized tributyrin agar media were poured by pour plate method aseptically and further processed similar to that of UV-treatment method.

***Mutagenesis by ethidium bromide treatment and selection of mutant strains*:** A stock of 5 μg/ml Ethidium bromide solution was prepared and 5 ml from the solution was added to 45 ml of sterile nutrient broth medium (40). These media were inoculated with 1ml of the respective prepared cell suspensions of lipase isolates B1, B3 & B4 aseptically and incubated them at 30^{O}C & 150 rpm in an orbital shaker incubator. Broth samples of 1.5ml from each culture flask were withdrawn aseptically at 30, 60, 90, 120, 150 and 180 min. of incubation periods and centrifuged them at 10,000g & 4^{O}C for 10 minutes. The

supernatant of each sample was discarded and the respective biomass pellets were re-dissolved with sterile saline water and further centrifuged at 10,000g and 4°C for 10 minutes for washing the cells and to remove the traces of ethidium bromide. In this way the cells were washed twice and then used the resultant biomass pellets to make the cell suspensions separately to a concentration of 1x 10^5 cfu/ml. Each cell suspension of 1 ml was taken in separate sterile petri plates and poured the sterile trybutyrin agar media for isolating the mutant strains using pour plate method. These samples were further processed similar to that of UV-treatment method.

***Screening for the best lipase isolates*:** The isolated positive mutants along with the wild strains of B1, B3 & B4 were further cultured in trybutyrin broth for 24hrs at 30°C & 150 rpm, and they were streaked on to trybutyrin agar plates by streak plate method for visual detection of lipase activity. The cell free supernatant of these cultures were prepared by centrifuging 1.5ml of broth at 10,000g & 4°C for 10 minutes separately and used them as crude lipase enzyme for agar well diffusion method (27-28) and titrimetric method (29-32) for quantitative lipase estimation. All the samples were tested in duplicates for both agar well diffusion method and the titrimetric method of lipase assay and the results were expressed as an average value for each strain. These results were also depicted in the Figures 1-4.

***Identification of bacterial soil lipase isolate B3*:** The bacterial soil lipase isolate B3 was cultured at 30°C and 150 rpm for about 12hrs and then isolated the genomic DNA form the biomass pellet, of 1.0ml broth sample centrifuged at 5000g for 10min, using the Xcelgen bacterial genomic DNA isolation kit (XG 2411-01) following the manufacturers protocol. The purified genomic DNA was further processed in Eppendorf Thermal cycler with 16S rRNA gene primers (41) and obtained the 1500bp 16S rDNA fragments that were subsequently purified using Mini Elute Gel Extraction Kit (QIAGEN) following the manufacturer protocol. These purified 16S rRNA genes of bacterial isolate B3 were subjected to automated DNA sequencing (42-43) on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA) and subsequently obtained the 16S rRNA gene sequences that were analyzed with

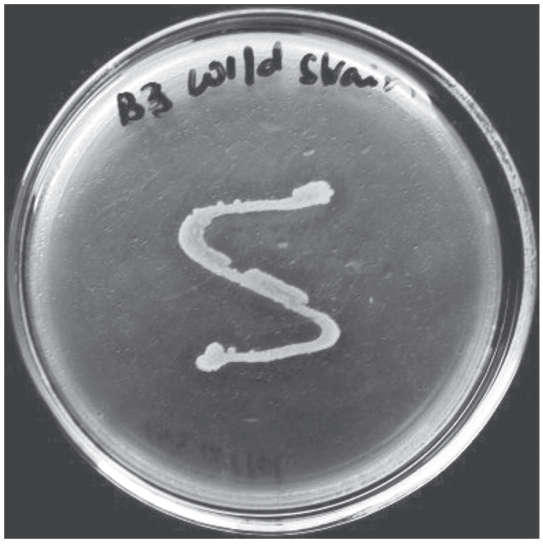

Fig. 1. Lipolytic activity of bacterial isolate B3 (wild) on trybutyrin agar plate.

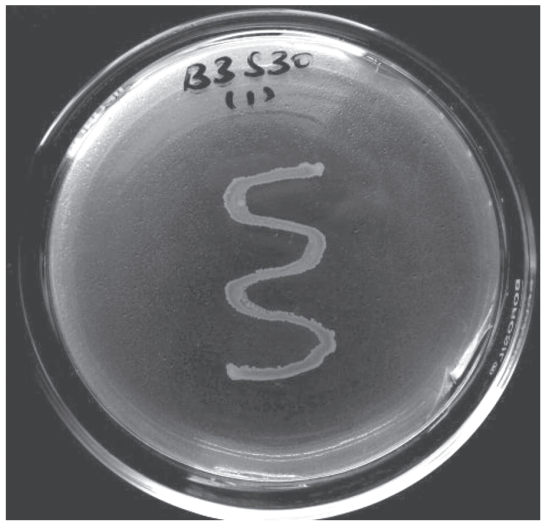

Fig. 2. Lipolytic activity of bacterial mutant isolate B3S30 on trybutyrin agar plate after UV irradiation treatment**.**

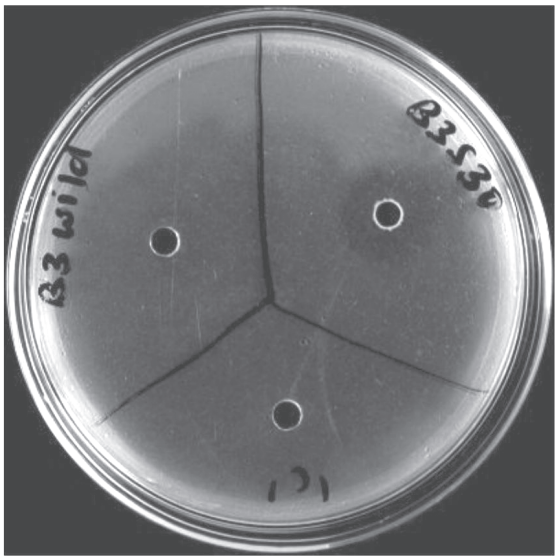

Fig. 3. Agar well diffusion assay of crude lipase from B3 wild and its UV irradiated mutant isolate (B3S30) on trybutyrin agar plate.

BLASTN, Clustal W and MEGA 4.0 software (44-45).

Results and Discussion

The microbial lipase isolates B1, B3 & B4 were further screened by strain improvement techniques of mutagenesis using UV radiation (254nm) and microwave radiation (800W) and the ethidium bromide (0.5mg/l) with an aim to enhance the lipase production and activity levels in their respective cultures. The results of these mutagenic strain improvement methods were depicted in the Tables 1 & 2. However ethidium bromide did not produce any positive lipolytic bacterial colonies of isolates B1, B3 & B4. In addition it was found that ethidium bromide killed most of the bacterial lipase isolates and the remnant colonies after 48hrs of incubation period lost their lipolytic activity.

The UV irradiation method (Table-1) produced considerable number of mutant isolates for B3 & B4, and a few mutants for B1. Nevertheless the number of selected mutant isolates based on the larger clear zone formation in the trybutyrin agar plates, were found more for the UV irradiation period of 30s among all the strains under the study. But in the subsequent

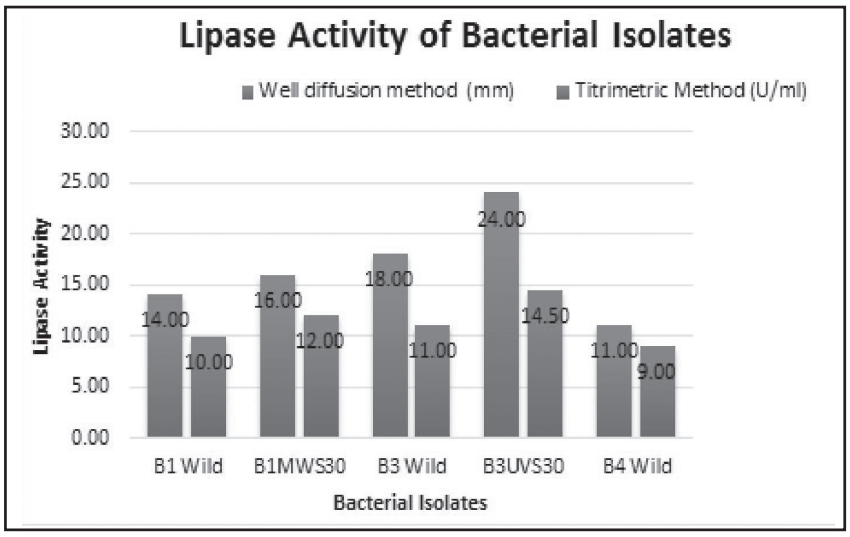

Fig. 4. Comparative lipase activities of wild and mutant bacterial isolates

generations the selected mutants B1S15 from 81.69% survived colonies and B1S30 from 39.44% survived colonies lost their higher lipase activity, indicating the unstable mutagenesis and the greater capability of repair mechanism of B1. Similarly the UV irradiated B4 produced the better lipase producing mutants B4S15 from 21.08% survived colonies, B4S30 from 9% survived colonies & B4M4 from 3.86% survived colonies, but then in the subsequent generations of experiments these selected mutants lost their lipase producing activity to a greater extent. On the other hand, the UV irradiated B3 produced the better lipase producing mutant isolates as B3S15 from 18.68% survived colonies & B3S30 from 11.49% survived colonies and in the subsequent experiments it was found that only one of the B3S30 mutants retained the higher lipase activity as shown in the Table-1 and the subsequent confirmatory results were shown in the Figures 2 & 3. Hence this stable mutant strain was named as B3UVS30.

The microwave irradiation for the generation of better lipase producing mutant strains was found to be moderate (Table-2) and could generate few mutants of B1 as B1S30 from the 57.26% survived colonies, B1S90 from 17.32% survived colonies & B1M2 from 11.45% survived colonies whereas for isolate B3, the selected mutants were only two as B3S60 from 19.83% survived colonies and on the least side, for B4, none of the mutants were found to be better lipase producing isolates. In case of the strain B3, the selected mutants either lost or decreased their lipolytic activity in the subsequent experiments. Conversely the strain B3 has produced one single stable mutant isolate named as B1MWS30.

The mutant strains, B1MWS30, B3UVS30 along with the wild strains B1, B3 & B4 were further studied for their lipolytic activity using well diffusion method and titrimetric method for screening and isolation of best lipase producing isolates and the subsequent results expressed as an average of triplicates were shown in Fig- 4. It may also be observed from Figures 3 & 4 that the strain B3UVS30 emerged as the best mutant strain with lipolytic zone of 24mm and lipase activity of 14.5U/ml. So the lipase producing bacterial soil isolate B3 from the 16S rDNA sequencing analysis was found closest to the *Pseudomonas* sp. strain WCH22 (HQ143645.1) and hence named as *Pseudomonas* sp. SPSU B3 (GenBank Accession Number JX524282) and its corresponding best mutant as *Pseudomonas* sp. SPSU B3UVS30 (46). So this novel lipase producing soil isolate *Pseudomonas* Sp. SPSU B3 was deposited to National Agriculturally Important Culture Collection (NAIMCC) of National Bureau of Agriculturally important microorganisms, Mau, Uttar Pradesh, India, and it is available with the culture accession number NAIMCC-B-01768.

Conclusions

The strain B3UVS30 was the best mutant isolate obtained from the parent isolate B3 using UV-mutagenesis. So, it may also be suggested that 30 seconds of irradiation (UV or Microwave) can bring out the lipase enhancing characters with minimum changes in the colony characters. However, microwave irradiation was mild on producing positive mutations with respect to lipolytic activity; on the other hand ethidium bromide treatment found to be of least response and more over altered the colony morphology resulting in the loss of lipolytic activity. Further, the lipase producing bacterial soil isolate B3 was identified by 16S rDNA sequencing and named as *Pseudomonas* sp. SPSU B3 and its mutant as *Pseudomonas* sp. SPSU B3UVS30. The novel isolate *Pseudomonas* sp. SPSU B3 was preserved and made available at national agriculturally important culture collection repository (Acc. No.: NAIMCC-B-01768) for further studies in the frontiers of biotechnology for their potential use as a lipase producing bacteria in the industry and academia.

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Table 1. The effect of UV irradiation method for generating better lipase producing mutant(s) from the bacterial isolates B1, B3 & B4.

Wild strain	Bacterial colonies against UV irradiation										No. of positive mutants isolated	Selected Stable Mutant
	Mutant Aspects	Time of Exposure										
		Seconds					Minutes					
		0	5	15	30	60	4	15	30	60		
B1	Total colonies	355	228	290	140	20	0	0	0	0	7	None
	Survived colonies (%)	100.00	64.23	81.69	39.44	5.63	0.00	0.00	0.00	0.00		
	Mutant (selected colonies)	Control	B1S5 (0)	B1S15 (0)	B1S30 (5)	B1S60 (2)	B1M4 (0)	B1M15 (0)	B1M30 (0)	B1M60 (0)		
B3	Total colonies	348	135	65	40	20	9	0	0	0	11	B3S30
	Survived colonies (%)	100.00	38.79	18.68	11.49	5.75	2.59	0.00	0.00	0.00		
	Mutant (selected colonies)	Control	B3S5 (0)	B3S15 (5)	B3S30 (6)	B3S60 (0)	B3M4 (0)	B3M15 (0)	B3M30 (0)	B3M60 (0)		
B4	Total colonies	389	128	82	35	18	15	4	0	0	10	None
	Survived colonies (%)	100.00	32.90	21.08	9.00	4.63	3.86	1.03	0.00	0.00		
	Mutant (selected colonies)	Control	B4S5 (0)	B4S15 (4)	B4S30 (4)	B4S60 (0)	B4M4 (2)	B4M15 (0)	B4M30 (0)	B4M60 (0)		

Table 2. The effect of Microwave irradiation method for generating better lipase producing mutant(s) from the bacterial isolates B1, B3 & B4.

Wild strain	Bacterial colonies against Microwave irradiation										No. of positive mutants isolated	Selected Stable Mutant
	Mutant Aspects	Time of Exposure										
		Seconds				Minutes						
		0	30	60	90	2	3	4	5	10		
B1	Total colonies	358	205	98	62	41	17	9	2	0	10	B1S30
	Survived colonies (%)	100.00	57.26	27.37	17.32	11.45	4.75	2.51	0.56	0.00		
	Mutant (selected colonies)	Control	B1S30 (5)	B1S60 (0)	B1S90 (3)	B1M2 (2)	B1M3 (0)	B1M4 (0)	B1M5 (0)	B1M10 (0)		
B3	Total colonies	343	247	68	51	38	21	4	0	0	2	None
	Survived colonies (%)	100.00	72.01	19.83	14.87	11.08	6.12	1.17	0.00	0.00		
	Mutant (selected colonies)	Control	B3S30 (0)	B3S60 (2)	B3S90 (0)	B3M2(0)	B3M3 (0)	B3M4 (0)	B3M5 (0)	B3M10 (0)		
B4	Total colonies	378	198	85	58	26	11	2	1	0	None	None
	Survived colonies (%)	100.00	52.38	22.49	15.34	6.88	2.91	0.53	0.26	0.00		
	Mutant (selected colonies)	Control	B4S30 (0)	B4S60 (0)	B4S90 (0)	B4M2(0)	B4M3 (0)	B4M4 (0)	B4M5 (0)	B4M10 (0)		

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piRNA: Basics and their Association with PIWI proteins

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Abstract

P element wimpy testis-induced (Piwi)-interacting RNAs, popularly known as piRNAs, are one of the most recently identified small non coding class of RNAs. Because of their association with Piwi proteins, they are known as piRNAs. These molecules are reported to have the sequence length of 23 – 31 nucleotides with abundant expression in the testis. The origin of these molecules takes place from the piRNA clusters located on the genome. The biogenesis of these molecules takes place in two steps beginning with Primary Pathway followed by Ping-Pong cycle, with certain exceptions. The molecules play an important role in germline stability by silencing transposable and non transposable elements, epigenetic regulations, DNA methylation, long term memory functions and canalization. There are many aspects of piRNA, including biogenesis and functionality, the clear understanding of which requires more studies. This review summarizes the facts of piRNAs, genomics of piRNAs, apart from their biogenesis and functionality part, reported till date.

Key words: piRNAs, genomics of piRNA, PIWI proteins

Introduction

Gene expression in individual cells of multicellular organisms is controlled by various mechanisms. As per the Central Dogma of Molecular Biology, RNA is considered as a passive intermediate in the flow of information from DNA to protein, which has been known for its regulatory capacity. For several decades, it has been known that non-coding RNAs (ncRNAs), such as prokaryotic ~100-nucleotide MicC and MicF, can modulate translation efficiency through base pairing to mRNA and altering its accessibility to ribosomes (1). However, with the discovery of RNA interference (RNAi), it has been found that certain much smaller classes of RNAs (20-30 nucleotides) are there, which are having much higher regulatory capacity (2). The presence of these small regulatory RNAs since ancient time and in wide range of organisms, it reveals that these classes of RNAs are highly conserved. Further, it has been found that their silencing principles are also widely conserved and these mechanisms typically involve a protein of the Argonaute family. Over the past few years, it has been revealed that there is a world of small non-coding RNAs that not only regulate gene expression on post-transcriptional and transcriptional levels, but also affect the organization and modification of chromatin. This review, describes about basics and genomics of piRNA, their association with Piwi proteins known till date along with the differentiating points between various small ncRNAs.

Basics of piRNAs : The studies during last few years have revealed a new class of small RNA i.e. P element wimpy testis-induced (Piwi)-interacting RNAs, which is known as piRNA. These piRNAs were first isolated in 2006 from mouse testis. About 30 nucleotide long piRNAs were initially recognized by simply looking at the total testis RNA on ethidium bromide stained gel (3, 4, 5, 6). The piRNAs are abundantly expressed in testis (7, 8). Subsequently many small RNAs, ranging from 26 to 31 nucleotides were discovered through cloning techniques (7).

Unlike miRNAs and siRNAs, piRNAs are produced by a Dicer-independent proceiing pathway and that interact with a subset of Argonaute proteins related to Piwi (3, 4, 5, 6), which is required for female and male fertility in *Drosophila* and other mammalian spp. (7, 9). Most of these RNAs correspond to the intergenic repetitive elements, including retrotransposons (10) and thus alternatively named as repeated-associated small interfering RNAs (rasiRNAs) (11). At present, all those RNAs which associate with Piwi proteins are termed as piRNAs and rasiRNAs are considered as a subspecies of piRNAs.

Genetic studies in mice, drosophila and zebrafish indicate that piRNAs are crucial to germline development as they are abundantly expressed in testis than other small RNAs (12, 13, 14). Most animal studies have revealed that Piwi proteins are expressed specifically in germline. In flies, Piwi proteins have been shown to be essential for the maintenance of germline stem cells. This suggests that piRNAs might be involved in stem cell development (15). Similarly, Piwi proteins associates with chromatin and interacts directly with heterochromatin protein 1a (HP 1a). This suggests the possible involvement of Piwi in epigenetic control of genome (16). Likewise, proteins involved in piRNA production have also been implicated in the control of gene expression in somatic cells (17, 18, 19) and in learning and memory (20), suggesting that piRNAs might have an impact on a broad range of biological processes (10).

Genomics of piRNAs : Many of the cloned piRNAs show an irregular distribution among chromosomes (3, 4, 5, 6, 7). The piRNA genes are mostly located on chromosomes 2, 4, 5 and 17 but seem to be absent on sex chromosomes in mice (7). The piRNAs can be divided into three major classes based on genomic localization: nonrepeats, simple repeat, and repeat associated piRNAs. The non-repeats piRNAs can be further divided into three subclasses: intergenic, intronic and exonic piRNAs. The majority of piRNAs sequences (34% in the ovary and 21% in the testis library) map to the sequences that are annotated as transposon. Similarly, more than 80% of the non-repetitive sequences map to intergenic regions (15). Most of the piRNAs are clustered in relatively short genome loci ranges from <1kb to >100kb. All sequences, both repetitive and nonrepetitive, originate from the same clusters. In turn, piRNAs in a given cluster are derived from the same orientation (7).

The sequences of piRNAs are more diverse than any other class of cellular RNAs. Li *et al.,* (21) reported that 8.8 million piRNAs reads in a deep sequencing library from adult mouse testis comprised 2.7 million different piRNAs; > 90% of piRNA species were sequenced just once.

Argonaute family of proteins : Small RNAs get incorporated with different types of Argonaute proteins to carry out their functions effectively. Argonaute proteins, the molecules of ~100 kDa, are highly specialized small-RNA-binding modules and are considered to be the key components of RNA-silencing pathways. Argonaute proteins were named after an AGO-knockout phenotype in *Arabidopsis thaliana* that resembles the tentacles of the octopus *Argonauta argo* (22). On the basis of sequence homology, Argonaute proteins can be divided into two subclasses. One resembles Arabidopsis AGO 1 and is referred to as the Ago subfamily; the other is related to the Drosophila PIWI protein and is referred to as the Piwi subfamily.

Members of the human Ago subfamily, which consists of AGO 1, AGO 2, AGO 3 and

AGO 4, are ubiquitously expressed and associate with miRNAs and siRNAs. Ago proteins are conserved throughout species and many organisms express multiple family members, ranging from one in *Schizosaccharomyces pombe*, five in drosophila, eight in humans, ten in Arabidopsis to twenty-seven in *C. elegans* (23). Argonaute proteins are also present in some species of budding yeast, including *Saccharomyces castellii*. It was recently found that *S. castellii* expresses siRNAs that are produced by a Dicer protein that differs from the canonical Dicer proteins found in animals, plants and other fungi (24). However, the model organism *Saccharomyces cerevisiae* lacks Argonaute proteins and none of the known small RNA pathways are conserved in *S. cerevisiae*. Argonaute proteins are also found in some prokaryotes (25), but their function in these organisms remains unclear.

The expression of Piwi subfamily members is mainly restricted to the germline, in which they associate with piRNAs. The human genome encodes four Piwi proteins, named HIWI (also known as PIWIL1), HILI (also known as PIWIL2), HIWI3 (also known as PIWIL3) and HIWI2 (also known as PIWIL4) (26). There are three Piwi proteins in mice, known as MIWI, MILI and MIWI2.

PIWI Proteins and Their Association With piRNAs : Out of the three Piwi members of mice, the expression kinetics of MILI and MIWI are different from each other. MILI is expressed in germline development up to the pachytene spermatocyte phase. MIWI has been identified to exist from mid-pachytene to the early spermatids stage (7). The two Piwi homologs Ziwi and Zili (also known as Piwil1 and Piwil2, respectively) have been reported in zebra fish (*Drosophila rerio*) (15). The Piwi proteins in flies are known as Aubergine (Aub) and Ago 3 which are capable to cleave their target mRNA (27). The Piwi proteins and their homologs in different animals, their associated piRNAs length, and possible mechanisms of action are summarized in Table 1.

Differences between piRNA vs. other small ncRNAs : The main differentiating features of piRNA with other small ncRNAs are in length, biogenesis, associated AGO proteins and localization. Lengthwise, piRNAs are longer than other small ncRNAs. Biogenesis of piRNAs is Dicer independent, which again differentiate them with other small ncRNAs. Other small ncRNAs are associated with Ago 1 and Ago 2 proteins while piRNAs are associated mainly with Piwi proteins. Though all the small ncRNAs are playing role in regulatory mechanisms at cellular level, there are certain differences amongst them, which are mentioned in Table -2.

Conclusions

piRNAs are most recently discovered and least characterized small non coding RNAs. They are named by their exclusive association with PIWI subfamily of Argounate proteins. They differ with other snRNAs by their size and produced through Dicer independent mechanism. piRNAs are abundantly expressed in germ cells. piRNAs are having their primary functions in germline stable transmission through silencing Transposable and non transposable elements. However, till date, their role has been extended to somatic cells by epigenetic regulation, canalization and functions of memory.

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Table 1: Piwi proteins homologs, origin and mechanism of actions in different animals.

Ago-family Protein	Origin of piRNA	Length o f piRNA (nts)	Mechanism of action	Species
MILI (PIWIL2 in human)	Transposon, and pi RNA clusters	24-28	Heterochromatin formation (DNA methylation)	Homo-sapiens
MIWI (PIWIL1 in human)	piRNA clusters	29-31	Unknown	Homo-sapiens
MIWI2 (PIWL4 in human)	Transposon, and piRNA clusters	27-29	Heterochromatin formation (DNA methylation)	Homo-sapiens
PIWIL3 in humans	Unknown	Unknown	Unknown	Homo-sapiens
AUB	Transposon, repeats, piRNA clusters and SU (ste) locus	23-27	RNA cleavage	*Drosophila melanogaster*
AGO3	Transposon and Rrepeats (unknown in testis)	24-27	RNA cleavage	*Drosophila melanogaster*
PIWI	Transposon, repeats and piRNA clusters	24-29	Heterochromatin formation	*Drosophila melanogaster*
AGO4 and AGO6	Transposon and repetitive elements	24	Heterochromatin formation	*Arabidopsis thaliana*

(28)

Table - 2. Differentiating features of piRNAs with other small ncRNAs.

Description	siRNA	miRNA	piRNA
Length (nts)	18 - 24	18-24	23 - 31
Biogenesis	Dicer dependent	Dicer dependent	Dicer independent
Associated AGO proteins	Ago 2	Ago 1	PIWIs
Origin	Long dsRNA	gDNA – miRNA loci	gDNA – piRNA loci
Localization	All somatic and germ cells	All somatic and germ cells	Mainly in germ cells

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Japanese Encephalitis: An overview of vaccine development

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Abstract

Japanese Encephalitis (JE) is a mosquito transmitted disease of pigs and humans that is caused by Japanese Encephalitis Virus (JEV). Annually around 50,000 cases are reported from Japan and other Asian countries where the disease is epidemic. But with rapid globalization and climatic shift, JEV has started to emerge in areas where the threat was previously unknown. There is no specific treatment for Japanese encephalitis and the available treatment is supportive. Vaccination is one of the most successful methods to combat infectious diseases like JE. Different vaccines for JEV have been available for many years and their use has reduced the incidence of JE in many countries. The first generation mouse brain derived vaccines are being replaced by cell culture and inactivated vaccines. The recent focus is to develop recombinant and subunit vaccines which may overcome all the side effects of the current vaccines. The present review focuses on the latest development in vaccine formulations with different approaches reported around the world.

Key words: Japanese Encephalitis, Therapy

Introduction

The leading cause of viral encephalitis in Asia is mosquito borne arboviral Japanese Encephalitis (JE) (1). JE is an inflammatory disease that affects the central nervous system causing acute inflammation of the brain. The virus is transmitted in an enzootic cycle among mosquitoes, primarily *C. tritaeniorhynchus*, and vertebrate- amplifying hosts, which include domestic pigs and Ardeid (wading) birds. Humans are considered dead end hosts as infected humans do not transmit the virus to the biting mosquitoes (2). This is because viremia is transient with low levels of circulating virus. Historically JE is known to have originated in Malay Archipelago (3). The virus evolved in several thousand years, into different genotypes (I–V) and spread across Asia. There are five genotypes which have been identified by the researchers. Their geographical distributions are: Genotype I includes isolates from Korea, India, Cambodia, Laos, and northern Thailand (4), Genotype II, from Malaysia, Sarawak, Indonesia, southern Thailand, and northern Australia (5), Genotype III, from Japan, Taiwan, China, India, Sri Lanka, Nepal, Vietnam, and the Philippines (6), Genotype IV from Indonesia (7), Genotype V, which includes isolates with a restricted distribution in India in addition to genotype I and II. It is the last genotype to be identified (8). The first clinical case of JE was recorded in 1871 in Japan (9). The first clinical case of JE in India was observed in 1955 at Vellore (10). There have been reports of several outbreaks, the most fatal outbreak was reported in 2005, wherein around 1700 people mostly children were killed and several thousands were disabled (11). Press Trust of India has reported the death toll in 2013 as 358 due to JE virus (12). The scientific survey conducted by the doctors who treated the JE virus affected areas shows that the affected patients were poor people hailing from mostly rural areas.

Virus Structure : JEV is a relatively small (~50 nm diameter) spherical virion that encapsidates a nonsegmented RNA genome of positive-sense polarity (13). The single strand of positive-sense RNA comprises of a single open reading frame capped by a 5'UTR 95 bp long and a 3'UTR 580 bp long (14). This ~11 kb viral RNA encodes a single polyprotein, which is cleaved by viral and cellular proteases into three structural proteins (capsid, C; membrane, M; and envelope, E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (15). The E glycoprotein (53-55 kDa) is the principal target for neutralization *in vitro* and *in vivo* by specific antibodies (16). The nonstructural protein NS1 (39 to 41 kDa) is a glycosylated protein, which is derived from the polyprotein by an N-terminal cleavage involving a novel host protease (15). NS1 is believed to be involved in the assembly and release of virions (17). NS2A and NS2B are low-molecular-weight proteins that are found to be involved in the processing of other viral proteins (18). NS3 protein (68 to 70 kDa) is conserved among flaviviruses and has protease and nucleotide triphosphatase/helicase activities (19). In JEV infected cells, NS3 is associated with microtubules and tumor susceptibility gene, and plays an important role in viral RNA packaging, intracellular trafficking of viral components and viral assembly. Therefore, NS3 has been considered to be responsible for development of novel potent therapeutic substances. NS4A and NS4B are small proteins whose functions are not clear, although they are involved in the membrane localization of NS3 and NS5 through protein-protein interactions (15), or in the formation of genomic RNA replication complex (20). NS5 protein (103 to 104 kDa) is the largest and most conserved protein and is considered to be the viral RNA polymerase with both methyl transferase and RNA-dependent RNA polymerase (RdRp) activities (21).

Clinical Implications : Based on serological studies in endemic areas and medical histories, JE virus infection may be asymptomatic in humans. It has been estimated that, 1 to 3 per 1,000 infected humans may show clinical manifestations in the form of illness that includes evidence of virus-induced inflammation in the cerebrum, cerebellum and spinal cord (22). Typically, symptoms start suddenly following a variable incubation period of 2 days to 2 weeks and a nonspecific viral prodrome. The earliest symptoms seen during the prodromal stage include lethargy, fever (23), headache (24), abdominal pain, nausea, and vomiting. Neurologic manifestations are seen in the late stage which include meningeal (meningitis) (25), parenchymal (encephalitis), or spinal cord (myelitis) involvement. Among infected children, 50%–85% develop focal or general seizures compared to 10% of adult cases. Seizures have been associated with poor clinical outcome. Although JE is often a mild disease, leading to an uneventful recovery, some cases rapidly progress to severe encephalitis with mental disturbances, general or focal motor abnormalities, and progressive coma (26).

Treatment : There is no specific treatment for Japanese encephalitis and the available treatment is supportive in nature (23). There are no reported cases of JEV being a communicable disease and therefore patients need not be isolated. Different approaches are being made for treatment at various stages of disease like design of chemotherapeutic drugs against virus and use of vaccines in preventing the disease.

Chemotherapy : An effective chemotherapeutic strategy against any viral infection is based on either 1) blocking the virus-coded function or 2) inhibition of cellular processes necessary for viral replication. The problem with the second approach is that it also hampers normal cellular function, but it is advantageous in a way that the therapy is active against all the viruses belonging to the same genus. Moreover, development of resistance against this type of chemotherapy is rare. The JEV chemotherapeutic drugs, which are tested, come under three different categories; viz; viral replication inhibitors, anti-inflamatory or anti-apoptotic drugs, interferon inducers (27).

Modern therapies *:* More modern therapies against the infection include the interferon therapy wherein the interferons induce production of effector proteins in cells, which inhibit various stages of viral replication, assembly, or release. *In vitro* studies in human trials have showed that interferon therapy is effective against JEV and other arboviruses, including West Nile virus (28). Anti-sense therapy is the use of RNA interference technology. It is also reported that a single siRNA (small interfering RNA) treatment could suppress viral infection across species (29).

Monoclonal antibody therapy has reported varied efficacy of MAbs targeted against specific JEV antigenic epitopes. Fully human or humanized MAbs against JEV might be practical and can generate cost-effective reagents for preventing or modifying the pathophysiological implications of JE (27).

Vaccination *:* Vaccination against infectious diseases is one of the most successful medical interventions in history. In the recent years, advances have been made in the understanding of the mechanisms underlying the induction of protective immune responses to infectious agents especially in viral infections. The most important factor is the identification of the key parameters that are responsible for the long lasting immune response induced by viruses, which has been identified and can now be exploited for the development of safe and efficacious vaccines. Four key parameters which form the basis of the strong immunogenicity of the viruses are i) particulate nature, ii) highly repetitive structures, iii) ability to induce innate immunity and iv) the relative length of time the immune system is exposed (30). For Japanese encephalitis, the aim of vaccination is to induce of circulating neutralizing antibodies that can prevent invasion of the central nervous system during the viremic phase of JE virus infection.

Different vaccines for JEV are available for many years and their use has reduced the incidence of JE in many countries. But with the decrease in the incidence of the disease and the appeal for mass immunization in the epidemic regions has triggered concerns regarding the adverse events following immunizations. The present review focuses on (a) existing vaccines for JEV with their merits and demerits, (b) the published literature on latest developments in vaccine development and (c) vaccines presently in various stages of development (Table 1). The vaccines available for JEV can be broadly classified as (i) inactivated vaccines (mouse brain derived, PHK and vero cell derived), (ii) live attenuated vaccines and (iii) recombinant vaccines.

I. Inactivated vaccines

(1) Inactivated mouse brain–derived vaccine: The first inactivated vaccines were prepared in 1954 and were based on formalin-inactivated Biken vaccine, grown in adult mouse brain. The Biken vaccine uses Nakayama-NIH strain of JE virus, originally isolated in 1935 from an infected human. Three- to five-week-old mice were chosen to grow the virus, as virus yields were very high and cell culture systems were very limited at that time. Each mouse brain produced the equivalent of 4–10 doses of vaccine. The vaccine derived from mouse brain is used both as a liquid or lyophilized product and has been available in Japan since 1973. For several decades, this JE vaccine was available in the United States and Europe (BIKEN, distributed by Sanofi Pasteur as JE-Vax).

(2) Inactivated vaccine cultivated on primary hamster kidney cells : This is an inactivated vaccine cultivated on primary hamster kidney cells. The Beijing-3 strain was the main variant of the vaccine used in the People's Republic of China from 1968 until 2005 (31). This vaccine only requires 1–2 doses to confer long-lasting immunity. The vaccine is made available at a highly competitive price to low-income countries.

There are problems associated with the presently used Japanese encephalitis vaccine and they include: a) Induction of unwanted adverse neurological reactions caused by the

nature of mouse brain-derived vaccine, b) Loss of follow-up for the third vaccination caused as a result of the long interval between vaccinations, c) The vaccines are expensive as there are only few manufacturers, d) JE vaccine is associated with local reactions and mild systemic side effects (fever, headache, myalgias, and malaise) in about 20% of vaccines, (32), e) Serious allergic reactions, including generalized urticaria, angioedema, respiratory distress, and anaphylaxis, have occurred within minutes of vaccination lasting upto one week after immunization, f) No data are available on the safety and efficacy of JE vaccine among infants less than 1 year of age, during pregnancy (33), on simultaneous administration with Diptheria, Tetanus and Pertussis (DTP) vaccine and effect of administration of other vaccines, drugs (e.g., chloroquine, mefloquine), or biologicals (34).

(3) Inactivated vaccines grown on vero cells: The IC51 (IXIARO; in Australia and New Zealand, JESPECT) vaccine is a purified, formalin-inactivated, whole virus JE vaccine developed by Intercell AG, Austria. The product was licensed for use in the United States, Australia, and Europe in 2009. The vaccine is based on a SA14–14-2 virus strain passaged 8 times in primary dog kidney cells, cultivated in Vero cells, and formulated with 0.1% aluminum hydroxide. Vero cells maintained in serum-free medium were selected as the manufacturing cell substrate. The absence of serum allows for a simplified purification process and, potentially, a superior safety profile. As an added safety advantage, this vaccine does not require additional stabilizers or additives (35).

JEEV™ is a second generation inactivated Japanese Encephalitis vaccine based on the SA 14-14-2 virus strain developed by Biological E, India, launched in 2012. It is a purified formalin inactivated vaccine adsorbed onto aluminium hydroxide. The vaccine's safety and efficacy has been established through multiple studies on Indian subjects and is licensed by the Drug Controller General of India (DCGI). JEEV™ is indicated for active immunization against Japanese encephalitis in adults and children.

JENVAC is another vero cell derived purified inactivated JEV vaccine manufactured by Bharat Biotech, India. JENVAC is the first vaccine to be manufactured in the public-private partnership mode. The virus strain for this vaccine was isolated from the blood sample collected from an encephalitic patient admitted to Government Hospital in Kolar district, Karnataka between Nov-Dec, 1981 and characterized by the National Institute of Virology at Pune (36). JENVAC received manufacturing and marketing approvals from the Drug Controller General of India after its successful clinical trails and the product is available in the market since late 2013. It can be administered as a single dose vaccine during epidemics for mass vaccination campaigns and as a two dose vaccine during the routine National immunization programme in endemic regions.

II. Live attenuated vaccines : A live attenuated JEV vaccine developed in China (SA14-14-2; Chengdu Institute of Biological Products) (31). It is reported that this vaccine strain was developed by passaging wild-type strain SA14 in primary hamster kidney cell culture. Following 114 passages of SA14 in primary hamster kidney cell culture, an attenuated derivative, 12-1-8, was isolated. This virus was given additional passages in primary hamster kidney cell culture in different laboratories to generate two sub strains, SA14-2-8 and SA14-5-3. The former was used as a veterinary vaccine in horses whereas the latter was evaluated in humans. Although both viruses were attenuated, neither was sufficiently immunogenic with seroconversion rates below 50% following one dose of vaccine. Subsequently, SA14-5-3 was given additional passages in primary hamster kidney cell culture to derive strain SA14-14-2. This virus has proved to be highly attenuated yet immunogenic. It has been successfully used as a vaccine in the People's Republic of China with over 100 million children vaccinated. Currently, the vaccine is administered as a two-dose regimen, one year apart, with the

first dose given at one year of age. The World Health Organization is currently developing criteria for the manufacture of live attenuated JE vaccines for its use anywhere in the world. Since the virus is attenuated; there is no clinical disease and there are no lesions following direct inoculation into the brains of monkeys or mice. There are no reported cases of reversion to virulence. The molecular basis of attenuation of SA14-14-2 has been investigated by comparing the nucleotide sequence of the genomes of wild-type SA14 and SA14-14-2. The two viruses differ by 57 nucleotides encoding 24 amino acid substitutions. Reverse genetics studies indicate that the envelope protein is a major determinant of the attenuation, in particular, amino acid 138 (37).

III. Recombinant Vaccines in various stages of development : The recombinant vaccines, presently at different stages of development can be classified into five categories namely, i) the recombinant protein based ii) the recombinant virus based iii) the DNA vaccine based iv) the bioinformatics based v) the nanobiotechnology based approach for vaccine development.

(1) The recombinant protein based vaccines : The structural and nonstructural proteins of JEV are being explored as vaccine targets. Different expression platforms like bacteria (*E. coli*), yeast (Saccharomyces and Pichia), baculovirus and mammalian cell lines are being used for developing new vaccine candidates. Mason et al in 1989 (38), synthesized small fragments of JEV E protein with trp fusion proteins in *E. coli* and reported that E protein segment containing residues from 303 to 396 was the shortest epitope capable of reacting with various JEV neutralizing monoclonal antibodies. Saini et al in 2003 (39), reported neutralizing antibodies in mice by expressing the epitopes of E protein in Jhonsongrass mosaic virus coat protein that formed virus like particles (VLP). Bhasker et al in 2009 (40), expressed E protein in *Saccharomyces cervisiae* and showed the production of antigen specific and non-neutralizing antibody response in mice. Xua et al in 2011 (41), have succeeded in construction of one recombinant baculovirus BacSC-E expressing His6-tagged E with the baculovirus envelope protein gp64, transmembrane domain (TM) and cytoplasmic domain (CTD). Vaccination of mouse and swine with recombinant baculovirus BacSC-E successfully induced neutralizing antibody response and protective immunity towards a lethal challenge of the JEV. Tafuku et al in 2011 (42), expressed JEV E domains (I, II and III as domains I through III (D1-3), domains I and II (D1-2) and domain III (D3) and nonstructural protein 1 (NS1) in *Escherichia coli*, and administered to BALB/c mice via the intranasal (i.n.) route. The E protein, but not the NS1, induced JEV-specific serum IgG with virus-neutralization capacity *in vitro*. When mice were lethally challenged with JEV, intranasal immunization with D1-3, D1-2, D3, or a mouse brain-derived formalin-inactivated JE vaccine conferred complete protection, while an 80% protection was observed in the NS1 immunized mice. Li et al in 2012 (43), concluded that purified JEV NS1 from Drosophila S2 cells in a native glycosylated multimeric form induced T-cell and antibody responses in immunized C3H/HeN mice. Mice vaccinated with 1 μg NS1 with or without water-in-oil adjuvant were partially protected against viral challenge and higher protection was observed in mice with higher antibody titers.

(2) Recombinant virus based vaccines : Recombinant viruses have the ability to induce both cell mediated and humoral immunity. The foreign gene product is amplified during virus infection thus increasing antigen exposure. Foreign antigens thus expressed are processed and presented to the immune effector cells resembling natural infection. Konishi et al in 1992 (44), constructed recombinant NYVAC (a highly attenuated recombinant vaccinia virus constructed by deletion of its 18 open reading frames) expressing JEV proteins prM and E with or without the NS1. Nam et al in 1999 (45), constructed a Modified Vaccinia Ankara (MVA) recombinant expressing the prM and the E proteins of JEV. Mice inoculated with NYVAC

generated Cytotoxic T Lymphocytes and mice inoculated with MVA recombinant produced JEV neutralizing antibodies and the immunized mice were completely protected from a lethal JEV challenge. Lipenga et al in 2008 (46), constructed and characterized the immune responses conferred by recombinant adenoviruses (rAd) expressing JEV E epitopes (six amino acid residues 60–68, 327–333, 337–345, 373–399, 397–403 and 436–445 in E, designated TEP). To prove the protective efficacy of the recombinants, it was administered on swines and found to be highly effective in insulating them from viral challenge with IM rAd-TEP. These findings indicate that rAd-TEP can be a potential vaccine for preventing JEV infection. Wang et al in 2012 (47), constructed a recombinant MVA carrying multi-epitope (B-cell, CTL and Th) gene of JEV (rMVA-mep) and demonstrated the vaccine efficacy in a mouse challenge experiment.

ChimeriVax-JE virus is produced using infectious clone technology based on insertion of pre-membrane (prM) and envelope (E) genes from SA JE, SA14–14-2 virus into the nonstructural genes of YF 17D viral strain as the viral "backbone". The resulting chimeric RNA was electroporated into Vero cells. Progeny virus particles contain JE-specific antigenic determinants that elicit neutralizing antibodies as well as cytotoxic T lymphocytes. YF 17D was chosen as backbone to the chimera because of its proven record of safety and efficacy (48). In 2011 Ishiwaka et al (49), demonstrated that a single-cycle West Nile virus (WNV) named RepliVAX WN could be used to produce a chimeric Japanese encephalitis (JE) vaccine (RepliVAX JE) by replacing the WNV prM/E genes with those of JEV. They also demonstrated that replacement of WNV NS1 gene in RepliVAX JE with that of JEV (producing TripliVAX JE) could produce a superior vaccine. TripliVAX JE elicited higher anti-E immunity and displayed better efficacy in mice than RepliVAX JE. Furthermore, TripliVAX JE displayed reduced immune interference caused by pre-existing anti-NS1 immunity.

(3) Plasmid DNA-based JEV vaccines : This vaccine is usually a plasmid DNA capable of synthesizing a protective immunogen from a given pathogen. DNA vaccines have ability to generate a broad range of immune responses which include the induction of antibodies, generation of CD4+ helper T lymphocytes and CD8+ cytotoxic lymphocytes. In 1998 Lin et al (50), showed that immunization of mice with DNA expressing NS1 alone was sufficient to protect mice but could not raise detectable neutralizing antibodies against JEV. Chang et al in 2000 (51), showed that intramuscular immunization of mice with plasmid DNA synthesizing the prM and E proteins of JEV elicited protective immunity in mice and 70% of them survived lethal JEV challenge. Chien et al in 2001 (52), concluded that intramuscular immunization induced the Th-1 type of immune responses, whereas the gene gun immunization induced Th-2 type response. Such vaccines have shown considerable success albeit with some shortcomings in terms of not evoking sufficient neutralizing antibody titers. Therefore the present attention has shifted towards the improvement of DNA vaccine modulated through several immunological adjuvants, such as the use of liposomes (53), inclusion of CpG motif (54), co-expressing cytokines and costimulatory molecules along with the target gene (55), exploring different routes of administration of vaccine(56), targeting the vaccine to specific cells (57) or endosomal/lysosomal compartment (58). In 2009 Bharti et al (59), have evaluated the efficacy of E gene as a DNA vaccine candidate in rhesus monkey and showed the generation of neutralizing antibodies and prime the immune system effectively against further JEV infections.

IV. Bioinformatics based approach for vaccines development : The development of different software tools in the field of immunology gave birth to the field of immunomics which is nothing but the abridging of immunology with bioinformatics (60). Immunomics gave rise to the reverse vaccinology approach as analysis starts with the information contained in a computer instead of with growing pathogens (61, 62). Role

Table 1

S.No	Type of vaccine	Substrate	Strain	Producer	Remarks
1.	Inactivated and purified	Mouse brain	Nakayama	Biken-Japan	Production stopped by 2005 and all stocks expired by 2011.
				Green Cross-Korea	
				Vabiotech-Vietnam	
			Beijing 1	Kaketsuken-Biken-Japan	Ceased production
				Kitasota-Japan	
		Primary Hamster Kidney (PHK) cells	P3 strain	Several manufacturers in China	Previously China's principal vaccine
			Beijing 1		
		Vero cells	SA-14-14-2	IC51-Novartis-Intercell	JESPECT-Australia IXIARO- Elsewhere
				Biological E,India	JEEV
			Beijing 1	Biken, Japan	BK-VJE. Kaketsuken, Japan
			821564 XZ, Indian isolate	Bharat Biotech,India	JENVAC
2.	Live attenuated	Primary Hamster Kidney (PHK) cells	SA-14-14-2	Chengdu Institute of Biological products, China	The use of PHK cells is gradually being replaced by vero cells
			Beijing-3 strain		
3.	Live attenuated, Chimeric	Yellow fever 17D vectored, Vero cells	SA-14-14-2	Acambis with Sanofi Pasteur	ChimeriVax-JE

of epitope mapping in finding a new vaccine for Japanese encephalitis virus is of great interest lately. Specific sequences coding for epitopes and cytokine enhancing factors can be incorporated into viral vectors or DNA vaccines. The focus can be on the immunogenic protein already used in vaccine production. Despite the protein's availability, it is still difficult to find a new epitope. Classical techniques are time consuming process hence bioinformatics tools like Epimatrix and Conservatrix can be of help in this direction. Conservatrix (63), is a sequence matching and counting tool, can be used to compare the sequence of every 10-amino-acid-long peptide in a given sequence database (e.g. one isolate of a virus) for identity with every 10-amino-acid-long sequence of another sequence database (e.g. another strain of the same virus). This can be used to identify broadly conserved (across-clade) epitopes. Conservatrix can be configured to allow amino acid substitution at non-anchor positions. The algorithm has been used to map highly conserved T-cell epitopes in variable genomes. Since Japanese encephalitis is a viral infection, the favorable epitope should be a specific T cell epitope which has an important role in

immunogenicity via the T cell immune system. Existing vaccines have been developed mostly against pathogens that show no or limited antigenic variation. With immunomics one can search the entire genetic repertoire for protective antigens, thus increasing by several orders of magnitude the number of antigens available for vaccine development. The combination of epitope-mapping informatics tools with new sensitive *in vitro* screening methods has driven many new vaccine approaches.

V. Nanobiotechnology based vaccines : Another upcoming approach is in the field of nanobiotechnology. This field in the domain of vaccinology is being used to develop synthetic vaccine carrier/delivery systems. These vaccine carrier systems mimic pathogen structure and are also engineered to show chemotaxis at immunization sites. There are two methods by which one can deliver the vaccines; the first being a method to provide a depot for an immunostimulatory compound that attracts APCs to immunization sites and the second method is to deliver the vaccines in the form of particles that are internalized by the infiltrating immune cells. Examples include the alginate microspheres used to deliver the chemoattractants and antigen entrapped nanoparticles. *In vitro* studies have demonstrated that the chemoattractant can readily diffuse out of the particles and attract dendritic cells. In contrast, the antigen-loaded nanoparticles do not leave the alginate microparticles, but antigen that is accessible at the surface of the particle can be extracted and presented by dendritic cells. The delivery of antigens is also being done by skin patches. Two general approaches have been used with skin patches, one by using a dry formulation in the patch and second the delivery of an adjuvant using the patch, followed by injection of conventional vaccine at the same site. Such approaches ensure that both antigen and adjuvant are delivered to the same population of antigen presenting cells. Particulate delivery systems can specifically target the adjuvant effect to the key cells of the immune system thereby reducing systemic distribution and minimizing induction of adverse reactions. Small unilamellar liposomes have a significant potential as delivery systems for the co-administration of antigens (peptides, lipopeptides) and of immuno-stimulatory adjuvants, including CpG oligonucleotides or DNA encoding antigens and/or immunostimulatory sequences. Advantages of nanotechnology include uniformity, reproducibility, and precision in the synthesis and manufacture of candidate compounds. Combined with novel pharmacokinetics and the possibility of targeted therapy, nanotechnology-based vaccines may prove superior to existing vaccines and have the potential to open therapeutic avenues for treating JEV.

Conclusions

JE has remained a tropical disease uncommon in the West. With rapid globalization and climatic shift, JEV has started to emerge in areas where the threat was previously unknown. Scientific evidence predicts that JEV will soon become a global pathogen and cause worldwide pandemic. A considerable percentage of JEV outbreaks occur in developing countries. Therefore it is the responsibility of the scientific communities, governments and WHO to find drugs that could reach the unprivileged masses to contain JEV. JE is a vaccine-preventable disease with numerous options now available for active immunization. Aggressive and responsible vaccination programs should greatly diminish the burden of disease. In India vaccination against Japanese encephalitis are administered in areas where the disease is hyper-endemic. Protection at personal level would also help to reduce the menace of JE.

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Comparative review of recessive genetic disorders occurrence in Indian Cattle

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Abstract

Holstein Friesian (HF) are high yielding animals therefore, dominates the global dairy industry. Because of merits of the breed, the Holsteins were extensively used for crossbreeding programmes in India. The availability of the bovine genome sequence and the advent of DNA technologies have widened our understanding for hereditary diseases in cattle and their early diagnosis. Some of autosomal recessive disorders are Holstein specific. The present review article describes prevalence of the most important autosomal recessive disorders in Indian Holstein and its crossbreds as compared to their occurrence worldwide. Mainly five disorders; BLAD (Bovine Leukocyte Adhesion Deficiency), DUMPS (Deficiency of Uridine Monophosphate Synthase), Bovine Citrullinaemia, CVM (Complex Vertebral Malformation) and FXI (Factor XI deficiency syndrome), are being screened in Indian cattle with the major objective to reduce the incidence of genetic disorders in cattle population and reduce the economic losses to the organized farms. Precisely, genetic disorders cause physical or functional anomalies with negative impact on viability. The old adage that prevention is better than cures is pertinent.

Key words: Holstein, BLAD, Citrullinaemia, DUMPS, Factor XI, CVM

Introduction

India has the largest cattle production in the world but most of them are considered to be either unproductive or poor producers as compared to exotic animals. The first systematic attempt in India to improve the quality of cattle was the 'Key Village Scheme' (KVS) initiated during the first five year plan. The programme was invigorated with the introduction of the Intensive Cattle Development Projects (ICDPs) in 1965. Among 30 major Indian breeds of cattle, there are only few milch breeds maintained for milk production and rest of them either neglected or endangered. Cross breeding programmes of nondescript Indian cattle with exotic germplasm of Holstein, Jersey and Brown Swiss breeds, on field scale started in 1964 with the launch of the ICDP of the Government of India and by 1969 it became the official policy of the government for increasing milk production. The crossbreeding was continued till late 19th century. Cross breeding gained momentum and economic relevance in the mid seventies. The governments had no intention to crossbreed pure Indian breeds of cattle, but the spectacular increases in milk yields in the crossbred progenies generated overwhelming demand for crossbred cattle from the farming community almost all over India that necessitated the expansion of the programme nation-wide. As most programmes have merits and demerits, crossbreeding gave us increase of milk production as merit and incorporation of new diseases in cattle as demerits. The availability of the bovine genome sequence and the advent of DNA technologies have widened our understanding of a number of hereditary diseases in cattle and their early diagnosis. By isolating

DNA from nucleated cell samples, followed by *in vitro* amplification of DNA fragments and digestion with endonucleases, it is now possible to detect the presence of lethal or mutant alleles for a specific phenotype. Genetic disorders especially autosomal recessive, are major concern worldwide as the carriers or heterozygous cattle population look normal and therefore, often used for breeding through artificial insemination (AI) based on their genetic merits. Holstein Friesian (HF), a breed that dominates the global dairy industry, is high yielding animals in the world therefore being used for crossbreeding programmes in India. Extensive use of Holstein bulls and unaware of their breed specific genetic disorders in past, autosomal recessive genetic disorders have been widely spread in Holstein and their crossbred cattle in India. The genetic disorders are caused by mutations, which could be because of change of nucleotide(s), deletion, and insertion of nucleotide(s) in a functional gene. Surveillance for genetic disorders should be an important in animal health programmes, therefore, an extensive screening of such disorders are going on at the research institutions, Universities and diagnostic laboratories all over India. As a result, many Holstein Friesians and their crossbreds especially bulls have been identified as heterozygous or carriers for genetic defects which are described herein. Besides, the article describes various mutations in the candidate genes causing genetic disorders, symptoms of diseases, techniques to detect mutations, mode of inheritance, economics of diseases, occurrence of disorders in Indian cattle and prevention of such disorders in future population of elite animals.

Bovine Leukocyte Adhesion Deficiency (BLAD) syndrome

***Back ground and prevailing worldwide*:** It is an autosomal recessive genetic disease (1) that affects especially Holstein breed. A human equivalent also exists, called human Leukocyte Adhesion deficiency (LAD). The carrier frequency of BLAD among US Holstein cattle had once reached approximately 15% among active breeding bulls and 8% among cows. In Taiwan, Holstein is the only cattle breed for milk production where the percentage of BLAD carriers was estimated in more than 5% of total Holstein (2). Many cases were reported in Hungary (3), Korea (4), Lithuania (5), Uruguay (6) Iran (7,8,9,10), Poland (11,12), Japan (13,14), Czech (15, 16), China (17), Argentina (18), Denmark (19, 203), Brazil (21), Pakistan (22), Germany (23), USA (1, 24) etc. Besides, owing to the wide – spread use of top breeding HF bulls imported from USA, many countries reported a high incidence of BLAD carriers in their black and white population (25). In the US alone, 80% of the 10 million dairy cows are Holsteins. It is estimated that 16000 calves are born with BLAD each year. The average economic loss per calf is roughly 300 USD annually. Many cases of BLAD affected were also reported in Japan (13, 26), Korea (27), USA (28), Republic of Macedonia (29) etc. Continuous screening of young bulls before entering in AI stations is reducing the incidence of BLAD carriers among HF animals. In Poland Holstein population, the frequency of carriers of the mutated allele showed a clear decreasing trend. The highest frequency (7.9%) was recorded while implementing the BLAD control program (1995–1997). Regular monitoring has enabled a great reduction of this threat to the tested population. Today only sporadic cases of BLAD heterozygotes are reported (12). BLAD was first identified in Holstein-Friesian cattle at the beginning of the eighties and no study has reported the occurrence and etiology of this disease in other breeds. The mutation found in the Holstein breed was traced back to a heterozygote bull (Osborndale Ivanhoé), which due to its elevated genetic merit for milk production has been widely used in artificial insemination. This bull and its offspring (Penstate Ivanhow star - son and Carlin M Ivanhoe Bell - grandson) founded one of the main Holstein lineages. Affected cattle with BLAD were linked to common ancestral sires that had been documented to be carriers. Several Holstein Friesian bulls were identified as BLAD carriers and the gene encoding impaired CD18 spread to

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many countries and responsible for spreading BLAD to several herd worldwide (1).

Animal with BLAD is characterized by recurrent pneumonia, ulcerative and granulomatous stomatitis, enteritis with bacterial overgrowth, periodontitis, loss of teeth, delayed wound healing, persistent neutrophilia and death at an early age (13, 30). Most of the calves positive for BLAD also exhibit stunted growth and a persistent progressive neutrophilia (often exceeding 100,000/ µl to as high as 300,000/µl). Neutrophils from positive calves have several functional deficits and most importantly, fail to exit the blood stream to combat the invading infection, as a result, these neutrophils gradually build-up in blood stream. However, the number of neutrophils and lymphocytes were within physiological limits and no differences were recorded between two genotypes, carriers and normal (31). Neutrophils are our body's first line of defense against bacterial infections. These cells recognize chemicals produced by bacteria in a cut or scratch and migrate towards the smell and eat (phagocytosis) the microbes. Neutrophils migrate and roll in the blood vessels and capillaries. Neutrophils require protein molecules called β-integrins that are a family of structurally and functionally related glycoproteins that occur on the surface of the neutrophils. These proteins help the Neutrophils to migrate to the site of inflammation. The β-integrins are now termed the CD11/CD18 family of proteins (32). Specific binding of β-integrins or Integrin, beta 2 (ITGB2) on Neutrophils surfaces with the intercellular adhesion molecule I (ICAM-I) expressed on vascular endothelium is required for neutrophil emigration into extra vascular inflammatory sites.

The molecular basis of BLAD is a single point, which replaces adenine to guanine at 383 of exon 5 of the ITGB2 gene that change amino acid, aspartic acid to glycine at amino acid 128 in the functional protein. The mutation ultimately leads to wrong protein that is impaired in function (1). The gene is located on chromosome number 1 (BTA1), which contains 15 coding out of 16 exons with a total length of 29161 bases (NCBI AC_000158.1).

The silent point mutations were also detected in CD18 gene (NCBI Accession No. M81233) at 775 bp position. However, in many cases BLAD-carriers were distinguished by a very high breeding value for selected milk performance traits (11). The new polymorphism at 348 position was found to be silent as it does not change amino acid (Asparagine, AAT>AAC) within exon 4 of CD18 gene (Accession No. KF840683).

***Occurrence in India*:** In India, where Holstein bulls and their semen doses are still maintained for extensive use for and crossbreeding programmes, it is obvious to detect BLAD carriers in Holstein and crossbreds. BLAD carriers were first detected in two young Holstein bulls (1.33%) which were born through artificial insemination with the imported semen (33). However, mutation was not detected in other breeds of cattle including Jersey and zebu and buffaloes. After that the routine screening of BLAD in selected Holstein and their crossbreds had been started. Later, the blood samples and semen samples of 1250 phenotypically normal individuals, including 377 HF, 334 HF crossbred, 105 Jersey, 160 zebu cattle breeds and 274 water buffalo (*Bubalus bubalis)* belonging to various artificial insemination stations, bull mother farms (BMFs) and embryo transfer (ET) centres across the country were tested by PCR-RFLP technique to detect possible carriers for BLAD (34). The results indicate that out of 1250 cattle and buffaloes tested for BLAD, 13 HF purebreds out of 377 and 10 HF crossbreds out of 334 appear to be BLAD carriers. In the HF and HF crossbred population, the percentage of BLAD carriers was estimated as 3.23%. A study on a group of 55 Karan Fries that is crossbred devolved in India by using Holstein Friesian and Tharpakar breed (35, bull calves were tested for BLAD by PCR and RFLP analysis. Result indicated that out of 55 examined calves, 2 (3.64%) were BLAD carriers and 1 (1.82%) was BLAD affected (36). In one more study, out of 42 HF and HF crossbred bulls, 2 bulls (4.76%) of HF were found to be heterozygous for BLAD (37).

Few more cases, 2 out of 126 HF bulls (1.59%) were also reported recently (38). An additional case of new silent polymorphism at 384 bp position was found in a heterozygous/carrier Holstein (39). The sequence was submitted to NCBI, and the accession No. KF840683 was allotted for new silent polymorphism. Sharma et al, (40) screened 150 buffaloes (*Bubalus bubalis*) and found no such mutation causing BLAD (BuLAD). However, the polymorphism studies using MspI restriction enzyme revealed two genotypic patterns viz. AA pattern (bands of 293, 141, 105, and 31 bp) and BB pattern (bands of 293, 105, 77, 64, and 31 bp) in buffalo gene. The sequences of A and B alleles were submitted to the GenBank with accession numbers; EU853307 and AY821799. The regular occurrence of BLAD carriers in Holstein and Holstein crossbreds emphasize to continue the genetic testing for BLAD.

Bovine Citrullinaemia

***Back ground and prevailing worldwide*:** Bovine Citrullinaemia (BCT) is a fatal inherited autosomal recessive disease reported in humans, dogs and Friesian cattle (41). Bovine citrullinaemia was reported in Australia in 1986 and the mutation responsible traced to a North American sire named Greyview Crisscross, the semen of whose son Linmack Kriss King (LMKK) was used extensively in Australia (42). About 8% of bulls considered for AI in Australia have proven to be heterozygous for the defective gene. Linmack-derived semen has been used extensively in New Zealand.

The occurrence of bovine Citrullinaemia was found high in Australia where this mutation is reportedly wide spread. Healy et al, (42) reported that 50% of Australian national Holstein herds and 30% of breeding bulls in AI centres were descendants of Linmack Kriss King (LMKK), which was carrier for Citrullinaemia. In other countries like USA and Germany, the incidence of the Citrullinaemia is very low (43, 44). Many cases of bovine citrullinaemia heterozygous were reported in Hungary (45), Taiwan (46), China (47, 48) etc. Many countries; Czech Republic (15), Turkey (49), Iran (50) etc., screened citrullinaemia but observed no heterozygous in their Holstein population. The reason of not observing any incidents in these countries could be because of screening of a small population or carrier bulls were not extensively used. The urea cycle involves a series of biochemical steps in which nitrogen, a waste product of protein metabolism, is removed from the blood and converted to urea. It has been established that bovine Citrullinaemia is a consequence of a deficiency of Argininosuccinate synthetase (ASS), one of the enzymes of the urea cycle. Consequently, the concentrations of citrulline become greatly elevated in tissues and body fluids due to decreased activity of the urea cycle enzyme arginosuccinate synthetase (ASS), which processes citrulline on the way to the production of urea (51). The deficiency of ASS occurs when a calf inherits a copy of the mutant gene encoding for ASS from each parent. The mutation responsible for citrullinemia has been characterized as a single-base substitution at 256bp (C>T) in coding exon 3 of argininosuccinate synthetase (*ASS*) gene, which converts the CGA codon that codes for arginine-86 to TGA, a stop codon. This conversion results in a truncated peptide product (85 amino acids instead of 412) which cannot participate in urea cycle (52). A second C > T transition at 525bp position represented a silent polymorphism in proline-175 (CCC > CCT). The gene is located on chromosome No.11 (BTA11). Clinical disease occurs only in homozygous recessive animals. Affected calves are born apparently normal but they develop head pressing, blindness and death usually by first week of age.

***Occurrence in India*:** In India, first carrier bull for citrullinaemia was observed when they screened a group of various breeds of *Bos Taurus* (n=200), *Bos indicus* (n=80), *Bos taurus* x *Bos indicus* crossbreds (n=50) and *Bubalus bubalis* (n=135) (33). The Holstein bull identified as carrier was imported from Australia. Although no proper pedigree was available for tracing the origin of mutation in the carrier animal, but they presumed

that the animal could be a 3rd or 4th generation descendant of LMKK. After the identification of carrier, an extensive screening of Holstein (n=337) and Holstein crossbreds (n=305) specially breeding bulls stationed in various sperm stations throughout the country was conducted (53), which indicated no heterozygous or carrier animal in Holstein and Holstein crossbred bull. It was then realized that Indian Holstein has no polymorphism for ASS1 gene. However, once again two Indian Holstein bulls out of 120 (1.67%) were found heterozygous for ASS1 gene during routine screening in a year (54). Detection of Citrullinaemia carriers compelled breeders to continue screening of Holstein and Holstein Crossbred breeding bulls. A fresh case of a carrier bull has been recently identified during routine investigation (55). To confirm the polymorphism in ASS1 gene, sequence of PCR product was performed along with a controlled Holstein bull. The sequence of a controlled Holstein revealed a silent mutation at 240bp that does not change amino acid (Sarine AGC>AGT) at 80 codon within exon 3 of ASS1 gene. The sequence with silent mutation was submitted to the NCBI (Accession No. KF933365). Detection of Citrullinemia carrier in Indian cattle population supports the programme for continuous screening.

Bovine Deficiency of Uridine Monophosphate synthase (DUMPS)

***Background and prevalence worldwide*:** Bovine DUMPS is a rare metabolic disorder of Holstein-Friesian and Wagyu breeds worldwide (56). Most of the DUMPS carriers identified in North America (n = 438) and Europe (n = 314), were the offspring of Happy Herd Beautician, a 5th best U.S. Holstein bull in 1987 (Holstein Association, 1987). The occurrence of DUMPS carrier is fewer worldwide except some of sporadic cases. The occurrence of bovine DUMPS was observed in China (47) where they tested around 436 cows and 93 bulls and found 1.17% carrier. Poli et al, (18) observed 0.96% and 0.11% DUMPS heterozygous out of 104 Argentinean Holstein bulls and 950 cows respectively. Ten mummified fetuses were tested for the deficiency of uridine monophosphate synthase (DUMPS), which is known to contribute to the embryonic and fetal mortality, in cattle (57) and observed two fetuses were heterozygous (carriers) for DUMPS. Two HF carriers were found among 314 AI bulls, 682 bull mothers and 155 young bulls in Hungary (45). Taiwan also recently reported two carriers out of 1468 HF animals screened for DUMPS (46). Many countries; Czech Republic (15), Turkey (49, 58), Iran (59), Pakistan (10), Romania (60), Poland (61) etc, tested their Holstein herds for presence of DUMPS carriers but none of Holsteins exhibited mutation in *UMPS* gene.

Uridine- 5´-Monophosphate Synthase is an enzyme which has key role in pyrimidine biosynthesis and is inherited as autosomal recessive locus (62, 63). The enzyme uridine – 5´-monophosphate (UMP) synthase catalyses the conversion of orotic acid to UMP, the precursor of all other pyrimidine nucleotides and a normal constituent in the milk of cows and other ruminants (64). Earlier heterozygous carriers used to be identified by measuring uridine monophosphate synthase (UMPS) activity in erythrocytes instead of molecular techniques (PCR-RFLP), as the activity is reduced to about half of the normal value (65). The single base pair mutation (C>T) changes arginine (CGA) to stop codon (TGA) in a gene for UMPS at 1213bp, codon 405 within exon 5 (66) leads to a functionally impaired UMPS enzyme (67). The UMP synthase gene was mapped to the bovine chromosome 1 (q31-36) (68). Embryos homozygous for DUMPS do not survive to birth and usually die early in gestation, so no homozygous recessive animal was detected so far. The embryos appear to be aborted or reabsorbed approximately 40 days after conception, leading to repeated breeding problems (27, 69). The genomic structure of UMP synthase gene as determined and a PCR- based diagnostic test for carrier detection has been established.

***Occurrence in India*:** The similar situation was also observed in India where 1250 including 976 cattle and 274 buffaloes were screened and no animal was found carrier for DUMPS (53) as a result it was decided to discontinue the screening of DUMPS in Holstein cattle. However, a case of DUMPS out of 86 Holsteins was observed first time in India (70) which has reinitiated molecular screening of DUMPS especially in Holstein and Holstein crossbred bulls being used for artificial insemination in the country.

***Bovine Factor XI (FXI) deficiency syndrome Background and prevalence worldwide*:** Factor XI (FXI) is one of more than a dozen proteins involved in blood clotting. It is an inherited bleeding disorder that has been seen in humans, dogs and cattle (71). The bovine form of disease was first discovered in Holstein cattle in Ohio in 1969 (72) and then in Canadian Holsteins in 1975 (73). It was later observed in Holstein of other countries. The prevalence of Factor XI is almost worldwide as many heterozygous and homozygous for recessive gene were detected in Holstein population. In Poland (74) 103 randomly selected cows were tested. Out of which, 28 cows with repeat breeding, and 9 cows with recurrent mastitis for the presence of an abnormal *FXI* allele were found. Besides, three related cows were diagnosed as carriers. Watanabe et al, (75), genotyped FXI deficiency in stunted Japanese black cattle and found homozygous recessive and heterozygous with different health problems. Marron et al, (76) reported frequency of the mutated allele has been determined to be 1.2% in a contemporary population of the USA Holstein sires. Turkey has also experienced the presence polymorphism in FXI gene in two of Holstein cattle (49, 77, 78). Thereafter no carriers were observed in Turkey (58). Similarly many countries; Iran (79) investigated FXI in their Holstein herd but could not observe any heterozygous. Bagheri et al, (80) tested 300 Khuzestan Buffalo in Iran for FXI but none of buffalo found to be carrier.

It also goes by the name of hemophilia C in human. FXI is trace protein in the blood. It is produced in the liver and plays a role in the coagulation cascade, the chain reaction that is set in motion when there is injury to a blood vessel. It helps in the activation of Factor IX, another blood protein important in the clotting process. Prolonged oozing of blood following dehorning and castration may also be observed. Affected cows frequently have pink coloured colostrums. Blood in milk led to the identification of the condition in British dairy herd (81). Affected cows have also been difficult to breed and are more susceptible to diseases such as pneumonia, mastitis and matritis (82). Affected cows have 50% higher frequency of being repeat breeders than normal mate as plasma progesterone concentration were slower to decline at the end of estrous cycle in affected cows. Studies revealed follicular diameter was smaller in affected cows. Heterozygous individuals show varying symptoms and degree of reduced FXI activity (83) but difficult to distinguish because of overlap in the normal and carrier activity ranges.

The insertion of 76bp segment [AT(A)28 TAAAG(A) 26GGAAATAATAATTCA] within exon 12 causes mutation in FXI gene (76). The insertion introduces a stop codon within the first four nucleotides of the insertion that results in a premature FXI protein lacking the functional protease domain. In normal case the PCR product would be 244bp (both copies of gene) where as in heterozygous condition one copy of gene would normal and another copy would be of 320bp because of 76bp insertion (mutation).

***Occurrence in India*:** Screening of FXI in Indian cattle and buffalo started during 2005 -2007 (84). Initially more than 1000 animals including 330 Holstein, 265 Holstein crossbreds, 105 Jersey, 69 Jersey crossbreds, 79 *Bos indicus* breeds and 153 *Bubalus bubalis,* were investigated for mutation due to 76bp insertion in candidate gene. Two Holstein bulls were found to be heterozygous for FXI deficiency syndrome which was confirmed by sequencing the PCR product of exon 12. None of other animal from cattle and buffalo breeds was found to be a carrier for FXI. The sequence comparison between normal and heterozygous

animals revealed that there is insertion of 77 base pair fragment [AT (A)29 TAAAG (A)27 GAATTATTAATTCT] instead of 76bp within exon 12 of factor XI gene. Similarly the PCR product of carriers was of 324bp instead of 320bp as observed in *Bos taurus* (76). Both polymorphic sequences of normal as well as heterozygous animals were submitted to the National Centre for Biotechnology Information (NCBI), and assigned the accession numbers DQ438908 and DQ438909 for normal and heterozygous HF animals respectively. After presence of heterozygous, screening of animals for FXI deficiency became mandate for breeding bulls in India. However, thereafter no carriers were observed in India (85).

Complex vertebral malformation (CVM)

***Background and prevalence worldwide*:** CVM is an autosomal recessively inherited disorder in Holstein cattle worldwide, which usually onsets during fetal development, leading to abortion of fetuses or prenatal death, and vertebral anomalies. The syndrome was first discovered in the Danish Holstein population in 1999 (86), but shortly thereafter reported in the Netherlands (87), United States (88, Holstein Association, USA, 2004), the United Kingdom (89), and in Denmark (90). The percentage of CVM carrier reported worldwide is very high. The analysis of a total of 957 sires showed that the frequency of CVM affected animals was 13.2% (91), of which the possibility of a CVM-homozygote calf was between 0.3 to 0.45% in German Holsteins. In Sweden, 228 bulls were genotyped for CVM during 1995-1999, of which 53 bulls, i.e. 23%, were confirmed CVM carriers (92). Rusk and Kaminski, (93) determined the actual carrier frequency of the CVM in Polish Holstein-Friesian cattle, they examined 202 proven bulls during 2001–2005, used by 4 domestic artificial insemination companies and 403 unproven bulls under evaluation. Out of the 605 bulls examined, heterozygotes were diagnosed, including 118 that were sons of known CVM carriers. In China, 10 CVM carriers were found among 68 at-risk Chinese Holstein bulls, and 282 carriers were found among 602 at-risk cows (94). Japan reported high incidence 32.50% of CVM (95). A screening was carried out for CVM in the cattle population of the Czech Republic. In 111 elite Holstein females, 21 (18.9%) were heterozygotes for CVM (15). One cow out of 84 Holstein cows and 6 bulls was found to be carrier for CVM in Republic of Mecedonia (29). To investigate the congenital complex vertebral malformation syndrome (CVM) in Danish Holstein calves, two breeding studies were performed including 262 and 363 cows, respectively (96). In both studies, significantly fewer CVM affected calves than expected were obtained; a finding probably reflecting extensive intrauterine mortality in CVM affected fetuses. Two Holstein cows out of 45 and two bulls (N=47) were found to be carrier for CVM in Slovakia (97).

The CVM was tested on 217 cows and 125 bulls selected randomly from a Holstein cattle population in south China. Five Holstein cows and five Holstein bulls were identified to be CVM carriers; the percentages of CVM carriers were estimated to be 2.3, 4.0 and 2.9% in the cows, bulls and entire Holstein cattle samples, respectively (98). However, many of studies exhibited no carriers of CVM detected in Iran (79, 99, 100, 101). Genealogical records traced the origin of the disease-causing allele to a common ancestral bull, Carlin-M Ivanhoe Bell, which has been used in dairy cattle breeding worldwide for two decades due to the superior lactation performance of his daughters. Coincidently Carlin-M Ivanhoe Bell was a carrier for two genetic diseases, CVM and Bovine leukocyte adhesion deficiency (BLAD). The BLAD and CVM genes are located on chromosomes 1 (1) and chromosome 3 (90) respectively. When the sire (father) of Carlin-M Ivanhoe Bell, a bull named Pennstate Ivanhoe Star, was tested he was found to be a carrier of both CVM and BLAD. Carlin-M Ivanhoe Bell's grandsire, Osborndale Ivanhoe, however, carried only BLAD. Scientists therefore believe that the mutation responsible for CVM occurred either in Pennstate Ivanhoe Star (Sire), or somewhere in his maternal family.

Disease symptoms have not been observed in carriers of CVM. Detailed clinical characterization of CVM demonstrated a composite phenotype with axial skeletal deformities such as hemivertebrae, misshaped vertebrae, ankylosis of mainly the cervico-thoracic vertebrae, scoliosis, and symmetric arthrogryposis of the lower limb joints, craniofacial dysmorphism, as well as cardiac anomalies (102, 103).

The Impaired protein molecules, a UDP-N-acetylglucosamine transporters or Golgi UDP-GlcNAc transporters (alternative name) in the Golgi apparatus membrane, causes CVM. These transporter proteins transport *Uridine diphosphate N-acetylglucosamine or UDP-GlcNAc* (a nucleotide sugar and a coenzyme in metabolism), from cytosol/Cytoplasm (synthesis site) into the Golgi lumen before these can be substrates for the glycosylation of proteins, lipids, and proteoglycans. The *UDP-GlcNAc* plays an important role in the structure of the cytoskeleton. The molecular cause of CVM is a substitution of guanine by thymine (G→T) in a solute carrier family 35 member 3 gene (*SLC35A3*), encoding a UDP-N- acetylglucosamine transporter. The gene is located on bovine chromosome BTA3 (90). This mutation results in the substitution of Valine by Phenylalanine (V180F) at position 180, impairing transporter membrane protein

***Occurrence in India*:** In India, sporadic studies conducted to investigate CVM in small group of Holstein and Karan Fries, crossbreds of Holstein and Tharparkar developed by the national institute. The occurrence of CVM carriers was reported very high i.e. 23.08% out of 52 Karan Fries bulls (104). Further a small group of 55 Karan Fries calves tested for presence of CVM carrier (105) but found no carrier. Kotikalapudi et al, (106) tested around 60 bulls for CVM. Their results show that one out of 60 bulls tested exhibited polymorphism (G→T) at position 559 in exon 4 of *SLC35A3* gene. Sequence analysis of PCR product also revealed the presence of two previously unknown homozygous mutations (TG→CT) at nucleotide positions 554 and 555 (accession No. KF679985) in addition to the previously reported heterozygous mutation at position 559.

***Effect of the diseases on breeding*:** If a bull is carrying one copy of mutant gene for any of mentioned disorders is mated with an unaffected cow, they will produce 50% heterozygous or carrier in the population but will not exhibit the symptoms of the diseases. If two heterozygous carriers are mated, they will produce 25% affected with the diseases, 50% carriers and 25% normal progeny in the population.

Conclusion

The results demonstrated a fewer cases of presence of disorders; BLAD, DUMPS, CVM, Bovine citrullinaemia and FXI in Indian Holstein and Holstein crossbred population which could have been enough to spread unwanted gene in the population. If no cases are observed in routine investigation even then an extensive screening programs for detection of genetic disorders seems necessary to ensure the utilization of bulls free from Genetic disorders for artificial insemination programmes in India.

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NEWS ITEM

New Health Beneficiary Pepper

A new pepper variety has been developed with a high capsinoid content to make it less pungent while maintaining all the natural health benefits of the fruit, according to researchers with the U.S. Department of Agriculture. The new small-fruited *Capsicum annuum* L. pepper through traditional breeding methods in an effort to make the health benefits of hot peppers available to more consumers.

In hot peppers, capsaicinoids are the compounds associated both with their signature heat and health benefits, which include being a source of antioxidants. However, their pungency can limit their use in foods and pharmaceuticals. Capsinoids, closely related compounds of capsaicinoids, provide the same benefits without the pungency.

Biopharmaceutical Discoveries

European scientists have made ground-breaking discoveries for improving the efficiency of the production of pharmaceuticals through plant biotechnology. Biotechnological production offers a cost-effective and environmentally friendly alternative to the chemical synthesis of rare and complex pharmaceutical compounds currently isolated from plants. Several expensive anticancer alkaloid blockbusters used in chemotherapy, such as terpenoid indole alkaloids - vinblastine and vincristine, are currently extracted from the plant *Catharanthus roseus* (Madagascar periwinkle) at high price. These compounds are used to treat Hodgkin's lymphoma, breast cancer, small-cell lung cancer and leukemia. Typically, very low levels accumulate in plant tissues, but chemical synthesis is not an economical alternative either due to their highly complex structures and specific stereochemical features. The biotechnological production of high-value plant-derived compounds using plant cell cultures is an attractive and sustainable alternative to extraction from whole plant material. However, the biosynthetic pathway leading to these compounds in plants is long and complex, with multiple enzymatic steps that are still largely uncharacterized at the genetic level and the best way is to unravel the metabolic pathway leading to the periwinkle terpenoid indole alkaloids. The complete pathway of twelve enzymes was reconstructed in tobacco plants, paving the way for cost-effective production of diverse therapeutic compounds. Moreover, cell culture technologies were developed, and the cultivation of the plant cells was scaled-up using bioreactors. The use of plant cells as real green chemical factories is now becoming feasible for the first time.

Genetically Engineered Potatoes

BASF Plant Science will again conduct field trials this year with genetically optimized potatoes which are undergoing the EU approval process. The field trials for genetically optimized potatoes which are in approval process at European Union are on the way in Germany, Sweden and the Netherlands, for starch potato Modena and late blight resistant variety Fortuna. These developed potatoes can be high performing varieties under different environmental conditions as well as the resistance characteristics of the Fortuna potato.

Vaccine for Dust-Mite Allergies

The University of Iowa has developed a vaccine that can combat dust-mite allergies by naturally switching the body's immune response. Dust mites are ubiquitous, microscopic buggers. They are found in 84 percent of households. Preying on skin cells on the body, the mites trigger allergies and breathing difficulties among 45 percent of asthma patients. Prolonged exposure to dust-mite can cause lung damage. Treatment is limited to getting temporary relief. This research explores a novel approach to treating mite allergy. Encapsulated miniscule particles are administered with sequences of bacterial DNA that direct the immune system to suppress allergic immune responses. The vaccine takes advantage

of the body's natural inclination to defend itself against foreign bodies. A key to the formula lies in the use of an adjuvant - which boosts the potency of the vaccine - called CpG. CpG sets off a fire alarm within the body, springing immune cells into action. Combining the antigen (the vaccine) and CpG causes the body to change its immune response, producing antibodies that dampen the damaging health effects dust-mite allergens generally cause.

Targeted Therapy and Pharmacogenetics - A New World of Medical Treatment

According to the Cornell University Research publication, "Targeted therapy will be the treatment of chose for cancer in future". It was explained that the genetic research showed that mutation in human gene MYBPC3 caused cardiomyopathy. The individuals with this genetic mutation are "sure" to have heart disorders and many suffer sudden cardiac failure. The gene provides the instructions to the cardiac muscle and its mutation caused the improper formation of the heart muscles. Targeted therapy blocks the growth of cancer cells by interfering with specific targeted molecules for tumor growth rather than just interfering with all rapidly dividing cells with the traditional chemotherapy.

Pharmacogenomics is an emerging branch of science. Some patients do not response to drugs because of their genes. These non-responders are also hyper-sensitive to the specific drugs which cause them to have multiple-organ failure. The Food and Drug Administration (FDA) was now advising patients to undergo "genetic testing" before using such drugs. "It was now possible for a person to get genes mapped for $ 1,000 and it is likely that every hospital may have this facility soon.

EU May Restrict Genome Editing of Crops

A fledgling technology to manipulate the genes of crops in order to make them less susceptible to disease and more productive is at risk of falling foul of the European Union's genetic modification rules. Genome editing is different to genetic modification. Genome editing typically involves finding the part of a plant genome that could be changed to render it less vulnerable to disease, or resistant to certain herbicides, or increase yields or other desirable traits. Researchers use "molecular scissors" to break apart the genome and repair it. This is a process that occurs naturally when plants are under attack from diseases and causes new mutations. This mutation enables the plant to survive future attacks. Now the relevant genes can be altered very precisely, without the need to import DNA from other organisms. These plants are indistinguishable from those that would occur through selective breeding. Gene editing could offer an alternative to genetic modification that could be much more palatable to consumers. Scientists believe there is huge potential for the technology because it avoids the slower, scattergun approach of selective breeding. It has only become possible to edit plant genes in the past few years following decades of work on mapping genomes and inventing ways in which they can be precisely altered. Under EU laws, however, it is unclear whether gene editing should be treated in the same way as genetic modification. The European Commission is expected to offer guidance on the technology soon.

Laser based Pain detectors

The forehead and fingertips are the most sensitive parts to pain, according to the first map created by scientists of how the ability to feel pain varies across the human body. A laser based method has been developed for detecting with a scale of one to 10. This method offers an exciting, non-invasive way to test the state of pain networks across the body. Chronic pain is often caused by damaged nerves, but this is incredibly difficult to monitor and to treat. The laser method may enable us to monitor nerve damage across the body, offering a quantitative way to see if a condition is getting better or worse.

Friendship through Genetic Analysis

In a genome-wide analysis, one can find that we are far more genetically similar to our

friends than we are to strangers of the same population. The researchers analyzed nearly 1.5 million markers of gene variations and compared pairs of unrelated friends against pairs of unrelated strangers. They found that, on average, one share about 1 percent of our genes with friends, which is a large number for geneticists. Friends shared enough genes to allow researchers to develop a "friendship score," which predicted who would be friends with the same level of confidence as genetic tests for predicting obesity or schizophrenia. The researchers also found that friends were most similar in genes controlling the sense of smell. Our sense of smell may draw us to certain environments, like a coffee house, where we meet people whose noses are tuned like ours. They added that our sense of smell could be one of the mechanisms we use to identify genetically similar friends. However, to be clear, more research is needed to explain how we find our genetically similar friends.

Implantable Gel that Would Help to Get Rid of Diabetes

Scientists have developed an implantable gel that contains genetically modified light-sensitive cells and it can be used to treat diabetes. Scientists developed an implantable gel that guided light under a mouse's skin. To control diabetes, the team shone light into the mouse and at the implanted gel using a fiber optic cable attached to its head. The light triggered cells in the gel to produce a compound that stimulated the secretion of insulin and stabilized blood glucose levels. The gel is in the prototype stage and attempts are made to make it more user-friendly.

High Fat Diets can Destroy Stomach's Signals to Brain.

Indulging in fatty foods could destroy stomach's signals to brain. Scientists found that the nerves in stomach, which propagates signal fullness to the brain papered to be desensitized after long-term consumption high-fat diet. The laboratory studies showed the stomach's nerve responses does not return to normal upon a return to a normal diet. A hormone leptin known to regulate food intake, can also change the sensitivity of the nerves in the stomach that signal fullness. Leptin acts to stop food intake however, in the stomach in high-fat diet induced obesity, leptin further desensitizes the nerve that detect fullness. The two mechanisms combined meant that obese people needed to eat more to feel full, which fuels their obesity cycle. The results had "very strong implications for obese people, those trying to lose weight, and those who are trying to maintain their weight loss."

Seedless Mango:

Bihar Agriculture University (BAU) scientist has developed seedless mango variety from hybrids of mango varieties Ratna and Alphonso. The mango is finely textured and juicy, with a rich, sweet and distinctive flavour when mature. Trials of the new variety, Named Sindhu, are under way at different locations in the country but the result of the one at (BAU) suggests it could be suitable for both integrated horticulture and kitchen gardening. An average fruit weighs 200 grams and its pulp is yellowish in colour, has less fibre than other mango verities. It generally grows in bunch and the fruit matures in the middle of July. According to BAU vice chancellor M.L.Choudhary the University has, on an experimental basis, decided to recreate plants of this variety and make them available to Bihar's mango growers during the next season. The seedless variety also has good export potential. The university would provide quality plants to mango grower in 2015 to exploit the export market. According to the national horticulture mission (NHM), Bihar ranks third in mango cultivation and covers about 50% a little over, 38,000 hectares-of the total fruit area in the state. The produce last year was in the region of 1.5 million tonnes. Malda, Mallika, Jardaloo, Gulabkhas, Bumbai, Daseri and Chausa are major mango varities grown in the state.

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