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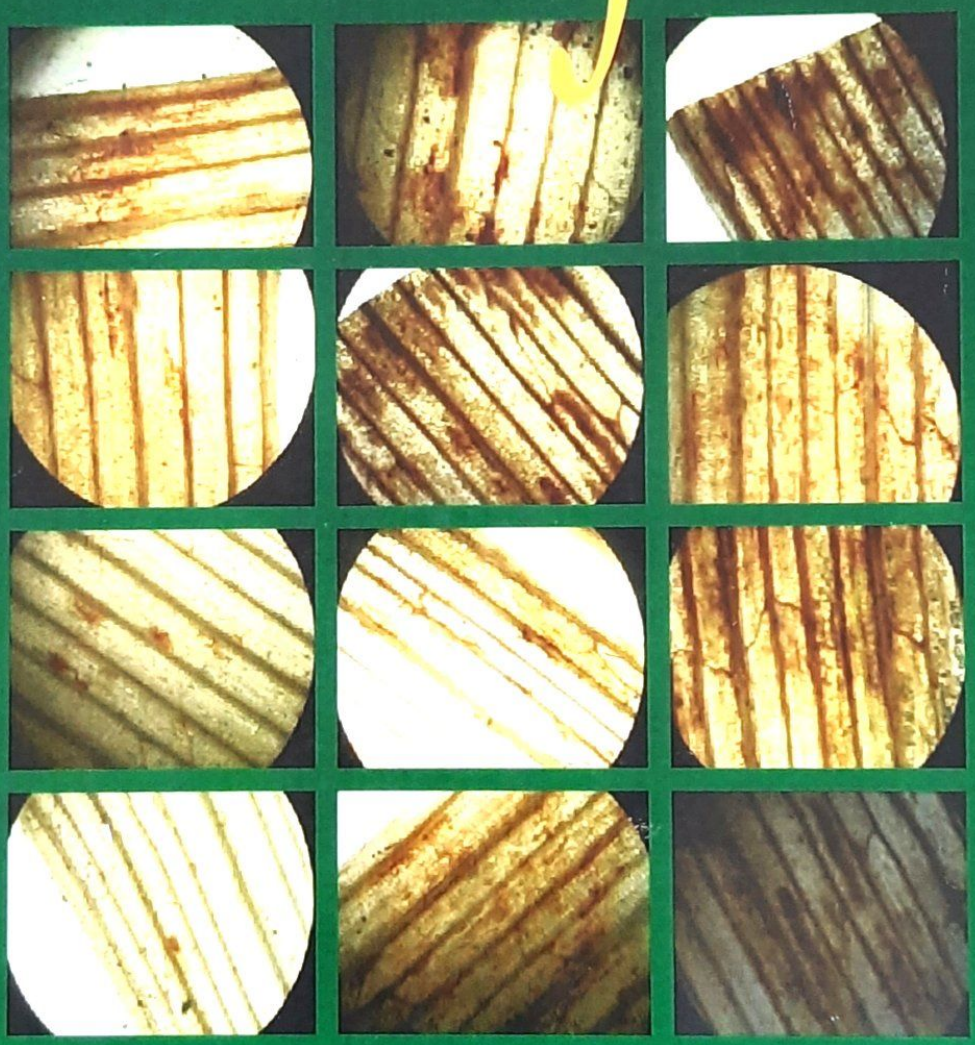

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Cover photograph: *In situ* detection of H_2O_2 in mid-portions of leaves in wheat (photo courtesy: **U Chakraborty**)

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Evaluation of traditional rice germplasms for alluvial zone of West Bengal –a review

Ashim Chakravorty and P.D. Ghosh*
Cytogenetics and Plant Breeding section, Biotechnology Research Unit, Department of Botany, University of Kalyani, Kalyani 741235, West Bengal, India

Abstract
Fifty one promising landraces of rice cultivars of three districts viz. Nadia, 24 Parganas(N) and Murshidabad of West Bengal were taken for characterization, estimation of genetic variability, genetic diversity and interrelation among them based on forty six agromorphological characters. The field experiment was carried out at the Zonal Adaptive Research Station, Krishnagar, Nadia, West Bengal during Kharif season of three consecutive years of 2006, 2007 and 2008. The land is mainly gangetic alluvium plain with neutral pH (pH=6), low availability of nitrogen and phosphate and potash content. Observations of various qualitative and quantitative traits were recorded at the different stages of growth. Distinctiveness, Uniformity and Stability (DUS) test was done on twenty eight qualitative traits following the National test guidelines, supplied by Rice Research Institute, Rajendarnagar, Hyderabad. Mean, standard error of mean, standard deviation, coefficient of variation were calculated for each quantitative trait to explore the variability, if any, among the cultivars (Singh and Choudhury, 1977). Genotypic correlations were, in general, higher than the phenotypic correlations and thus suggested that the observed relations among the characters were due to genetic factors. Genetic divergence was assessed among 51 landrace genotypes which were grouped into eleven clusters. Cluster mean was done and the characters which contributed maximum towards genetic divergence among the genotypes were culm diameter>culm length>grain length>Plant height (seedling)>sterile lemma length>grain length/breadth ratio>ligule length>flag leaf angle>culm number in the descending order.

Keywords: Landraces, characterization, DUS test, variability, diversity, D^2analysis

Rice (*Oryza sativa* L 2n =24) is one of the most important cereal crops in the world and also life blood of southeast Asia where more than 90 percent of rice is produced and consumed. It is also a major source of livelihood for than 250 million of households.

In Asia 92 percent of the world's rice is grown in 136.642 million hectares and consumed, provides 35-37 percent of the calories to more than 3 billion Asians. In India rice contributes around 45 percent of cereal production and is the main food source for more than 60 percent of the population in the country (Siddiq, 2002) covering 44.6 million hectares of total geographical area.

Green revolution: the beginning of genetic erosion

The so-called 'green revolution' in rice started in late 1960s due to cultivation of semi- dwarf cultivars carrying Dee-geo-woo-gen. With application of heavy dose of fertilizer costs are beginning to rise and rice farmers are facing declining profits. Scientists all over the world have tried to transfer the desirable genes from traditional varieties to modern varieties. The present investigation aims at collection, conservation and characterization of collected germplasms for exploitation in breeding program. For this purpose morphological and physiological characters with respect to growing habit, plant height, shape size and colour of the culm, leaf blade, panicle, hull, apiculus and dehulled grain presence or absence of pubescence, grain shape, size growth duration, resistance or tolerance to disease and insect pests, grain quality etc. will be studied to differentiate the land-races from one another for future breeding programme.

History of rice breeding

Systematic rice breeding was initiated at the beginning of the 20th century. Early breeding in tropical Asia was aimed at improvement of locally adapted popular varieties largely by pureline selection. Selection and evaluation process was confined to a single center in each region, as breeders then had no idea about the significance of multilocation testing and, as a result, a very large number of improved varieties were under commercial cultivation. Indonesia was the first country, where multilocation testing was started with the objective of reducing the number of varieties. Its successful experience not only prompted many countries to follow suit but also made the breeders realize the need for improving the method of varietal testing. Whereas the use of statistical parameters, such as, variance and standard error helped improved field testing technique, the application of the principles of randomization and analysis of the variance that decide on the field plot design enabled evaluations of test entries more accurately. Rediscovery around this period of Mendel's laws of inheritance, in spite of its potential for unfolding new variability, could hardly change breeding–selection

*Corresponding author:
E-mail: pdggene@rediffmail.com

approaches, as breeders relied excessively on pure line selection. Nevertheless, it helped to understand the mode of inheritance of scores of agro-botanic traits and floral biology as well as acquire emasculation-pollination skills, which proved valuable when recombination breeding assumed importance.

Irrespective of the method of selection, emphasis of breeding in the beginning was for improvement of traits other than yield. The traditional rice exporting countries, like Burma and Thailand, for instance, placed their breeding thrust on improvement of grain quality, while breeding for resistance to rice blasts, the only killer disease known then, received importance all over Asia. Identification of CO4, a pureline selection from the local variety 'Anaikomban' found resistance to the disease served as the donor to develop the most popular variety CO5, by hybridization. Breeding for high yields was confined to lowland varieties, as breeders believed late maturing varieties to yield more. Thus, single location selection /evaluations under low levels of nutrient management with emphasis largely on improvement of traits other than yield was the general breeding practice until the end of the world war II.

Rice breeding in India was started in 1911 at Dacca (now in Bangladesh) and in the following year at Coimbatore in the old Madras province. Recognizing the importance of rice to India's economy, The Indian Council of Agricultural Research, founded in 1929, sponsored, rice breeding projects in all the major rice growing states and as a result the country had as many as 82 research stations exclusively for rice research by 1950. It was around this period that ICAR's land mark decision led to the establishment of the central and national Rice Research Institute at Cuttack, in Orissa in 1946 for addressing problems of national importance. More organized breeding research that followed at central and state levels led to the release of more than 450 improved varieties all of which, except 27 cross bred varieties, were pure line selections from popular local varieties. Among varieties that remain popular till now, MTU1, MTU15 and HR 19 of Andhra Pradesh; Chin 7 of west Bengal; Columba strains of Maharashtra; GEB 24, CO 25 ASD 1 of old Madras Province; T141 and SR 26b of Orissa; Basmati 370 of Punjab and t1366 of Uttar Pradesh are important. Breeding for adverse growing conditions assigned to problem specific regional centers had also led to the development of varieties, which have hardly any alternative even today. They include among many ARC 353-648 of Assam;DWP-1311 of Andhra Pradesh; ADT 17, PTB 15 and 16 of Madras Province FR 13A and FR 43B of Orissa; Hybrid 84 of West Bengal for deepwater and flood - prone areas; Kalarata 1-24 and Bhurarata 4-10 of Bombay Province, SR 26 B of Orissa; Chin13 and Chin 19 of West Bengal for saline conditions and AKP 1, AKP 3, BCP 2 and BCP 5 of Andhra Pradesh; ASD 4, ASD18 and PTB18 of Madras Province; BAM 15 of Orissa; N22 of Utter Pradesh; Chin 25 and Chin 27 of west Bengal for drought prone situations. Resistance breeding against biotic stresses led similarly to the development of CO25 against blast and MTU 15, TKM 6, SLO 12 and CH 47 against stem borer. There are many more time – tested varieties that came out of resistance breeding. In spite of such an impressive record of breeding achievements the fact remains that the efforts made for yield improvement were of no consequence as compared to what had been done for catering to other agro-ecological and socio-economic needs.

It was immediately after the World War II, when food grain supply became far short of the demand and threatened the word community, that Food and Agriculture Organization (FAO) founded the international rice commission (IRC) within the FAO frame- work to find ways of increasing rice production. The IRC recognized non-lodging habit, fertilizer responsiveness, early maturity and wide adaptability as the important varietal characteristics for achieving higher and stable yields. Accordingly, the FAO launched several regional networks projects such as cataloguing and maintenance of rice genetic stocks, *Indica-Japonica* Hybridization, cooperative varietal Trials, Wide adaptability Test, Variety – fertilizer Interaction in *indica* varieties, Uniform Blast Nursery and International Training Programme in Rice Breeding during 1950-1956.

The international cooperative was instrumental in cataloging major rice varieties of the world and establishing center for maintenance and exchange of germplasms. The ambitious *indica/japonica* hybridization programme launched in 1952 was the early international effort to explore the possibilities of breaking the yield barrier in *indica* rices. Relatively higher yielding ability of japonicas believed them to be due to their non-lodging habit and responsiveness to higher levels of fertilizer, was what prompted the formulation and execution of this massive programme in Asia. Extensive hybridization programme with CRRI at Cuttack as the primary hybridization center and countrywide screening of the segregating material lasting for ten years proved a major disappointment. Except for three varieties viz., ADT 27, an early maturing type found suitable for *kuruvai* season in Tamilnadu and two medium late maturing types viz., Mahsuri and Malinja identified in Malaysia that became widely popular in India and Malaysia respectively, the project hardly yielded any good variety combining the desirable features of Japonica and Indica. Partial sterility and skewed segregation towards parental types have been attributed to low frequency of stable fertile recombinents. Had the breeders been careful to choose tropical *japonica* parents, instead of temperate *japonica* types like Norin 6, Norin 8, Norin 16, Rikue 12, Asahi etc., the genetic hurdle could have been averted. The realization that inter subspecific hybridization strategies would not yield anything substantial led to the abrupt termination of the project, which nearly coincided with the advent of the short statured *indica* variety Taichung (Native) 1 (T(N)1) and *Ponlai* varieties (Taichung 65,Tainan 3 etc). Taichung (native) 1 that heralded the era of plant type- based high yielding varieties is a derivative of the cross between the spontaneous dwarf's

mutant Dee-Geo-Wu-Gen and Taiwanese tall variety Tsai-Yuan-Chung. The early maturing dwarf variety well adapted to round- the- year cultivation provided the base for developing the 'plant type' concept and development of a series of high yielding dwarf varieties including the miracle yielder IR 8.

An important development that accelerated breeding research at global level was the establishment of the International Rice Research Institutive (IRRI) in the Philippines in 1960 by the ford foundation and Rockefeller Foundation in cooperation with the Government of the Philippines. Breeding activities began in 1961-62 with the sole objective of evolving non-lodging varieties with high yields, which led to the release of IR 8, the widely adopted miracle yielder suited to irrigated ecology and IR 5, a semi tall variety bred for less favorable environments. It was IR 8 that provided the momentum for development of a series of high yielding dwarf varieties, which included IRRI bred IR 20, IR 36, IR 42, IR 64, IR 72 etc, besides large number by the National Agricultural Research System (NARS). Thrust of the breeding research has been changing over the years with yield, quality and disease pest resistance receiving successively priority attention in the case of irrigated rice, while yield maximization and adaptation to moisture stress and submergence in rain fed rice, involving actively the NARS, most the thrust area research programmes were sponsored and coordinated by IRRI. The multidisciplinary genetic Evaluation and Utilization (GE) programme initiated in 1974 while broadening the breeding efforts at IRRI and NARS led simultaneously to the launching of global testing network called International Rice Testing programme (IRTP). The programme, later renamed as International Network for Genetic Enhancement of rice (INGER), involving over 75 countries and no less than 1000 scientists facilitated free exchange of germplasm (improved and unimproved) among the participant countries by IRRI's germlasm bank to an unprecedented scale. This one activity not only brought visibility to IRRI but enabled NARS to benefit the maximum. Several varieties named by IRRI were from IRTP/INGER nurseries. In the recent years, IRRI, in partnership with NARS and advance Research Institutes in the developed world, is engaged in strategic research towards achieving food nutrition security on a sustainable basis. Search for new yield thresholds through hybrid technology, new plant type, development and application of Biotechnology- Genetic engineering tools for adding value and finding solution to still unsolved biotic and abiotic stresses and functional genomics are important among them. Continued enrichment and maintenance of the gene bank which remains the 'gene source', for breeders all over, is the most laudable activity of IRRI.

It is the unique combination of short stature, non-lodging habit with profuse tillering and upright foliage, photo-insensitivity, high fertilizer responsiveness and early maturity in the new plant type that has helped break the centuries long yield barrier of the tropical rices. Short duration coupled with period bound maturity has made possible to raise 2-3 crops a year and increasing thereby the total productivity by 2-3 times over the late maturing, season bound traditional tall varieties. The impact of the plant-type based high yielding varieties is evident from various indicators. Many chronically rice deficit countries have become self sufficient and some with sizeable surplus within 10-15 years of their introduction. Growth of rice (paddya) production in Asia has risen to 540.62 million tones by 1997-98 from 292.17 in the triennium ending 1971, while productivity from 2381 kg/ha in 1971 to 3900 kg/ha in the triennium ending 1997-98. Yield and production have registered from two times increase in India to four times increase in the countries like Indonesia and Phillipines during the corresponding period. Largely development of high yielding varieties and their extensive adoption enabled many countries in Asia to register high and steady production growth.

As for India, more than 550 high yielding dwarf varieties have been released for all the major ecologies, the maximum being for irrigated environment. Evolution of varieties for different ecologies at such a rapid pace has been possible because of the coordinated network approach conceived and adopted at national level for material generation and multi location testing. The realization that the entire growth has been on account of top sided attention given to irrigated rice, prompted at national and international levels a shift in breeding emphasis towards long neglected rain fed ecologies, which account for 50% at global level and 55% in India. Rain fed ecology being the most complex and diverse, eco-regional models were conceived by IRRI for characterizing such ecologies and addressing the problems unique of them. The rain fed low land and rain fed upland Consortia – the multi-country projects are close to finding solution to the problems of handicapped ecologies in Asia. The impact of this, coupled with national initiative, is seen today in the steadily increasing productivity and production in the predominantly rain fed eastern part of India.

Ever since the development of the dwarf high yielding varieties, breeders all over have been looking for technologies that would help raise to further the genetic ceiling to yield. Of the two strategies contemplated *viz.* tailoring of physiologically still more efficient plant type and exploitation of hybrid vigour, the later has become a reality since the development in the late seventies of commercially viable hybrid rice technology in China. Its extensive adoption over 18 million ha (55% of the rice area) has helped China add annually 20 million tones to the national rice production making thereby the chronically food deficient countries self sufficient. The knowledge gained from the experience and material support form IRRI and China, proved handy fifteen years later for India to develop hybrid technology suited to its agro-climatic conditions. The success stories of China and India have motivated a few more countries in the region to initiate hybrid rice research.

Thus the nine decade long history of rice breeding comprises three major phases viz., the period of tall varieties with slow yield growth till early fifties, the period of semi-dwarf varieties breaching the yield

barrier since mid sixties and the period of hybrid technology marking the second yield breakthrough since eighties. The current breeding emphasis at IRRI and a few other countries like China, Japan and India include (i) tailoring of new plant type varieties capable of yielding 15-20% higher than the best variety available (ii) engineering future rice varieties with novel genes conferring resistance to major insect pests and diseases and (iii) improving the nutritional value of rice through conventional and molecular breeding approaches.

Development of DUS test guidelines in rice

Globally, India stands first in rice area (44.6m.ha), second in production (93.0 m.t) after China and contributes to 23.5 % of world rice production. Within the country rice occupies 22.8% of total cropped area, 46.3% of area under cereals and contributes to 42% of total food grain production and continues to play a vital role in the national food security, as it constitutes staple food for two thirds of the population supplying about 33% of food calories. Also being the one of the secondary centres for the origin of rice the country is blessed with a rich diversity with the guess estimate being around 1,20,000 accessions of land races, farmers varieties and wild relatives. Several among the rich pool or rice landraces would come for registration on account of their special qualities, pest/disease resistance, quality features and medicinal use. Further 714 rice varieties are released so far of which 333 varieties are suitable for irrigated areas, 101 for rainfed uplands, 131 for shallow lowlands, 49 for semi deep and deep water areas, 37 for high altitudes, 19 for saline/alkaline and 42 in aromatic scented categories. Had the laws of registering our varieties existed earlier, the whole episode of Basmati patent by Rice Tec, USA would not have arisen, as basmati is a unique quality rice endemic to sub Himalayan tracts of Indian sub-continent, conserved by farmers from a long time and several research efforts made by scientists resulted in the release of many varieties in India. Now that we made a good headway in making our laws many of the breeders varieties and also farmers varieties could be protected through PBR, once they confirm to the basic Distinctiveness, Uniformity and Stability (DUS) test requirements.

The first step to implement our PPVFR provisions is formulation of National Test Guidelines for conducting DUS tests. National Test Guidelines have been developed for 35 crops representing harmonized approach for the testing of new varieties which will form the basis for DUS examination. These contain details on i) subject of guidelines ii) material required iii) conduct of tests, iv) methods and observation, v) grouping of varieties, vi) characteristics and symbols, vii) table of characteristics, viii) explanations on table of characteristics, ix) literature and x) technical questionnaire. The characteristics in the table follow the botanical and chronological order of recording from seed (submitted), seedling, plant (growth habit etc), stem, leaf (blade, petiole, stipule) inflorescence, flower and fruit.

Directorate of Rice Research (Rajendarnagar, Hyderabad) has played a key role in developing National Test Guide Lines for DUS test in Rice in consultation with the National Core Group Experts for development of National Test Guide Lines in crop plants and also with rice crops experts. The National Guidelines for the conduct of Test Distinctiveness, Uniformity and Stability for rice include descriptors to be observed for establishing the distinctiveness, explanation to the descriptors (essential, additional and stress related traits) procedures to be followed and technical questionnaire.

Genetic variability of yield and yield attributes, character association, heritability and genetic advance

Burton (1952) has suggested that genetic variability along with heritability should be considered to judge the effect of selection. Studies on genetic variability parameters like GCV, heritability and genetic advance and association of different traits should be considered for identification of suitable genotypes to be used as elite cultivars or for the breeding improvement involving the selected genotypes.

Majumdar *et al.* (1971) studied the genetic variability in respect of different characters in a collection of 10 varieties of rice. Genetic parameters like genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), hertitability, and genetic advance were computed. The result revealed that a large proportion of phenotypic variability was genetic and highly heritable for almost all characters like *plant* height, number of effective tillers, length of panicle, grain yield per plant, weight of 100 grains and grain size (length breadth ratio). They found high GCV values in some characters like grain yield per plant and for plant height which may prove the existance of justifiable genetic distance among the different varieties, a fact, indicative of the efficiency of these strains to be involved in rice improvement programme.

High mean associated with high variability was considered as a better index for selection. It was reported that high GCV and PCV was revealed for panicle length, number of productive tiller per plant (Das and Borthakur, 1974; Unikrisnan, 1980; Sukanya, 1984 and Vijay Kumar, 1990). High heritability coupled with moderate genetic advance was observed in all population for plant height in rice. This indicated the involvement of both additive as well as dominant gene-action in controlling plant height and hence this trait does not offer great scope for improvement by selection (Minidas, 1990).

Chaudhury *et al.* (1980) suggested that grain yield could be improved by exercising selection for high number of spikelets per panicle along with genetic advance were recorded for these characters.

Ghosh *et al.* (1981) reported that the genotypic coefficient of variation was high for grain yield, plant height, ear bearing tillers, number of grain per panicle and low for days to flowering.

According to Shumsuddin (1982), heritability was highest for total number of spikelets followed by grain yield per plant, 100 grain weight and lowest for panicle length. Amirthedevarathinam (1983) observed wide range of genotypic and phenotypic variation in the

eleven characters considered in the genetical study on rainfed rice.

Rabindranath *et al.* (1983) recorded the range of variation was highest in number of grains per panicle and lowest in 100 grain weight. PCV was higher than the GCV for all characters.

In 1984, Chauhan and Tandon experimented with 30 rice varieties to measure the extent of genetic variability under two cultural environments *viz.* rainfed upland (direct sown) and irrigated (transplanted). Plant height, effective tiller per plant and grain yield had high heritability and GCV under both the environments. However, the magnitude was higher in upland situation.

Gomathinagasam *et al.* (1988) observed that plant height contributed indirectly to the yield through the all characters considered in his experiment except 1000 grain weight. Loknathan *et al.* (1991) observed that plant height showed high genetic advance and heritability which registered its high potentiality for selection which contradicted the observation of Minidas, 1990.

Rahangadale and Khorgade (1988) found high heritability associated with high genetic advance for the characters like 1000 grain weight and plant height in their evaluation study with 25 indigenous upland rice varieties.

Ruben and Kishanga (1989), recorded that panicle per m^2, mature grain per panicle, panicle weight and panicle length were positively correlated with the grain yield.

Panwar *et al.* (1989) found that spikelet number was the main component affecting yield directly.

Bapu and Sundarapandian (1992) observed from the F_3 population of the cross between CO-37 and CO-41 and its reciprocals that number of productive tillers, panicle length and plant height were positively correlated with grain yield.

Estimation of GCV, PCV, heritability, genetic advance at 5% selection index for grain yield and other attributes were computed by Rema Bai *et al.* (1992). In all the cases the PCV was higher than GCV indicating environmental influence on the characters like plant height, flag leaf angle, number of panicles bearing tillers per plant, panicle length, number of grains per panicle *etc.* Plant height exhibited a higher value of heritability with a moderate value of genetic advance.

Yadav (1992) found high heritability for the characters like plant height, yield per plant, sterility, harvest index, days to 50% flowering, days to maturity, panicle per plant and seeds per plant.

After studying variability, heritability and genetic advance, Lokaprakash *et al.* (1992) suggested that 1000 grain weight, panicle weight, and number of filled spikelets per panicle were important for the breeder to construct selection indices as they showed high heritability along with high genetic advance for these traits. He also found that the variability was higher for panicle weight, number of panicles bearing tillers per plant and yield per plant but it was low for plant height, panicle length and test grain weight,

Mahajan *et al.* (1993) in their experiment with upland rice genotypes indicated that grain yield per plant was positively and significantly correlated with straw yield per plant and filled grains per panicle and suggested that the filled grains per panicle should be considered as a most important yield contributing character because it exerted a positive direct effect on yield.

Manuel and Rangasamy (1993) reported that the yield was positively correlated with the characters like plant height, panicle per plant, grains per panicle and dry matter production in case of hybrid rice. These results were in agreement with the finding of Bhattacharya (1981) and Ramalingam *et al.* (1995).

Chaubey and Richharia (1993) provided information on heritability, genetic variance and correlation considering 8 yield components of 80 indica rice varieties and found that broad sense heritability was high for all characters except harvest index and panicle weight was the highest contributor to grain yield, but Marwat *et al.* (1994) reported that the productive tillers, panicle length and 1000 grain weight had the highest direct effect on grain yield per plant.

Abd-E1-Samie and Hassan (1994) observed highly positive and significant phenotypic correlation between grain yield per hill and number of panicles per hill, grain yield per hill also showed positive and significant correlation with all the yield components except 100 grain weight.

Ganesan *et al.* (1995) observed that grains per panicle, grain yield per plant and dry matter production had high GCV, heritability, and genetic advance and suggested predominance of additive gene effects for the expression of these characters. Days to panicle emergence showed moderate genetic variability along with higher heritability and genetic advance, indicating the extent of scope for further improvement through phenotypic selection.

Sawant *et al.* (1995) reported high expected genetic advance, along with high heritability for panicle length, grains per panicle and 100 grain weight which indicated the predominance of additive gene effects in controlling these traits.

Sawant *et al.* (1996) and Padmavathi *et al.* (1996) studied character association among yield and yield contributing traits and indicated that ear bearing tillers per panicle, 1000 grain weight, panicle length and grain per panicle were positively and significantly correlated with grain yield.

Rao *et al.* (1997) studied genetic variability and character association between yield and its contributing traits in two environments. Irrespective of the environments, high PCV and GCV, coupled with high heritability and high genetic advance were found for spikelet sterility and leaf area index which indicated the predominance of additive gene effects in controlling of these traits. Productive tillers number, harvest index and dry matter production had positive and significant correlation with yield in both the environments.

Rather *et al.* (1997) reported that days to 50% flowering,

1000 grain weight etc. were the important characters which may be considered as direct contributors to yield. Sarawgi *et al.* (1997) indicated that grain yield in rice had significant positive correlation with number of fertile spikelets per panicle, 100 grain weight and harvest index etc.

Luzi-Kihupi (1998) showed that grain yield per hill was positively correlated with all the yield components except percentage of unfilled grains and days to 50% flowering. Plant height, number of filled grains per panicle and grain weight were highly heritable characters. He suggested that number of filled grains per panicle and grain weight could be of use as selection criteria for screening high yielding rice lines. Gholipoor *et al.* (1998) observed that positive genotypic correlation between yield and 100 grain weight, days to maturity but negative significant genotypic association with plant height and suggested that 100 grain weight could be considered for selection of lines with high grain yield.

Meenakshi *et al.* (1999) also observed positive correlation between yield and harvest index along with the characters like tillers per plant, grains per panicle, dry matter production.

Balan *et al.* (1999) evaluated 20 rice genotypes through estimation of genetic parameters under upland condition. High heritability combined with high genetic advance was observed for grain yield indicating the presence of additive gene action for the control of the trait.

Gupta *et al.* (1999) conducted an experiment involving 95 land races of hill rice for variability analysis of yield and its components. The GCV estimate was high for grain yield per plant and grains per panicle whereas for other characters it was low to moderate in magnitude. In general, estimates of PCV were higher than GCV, however, the closeness for GCV and PCV for 100 grain weight, plant height suggested that these characters were least influenced by the environmental factors. Grains per panicle showed highest estimate of heritability and genetic advance.

Kaw *et al.* (1999) took 94 rice genotype (38 japonica/ indica F_1, 36 indica/ japonica F_1 and 20 parents) for evaluating genetic variability under 3 cold stressed environments. Heritability estimate was highest for plant height and lowest for panicle length.

Genetic divergence

Singh (1983) estimated genetic divergence of 32 varieties of rice and grouped them into 9 clusters. He observed that genotypes even chosen from the same eco geographical region were found to be scattered in different clusters. Plant height contributed maximum role in total genetic diversity followed by sterile grain and grains per panicle. Utilizing the genotypes within clusters which are highly divergent, wide spectrum of variability may be created through hybridization.

Pandey and Ghorai (1986) studied genetic divergence employing 48 improved varieties of rice grown in different geographical regions and observed the parallelism between genetic diversity and geographical distribution. But these genotypes were grouped into a number of clusters and clustering was greatly influenced by the environments. Genotypic variance was high for grains /panicle and culm length.

Singh *et al.* (1986) assessed genetic divergence among 50 cultivars of low land rice and grouped them into 10 clusters. He also opined that genetic diversity was not related to geographical distribution. He found that plant height, sheath length, kernel length and breadth, test weight, panicle length and number of spikelets were mainly responsible for genetic divergence.

De *et al.* (1988) grouped 75 strains of rice into 13 clusters and found 100 grain weight and number of grains per panicle were the highest contributors to D^2 values.

Roy and Panwar (1993) estimated genetic divergence between 99 genotypes of rice on the basis of grain yield along with 9 related traits and grouped them into 16 clusters. The results indicated that significant genetic divergence was created in the genotypes mainly by panicle per plant, grains per panicle, grain yield per plant, spikelets per plants.

Sawant *et al.* (1995) carried out divergence study in 75 genotypes of rice and grouped them into ten clusters on the basis of eight yield component characters. He found sufficient inter cluster distance and suggested that the genotypes in different clusters were diverse from each other.

Singh *et al.* (1996) measured genetic divergence among 40 genotypes of rice on the basis of yield component data and grouped them into 6 clusters. Grain yield contributed the most, 40.6% of total divergence and plant height contributed 16.5%. The genotypes between clusters having better cluster distance were recommended for inclusion in hybridization programme as these were expected to produce variable segregants.

Rao and Gomathinayagam (1997) assessed divergence among 40 drought tolarent rice genotypes by growing them under semi-dry conditions at two different locations and found the importance of genotype-environment interaction in clustering pattern of the genotypes.

Mokete *et al.* (1998) estimated genetic divergence within 25 genotypes of rice on the basis of yield component data and grouped them into 5 clusters. The genotypes belonging to the clusters revealed substantial difference in the means for important yield contributing characters. So the genotypes belonging to these clusters from ideal pairs for planning a hybridization programme.

Kandola and Panwar (1999) studied genetic divergence among 52 endogenous and exotic genotypes of rice on the basis of 60 agro-morphological and quality characters and grouped them into 11 divergent clusters and found no association between genetic and geographic diversity and concluded that hybridization among genotypes drawn from widely divergent clusters with high yield potential is likely to produce heterotic combinations and wide variability in segregating generations.

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Bioinformatics of pathogenic food bacteria

Uttam K Mondal[1], Arnab Sen[2] and Asim K Bothra[1]*

[1]Cheminformatics Bioinformatics Laboratory, Department of Chemistry, Raiganj College (University College), Raiganj-733134, Uttar Dinajpur, West Bengal, India. [2]NBU Bioinformatics Facility, Department of Botany, University of North Bengal, Siliguri-734013, West Bengal, India.

Abstract

Cholera is a fatal water borne disease caused by *Vibrio cholerae*. The primary symptoms include profuse watery diarrhea, muscle cramps and vomiting of clear fluid leading to rapid dehydration and electrolyte imbalance, and death in some cases. The causal organism i.e. *Vibrio cholerae* was first isolated by an Italian anatomist, Filippo Pacini in 1854. With the advent of genome sequencing technology, several *Vibrio cholerae* strains have been sequenced and available in public domain. This provided the opportunity for the scientists to study their genome in details. In the present review we have looked into the genetics of food pathogen as a whole, with special reference to research on *V. cholerae*. We have also critically reviewed various aspects of Bioinformatics like codon usage patterns, phylogenetic studies, studies on structural bioinformatics etc. on food borne bacteria. We are confident that the present review will help the researchers of food pathogenic biology to obtain valuable information about the present status of bioinformatics of food pathogenic bacteria and future outlook of these aspects.

Keywords: *Vibrio cholerae* , food pathogenic bacteria, codon usage, functional genomics

Cholera is a fatal disease caused by infection with the bacteria *Vibrio cholerae* (Ryan 2004) which is transmitted through contaminated water. Prior to the discovery of an infectious cause, the symptoms of cholera were thought to be caused by an excess accumulation of bile in the patient. The disease Cholera gets its name from the Greek word *choler* meaning bile. This was consistent with medical thought at the time, which held that four liquids or *humors* controlled health, and lead to such medical practices as bloodletting as a method of curing illnesses. The bacterium was first reported in 1849 by M. Gabriel Pouchet, who discovered it in stools from patients with cholera. But he did not appreciate its significance (William 1979). The first scientist to understand the significance of *Vibrio cholerae* was an Italian anatomist Filippo Pacini, who published detailed drawings of the organism in "Microscopical observations and pathological deductions on cholera" in 1854. He went on to publish additional papers in 1866, 1871, 1876 and 1880, all of which were ignored by the scientific community. He correctly described how the bacteria caused diarrhea, and developed treatments that were found to be effective (Bentivoglio & Pacini 1995). But his findings did not influence medical opinion. In 1874, scientific representatives from 21 countries voted unanimously to resolve that cholera was caused by environmental toxins from *miasmatas*, or clouds of unhealthy substances which float in the air (Howard-Jones N 1984).

Reports of pathogenic E. coli appeared in medical literature way back in 1947 (Ruchman & Dodd 1947). Publications regarding variants of E. coli which cause disease appeared regularly in medical journals throughout the 1950s, 60s, and 70s, (Macqueen 1954; Mc-Clure 1955; Gronroos 1957; Cowart & Thomason 1965; Linde *et al.*, 1966) with fatalities being reported in humans and infants starting in the 1970s (Glantz 1970; Drucker *et al.*, 1970; Smith & Gyles 1970). Infection with the bacteria *Helicobacter pyoli* is the cause of most stomach ulcers. The discovery is generally credited to Australian gastroenterologists Dr. Barry Marshall and Dr. J Robin Warren, who published their findings in 1983. The pair received the Nobel Prize in 2005 for their work. Before this, nobody really knew what caused stomach ulcers, though a popular belief was that the "stress" played a role. Some researchers suggested that ulcer was a psychosomatic illness (Paulley 1975; Kellner 1975; Aitken & Cay 1975). Besides, a number of food borne pathogens causing bacteria like *Salmonella* (non-typhoidal), *Listeria monocytogenes*, *Campylobacter*, (Mead *et al.*, 1999) were discovered. Genomic islands may contain large blocks of virulence determinants (adhesins, invasins, toxins, protein secretion systems, antibiotic resistance mechanisms, etc) and thus are referred to as pathogenicity islands. Pathogenicity islands were first described in pathogenic species of *E coli*, but since then have been found in the genomes of numerous bacterial pathogens of humans, animals, and plants (*Salmonella*, *Vibrio*, *Shigella*, *Yersinia*, *Listeria*, *S aureus*, etc) (Garcia *et al.*, 1999; Lindsay *et al.*, 1998). Pathogenicity islands are believed to have been acquired as a block by horizontal gene transfer because of their G+C content is significantly different from that of the genomes of the host micro-organism and they are often flanked by direct repeats. Many scientists are currently beginning to gain understanding of the molecular mechanisms involved in

*Corresponding author:
E-mail: asimbothra@gmail.com

the action of toxins. The good news is that there are a limited number of major toxin families that display common structural and biochemical motifs which may be exploited for future therapeutic development and these may be effective against multiple organisms.

Genetics of food pathogen

In 1906 the term "genetics" is introduced. Various techniques like mutations, deletion mapping, cloning vectors etc. have facilitated the identification of genes associated with food pathogens. The first genome of a food-borne bacterium to be sequenced was that of *C. jejuni* and it led to the discovery of important new aspects pertaining to the biology of this organism (Parkhill *et al.*, 2000). PAIs were first discovered in pathogenic *E. coli.* Pathogenicity islands of various pathogens are cag (*H. pylori)*, SPI1-5 (*Salmonella* spp) VPI (*V. cholerae) etc* are responsible for different diseases (Hentschel & Hacker 2001). Kim *et al.*, designed a method for the Microarray detection of food-borne pathogens using specific probes prepared by comparative genomics (Kim *et al.*, 2008).

Research trends in food pathogenic bacteria (FPB)

The discovery of pathogenic organisms throughout the globe, studies concerning their gene products as well as the development of molecular biology helped FPB research enter a new stage where basic research blended well with latest sophisticated techniques. Techniques such as immune magnetic separation (IMS) and polymerase chain reaction (PCR) have paved the way for rapid and sensitive detection of food borne pathogens, and advances in nano-biotechnology have allowed for the miniaturization of devices. Collaborations between workers in the fields of engineering, nanotechnology and food science have introduced new lab-on-a-chip technologies permitting development of portable, hand-held biosensors for food pathogen detection. Despite the recent advancements in food pathogen detection, there still exist many challenges and opportunities to improve the current technology.

Beginning of interdisciplinary research and dawn of bioinformatics

It was in the year 1986 that the Department of Energy (DOE), USA and National Institutes of Health (NIH) started the Human Genome Project (HGP). It was one of the most happening experiments in the late 20th century. The objective of the project was to identify all of the genes in humans and craft a database containing the information (Ideker *et al.*, 2001). A number of other genome projects also started in major industrialized countries of Europe and Japan. The scientists were, at first, skeptical about the HGP, since huge amount of money had to be spent that would hamper basic research and as the project moved scientists worried about the massive amount of data and its interpretation (Bloom, 2001) In the year 2000, Prof. Collins of the National Human Genome Research Institute and Prof. Craig Venter of Celera Genomics appeared in a press conference and stated that they had achieved what was thought impossible and published the draft sequence of human genome (Wade 2000). That was the beginning and it opened the flood-gates for other genome sequencing projects. Gradually sequences of mouse, rat, worms and yeast were completed (Miller *et al.*, 2004). Kyrpides (1999) reported that in the end of the 20th century, there were 24 complete genomes that included 16 bacterial, 6 archaeal, and 2 eukaryotic genomes and currently there are more than two thousand genomes available in public databases (Kyrpides 1999). The large numbers of genomes resulted in the generation of huge amount of information concerning the genetic nature of biological organisms spanning different kingdoms, groups and lineages etc. Bloom proposed that the greatest problem appeared with the interpretation of underlying information from genomes leading to materialization of the new science of bioinformatics. Bioinformatics revolutionized the science of biology and directed it towards a more holistic approach compared to the reductionism visible in molecular biology research in the late 20th century (Bloom 2001). Now an organism is viewed as a system comprising of the information associated with genes and proteins that are responsible for maintaining day to day functions and networks of regulations that spell out how gene expression occur (Ideker *et al.*, 2001). In 1995, the publication of the complete genome of *Haemophilus influenzae* marked the beginning of another revolution in the field of bioinformatics (Fleischmann *et al.*, 1995). Currently there are about two thousand bacterial and archaeal genomes in the public domain. The publication of huge amount of sequence data helped in the development of high end computers, smart computing tools, for large-scale annotation, functional classification of the proteins (Searls 2000) and development of specific databases (Birney *et al.*, 2002) for availability to the broad scientific community. As the science of bioinformatics developed, computation became cheaper and cheaper and was duly complemented with the growth of Internet since the late 90s (Perez-Iratxeta *et al.*, 2006). High throughput tools were also developed by the beginning of the 21st century as genetic data became a gold mine for researchers (Perez-Iratxeta *et al.*, 2006). New software started developing for more efficient and comprehensive analysis of the genomes, proteomes and proteins. The stage was set and bioinformatics had already established itself as the leading science of the 21st century.

Post genomic era and FPB

The amalgamation of the knowledge of physiology, biochemistry, genetics and molecular biology gave idea about the understanding of the mechanism of host-pathogen interaction in pre-genomic era. The completion of the genomes of *Salmonella enterica (*McClelland *et al.*, 2001), *Helicobacter pylori Strain* G27 (Baltrus *et al.*, 2009) and sequences for a number of pathogenic bacteria. Pathogenicity islands were first described in pathogenic species of *E coli*, but since then have been found in the genomes of numerous other bacterial pathogens of humans, animals, and plants (*Salmonella, Vibrio, Shigella, Yersinia, Listeria, S aureus*, etc)

(Garcia *et al.*, 1999; Lindsay *et al.*, 1998). The first genome of a food-borne bacterium to be sequenced was that of *C. jejuni* and it led to the discovery of important new aspects (Parkhill *et al.*, 2000). The studies on the genomes exposed new evidences pertaining to evolution and structure, interactions between host-pathogen interactions.

The research on functional genomics and proteomics with a focus on food pathogenic bacteria has been greatly significant in the 21st century. It has become necessary to investigate much on the comparative codon usage patterns, whole proteome analysis and molecular phylogeny using bioinformatics tools. Comparative genomics particularly focusing on codon usage using different parameters is expected to provide insight into the inherent molecular nature of the genomes of food pathogenic bacteria.

Previous works on codon usage patterns

In the post genomic era increasing number of genomes generated a concept among computational biologists that each and every genome has its own story. Since the time when the first nucleic acid sequences were obtained a number of hypothesis on the evolution of genomes have been put forward. The genetic code has been one of the most interesting aspects of biological science. The code is degenerate with multiple codons coding for a particular amino acid. Groups of codons coding for a particular amino acid are synonymous ones. It has been reported that these synonymous codons are somewhat conserved across species (Peden 1999). The increase in sequence information *albeit* partial in the 1980's facilitated the studies concerning the usage of synonymous codons in organisms. Majority of work on codon usage patterns at that period focused upon *E.coli* (Peden 1999). Gradually the techniques for codon usage were applied on mammalian, bacterial, bacteriophage, viral and mitochondrial genes (Grantham *et al.*, 1980a; Grantham *et al.*, 1981; Grantham *et al.*, 1980b). On the basis of studies conducted on mRNAs from a number of prokaryotic and eukaryotic species, Grantham *et al.* (1980a) proposed the "Genome Hypothesis" which hypothesized that codon usage pattern of a particular genome was an explicit attribute of that organism. Grantham *et al.*, (1981) reported that difference in codon usage pattern might be associated with the tRNA content. More work on the codon usage patterns (Gouy & Gautier, 1982) in *E.coli* regarding codon usage and tRNA abundance led to the conclusion that highly expressed genes exhibited non-random codon usage and used a small set of codons that corresponded to abundant tRNAs. However, it was not clear why specific synonymous codons were used preferably. Grosjean & Fiers (1982) opined that optimal codon choice is the outcome of the necessity imposed by interaction between codon and cognate tRNA. Ikemura (1981) defined optimal codon as one that was translated by the most abundant cognate tRNA which he later amended (Ikemura, 1985). These optimal codons are under the influence of translational efficiency. Kurland (1991) reported that translational efficiency is shaped by highest turnover of ribosomes, effectiveness of aminoacyl-tRNA and ternary complex conditions. Sharp *et al.* (1993) reported that the preference of some synonymous codons were the outcome of translational selection i.e., for increasing efficiency and accuracy a codon is used that is translated by the abundant tRNA species. Rocha (2004) correlated codon usage bias from the tRNA point of view. He proposed that co-evolution of tRNA gene composition and codon bias in genomes from tRNA's point of view concur with the selection-mutation-drift theory. A number of studies (Sharp *et al.*, 1993; Carbone *et al.*, 2005) revealed that codon bias is influenced by effective population size, translational selection, mutational pressure, compositional bias and genetic drift. Chen *et al.*, (2004) postulated that codon bias is first and foremost influenced by mutational pressure and then translational selection. Studies on translational selection helped in the detection of highly expressed genes in genomes (Karlin & Mrazek, 2000). Majority of the highly expressed genes are associated with cell growth and cell division. It has been found that evaluating translational selection is quite difficult in genomes with a high or low G+C content owing to the consequence of intense levels of G+C content on codon usage. Medigue *et al.*, (1991) applied the principles of codon usage, cluster analysis and correspondence analysis to study horizontal gene transfer mechanisms. A number of workers have suggested that codon usage and amino acid usage (Goldman & Yang 1994; Nesti *et al.* 1995; Pouwels & Leunissen 1994; Schmidt 1995) helps in determining the phylogenetic relationships between organisms. Besides codon usage, amino acid usage has also been studied extensively (Peden 1999). Ikemura (1981) was instrumental in reporting good correlation between amino acid composition and codon bias. Hydrophobicity, aromaticity and amino acid charges are testified to be influencial in amino acid usage (Lobry & Gautier, 1994). The prediction of open reading frames has been performed utilizing the information of codon usage (Krogh *et al.* 1994; Borodovsky *et al.* 1995). Peden (1999) provided some classic examples of the utilization of GeneMark prediction programme (Borodovsky *et al.*, 1994) for identification of coding sequences from shotgun genome sequencing projects.

Sharp and Li (1987) were the pioneers in developing the Codon Adaptation Index (CAI) to assess the similarity among the synonymous codon usage of a gene to that of the reference set. This particular index has been commonly used as a parameter for predicting the highly expressed genes in an organism. Besides CAI, a number of indices are regularly used to investigate codon usage patterns in a number of organisms. Peden (1999) opined that two types of indices one evaluating on the whole deviation of codon usage from the expected set and the other determining bias towards a specific subset of optimal codons are used. Indices such as GC content, GC3 content (Peden 1999) effective number of codons (Nc) (Wright 1990), relative synonymous codon usage (RSCU) (Sharp *et al.*, 1986) scaled chi-square and G statistic, P2 index (Gouy & Gautier, 1982) measuring the codon-anticodon interaction, codon bias index (CBI) (Bennetzen & Hall, 1982), Fop (frequency of optimal

codons) (Ikemura, 1985) and codon adaptation index are very significant in studies concerning codon usage patterns. Besides these indices, correspondence analysis (Benzecri, 1992) a type of multivariate statistical analysis has also been commonly used to find out the degree of associations between different genes and amino acids. It is a technique that explores non random synonymous codon usage.

This important field of study starved due to the scarcity of ophisticated software in the 1980's. The basic problem was the integration of different indices into one program that would serve the researchers well. The foremost software was CODONS (Lloyd & Sharp, 1992). The GCG package (GCG, 1994) consisted of programs for analysis codon usage. CORRESPOND was used for performing multivariate statistical analysis (Peden 1999). NetMul (Thioulouse *et al.*, 1995) was designed as a subset of ADE software for studying of codon usage and multivariate statistical analysis. CODON W (Peden 1999) took over the stage and became very popular because of its robust nature and error free analysis. INCA (Supek & Vlahovicek, 2004) is also being used by researchers. Software was also developed for codon usage optimization. This involved alteration of rare codons in target genes so as to imitate the codon usage of host with no modification of the amino acid sequence of encoded proteins (Gustafsson *et al.*, 2004). GeneDesign (Richardson *et al.*, 2006), Synthetic Gene Designer (Wu *et al.*, 2006), Gene Designer (Villalobos *et al.*, 2006) are some of the useful software packages providing a platform for synthetic gene design and codon optimization. There has been a number of softwares for estimation of the highly expressed genes employing CAI. CODON W (Peden 1999), JCAT (Grote *et al.*, 2005), CAI CALCULATOR (Wu *et al.*, 2005), ACUA (Umashankar *et al.*, 2007) and e CAI server (Puigbo *et al.*, 2008) are commonly used. However, the e CAI server (Puigbo *et al.*, 2008) has proved to be the most powerful and efficient tool for estimation of expression levels of the genes.

Although a lot of work has been performed and is going on in codon usage of different microorganisms but very little work has been performed on codon usage of food pathogenic bacteria. The first species in which codon usage was examined in detail, the bacterium Escherichia coli (Post & Nomura 1980; Ikemura 1981) and the yeast Saccharomyces cerevisiae (Ikemura 1982; Bennetzen & Hall 1982), were both found to show strong evidence of natural selection on codon usage. All these are just very little and a comprehensive work with focus on lifestyle patterns is the need of the hour.

Phylogenetic studies on FPB

Nothing in biology makes sense without evolution. This also applies for food pathogenic bacteria too. To establish the differentially evolved genes Eswarappa *et al.*, compared the phylogeny of the nine potential differentially evolved genes with phylogeny of the S. eneterica species and with the phylogeny of the five pathogenecity island of Salmosella (SPI-1 to SPI-5) (Eswarappa *et al.*, 2008). This also applies for other pathogenic bacteria.

Structural bioinformatics of FPB

High throughput sequencing of a number of eukaryotes and a number of bacteria has paved way to the field of structural bioinformatics. Structural bioinformatics is expected to give rise to a large number of protein structures. The technological benefits of this field are already yielding results and have a substantial impact on structural biology research for prokaryotes (Burley & Bonnano, 2002).Metropolis *et al.*, introduced the Monte-Carlo technique to the solving of physical equations. It described the idea of using random numbers to project a representative subset of conformational space, whilst using the exponent of the energy as a probability filter (Metropolis *et al.*, 1953). Levitt and Warshel simulated the folding of the Bovine Pancreatic Trypsin Inhibtor (Levitt & Warshel 1975). Case and Karplus work on "Dynamics of ligand binding to heme protein" in 1979. This is arguably the first simulation of ligand moving through the protein. At the early stage, either the protein could be made stationary allowing the oxygen to bounce around, or let individual sidechains could be allowed to hit by the oxygen. Each oxygen atom was simulated for 3.75 ps. For these pioneers it was a surprise to see that the oxygen bounces around the inside of the myoglobin, without getting too far. Nevetheless, they identified 2 different pathways for the oxygen to travel into the binding site (Case & Karplus 1979). Umbrella sampling is the most popular method of exploring large conformational changes in MD. In 1998 Notthrup et al show "Dynamical theory of activated processes in globular proteins". In this paper, Karplus and friends modeled a rather more modest conformational change: the swinging of an aromatic residue sidechain. From the simulations, they generated a free-energy surface, from which they calculated a sidechain flipping rate. This paper is important not just for simulating the first sidechain flip but also for introducing proteins to "umbrella sampling" (Northrup *et al.*,1982). First application of normal modes to identify low-frequency oscillations using the energy minimum of the molecular mechanics force-field of a protein was deseribed by Brooks & Karplus in 1983 (Brooks & Karplus *et. al.*, 1983). This is the basic technique to identify domain-level motions in a protein. First simulation of a protein in explicit waters was done by Levitt & Sharon (Levitt & Sharon 1988). Suddenly, acceptable computer resources got a whole lot more expensive. Guilbert *et. al.* gave first description of the RMSD potential, a powerful method for identifying low-energy pathways in the neighborhood of a given static structure (Guilbert *et al.*, 1995). Israelachvili & Wennerström showed that water molecules can have structuring effects of several Ångstroms flagging the importance of using explicit water molecules (Israelachvili & Wennerström 1996). The first reported 1 microsecond MD simulation, it was a mamoth effort for the late 90's, really pushing the technology of parallel clusters, a technology which the whole scientific community accepts. They tried to fold a tiny protein, the villin headpiece subdoamin, and got some of the way (Duan & Kollman 1998). Bernèche and

Roux uses umbrella sampling to identify all the positions of the K+ ion along the KcsA K+ membrane channel. They show that the channel for K+ is virtually barrierless, hence it is a diffusion controlled process but more impressively, they identify two K+ sites just outside the channel, which was subsequently identified by electron density in a high-resolution structure (Bernèche & Roux 2001).

Rapid developments of technological know how in proteomics coupled with the improvement of bioinformatics tools have resulted in a deluge of structural information that guarantees acceleration in research. *In silico* identification of potential therapeutic targets in *Clostridium botulinum* by the approach of subtractive genomics was carried out by Koteswara *et al.*, (2010). Evolutionarily related proteins have similar sequences and naturally occurring homologous proteins have similar protein structure. It has been shown that three-dimensional protein structure is evolutionarily more conserved than expected due to sequence conservation (Kaczanowski 2010).

Proteins fulfill several crucial functions, having catalytic, structural and regulatory roles in all organisms. Knowledge of the three dimentional structure of proteins is a basic prerequisite for understanding their function. It provides a basis for studies of substrate or ligand interactions with a particular enzyme or protein. Three dimensional structures of proteins are determined by X-ray crystallography and NMR spectroscopy. By 1st June 2007, more than 40000 sets of atomic coordinates for proteins have been deposited in the Protein Databank (Berman 2008).

The first modeling studies, carried out in the late 1960s and early 1970s, were based upon the construction of wire or plastic models. The later studies were performed using interactive computer graphics. Browne *et al.* published the first report on homology modeling (Browne *et al.*, 1969). The model was bovine α-lactalbumine on the known 3D structure of hen egg white lysozyme. Later on Warme *et al.* produced a model for α-lactalbumine on the basis of the crystal structure of lysozyme (Warme *et al.*, 1974). These models were constructed by taking the existing coordinates of the known structure, and mutating side chains not identical in the protein to be modeled. This approach to protein modeling is still employed today with considerable success, especially when the proteins are similar (May & Blundell 1994).

McLachlan and Shotton modeled α-lytic proteinase of the fungus Myxobacter 495 on the basis of the structures of the mammalian chymotrypsin and elastase (McLachlan & Shotton 1971). This was a difficult task because the sequence identity between the protein to be modeled and the known structures was of the order of 18%. Subsequently, the crystal structure of α-lytic proteinase was determined and comparison was made between the X-ray structure with the homology model. It was found found that although segment of both domains of the model were built correctly, misalignment of the sequence led to local errors.

Greer introduced the modeling of variable regions in proteins on the basis of equivalent region from homologous proteins of known structures. In order to construct the homology models of a number of different serine proteases, he superimposed the structures of trypsin, chymotrypsin and elastase and found many equivalent Cα atoms within 1.0 A of one another (Greer 1980, 1981). The regions comprised of the amino acids of these Ca atoms were described as structurally conserved regions (SCRs). All of the remaining positions corresponded to structurally variable or loop regions (VR) where the insertions/deletions were located. The main chain of both structurally conserved (SCR) and variable regions (VR) were built from the fragments of known serine proteases. The side chains were modeled according to the conformation found at the equivalent positions for those identical side chains in the known structures.

Among the aspartic proteinases, initially models were constructed for rennin and rennin inhibitor complexes using the 3D structure of the distantly related fungal proteinases (1985; Akahane *et al.*, 1985). Later on, the homology models for rennin were built using the structures of mammalian aspartic proteases, pepsin and chymosin (Fragao *et al.*, 1994; Hutchins and Greer 1991). Comparison of the rennin models constructed from fungal and mammalian enzyme revealed that errors in the models arose from the difference in the arrangement of helices and strands between the mammalian and fungal aspartic proteinases, as well as the different variable regions. Nevertheless, the active of rennin was modeled reasonably correctly.

In the early eightes, manual homology modeling was facilitated by manipulation of protein molecules on the graphics terminal that was made possible by computer programs such as FRODO (Jones 1978). Since mid 1980s, a large number of homology models of proteins with different folds and functions have been reported in the literature (Johnson *et al.*, 1994; Sali 1995).

The sequence alignment and template structure were then used to produce a structural model of the target. Because protein structures are more conserved than DNA sequences, detectable levels of sequence similarity usually imply significant structural similarity (Marti-Renom *et al.*, 2000).

The quality of the homology model is dependent on the quality of the sequence alignment and template structure. The approach can be complicated by the presence of alignment gaps (commonly called indels) that indicate a structural region present in the target but not in the template, and by structure gaps in the template that arise from poor resolution in the experimental procedure (usually X-ray crystallography) used to solve the structure. Model quality declines with decreasing sequence identity; a typical model has ~1-2 Å root mean square deviation between the matched C^{α} atoms at 70% sequence identity but only 2-4 Å agreement at 25% sequence identity. However, the errors are significantly higher in the loop regions, where the amino acid sequences of the target and template proteins may be

completely different.

Homology modeling can produce high-quality structural models when the target and template being closely related, which has inspired the formation of a structural genomics consortium dedicated to the production of representative experimental structures for all classes of protein folds (Williamson 2000). Like other methods of structure prediction, current practice in homology modeling is assessed on a biannual large-scale experiment known as the Critical Assessment of Techniques for Protein Structure Prediction, or CASP.

Future prospects

Food pathogenic bacteria research presents a completely new outlook in the post genomic era. The wealth of information obtained from the genome projects needs to be mined. New insights are gained with the discovery of novel toxin genes. Bioinformatics, in combination with metagenomics as well as metaproteomics based approaches, has the potential to give a more detailed scenario that underlies the mechanisms of pathogenicity as well as diseases. In absence of crysllographic or NMR structure Homology modeling will enlighten us about three dimensional structure and molecular dynamics simulation providing newer possibilities for exploring the molecular mechanism and activity of that toxin. Thus scientific perception will continue to play a vital role in creating models that clarify the functions of pathogens in improved manner.

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Influence of tungsten and molybdenum on seed germination and early seedling growth of wheat - a comparative study

Anil Kumar and NC Aery*
Laboratory of Geobotany and Biogeochemistry, Department of Botany, Mohanlal Sukhadia University, Udaipur-313039, Rajasthan, India

Abstract
Fifty one promising landraces of rice cultivars of three districts viz. Nadia, 24 Parganas(N) and MA comparative study on the effect of tungsten and molybdenum on seed germination and early seedling growth of wheat was carried out. Both W and Mo influenced the seedling growth in similar fashion. W and Mo enhanced the germination relative index at lower application. Relative yield increased at lower applied doses of W as well as Mo whereas at higher application it decreased. Lower applied doses of W and Mo showed maximum index of metal tolerance. Higher applied doses of both metals showed toxic effect on plant growth. A highly significant (p = 0.01 in W and p = 0.001 in Mo) increment in grade of growth inhibition (GGI) at the higher administration of W and Mo was observed. The toxic effect of tungsten on wheat was more marked than molybdenum.

Keywords: Tungsten, molybdenum, germination, relative yield, tolerance index, GGI

Tungsten (W) and molybdenum (Mo) are trace elements and rank 54th and 53rd in natural abundance. Both metals are similar in chemical properties, electron negativity and atomic and ionic radii. The fact that W and Mo can perform similar functions in different organisms reflects their chemical similarity. Tungsten can compete with molybdenum for incorporation into the enzyme complex and results in enzyme inactivation. Plants absorb W and Mo in the form of WO_4^{2-} and MoO_4^{2-} respectively (Wilson and Cline, 1966; Mengel and Kirkby, 1996) and accumulate it in different plant parts (Aery, 2000). Whereas the role of Mo is well studied in plants and considered as essential mineral nutrient (Arnon and Stout, 1939) W is less studied and generally known to be a competitive inhibitor of Mo function *in vivo* in bacteria (Brill *et al.*, 1974), in green alga *Chlorella* (Cardenas *et al.*, 1972) and the higher plants such as spinach (Notton and Hewitt, 1972).

Though the biological importance of tungsten has been fully proved by the isolation of a number of tungsten containing enzymes (L'vov *et al.*, 2002), it has been found to be beneficial for plant growth only in some instances (Davies and Stockdill, 1956; Kumar and Aery, 2010b).

Tungsten and molybdenum are similar in their natural abundance. Their physical and chemical properties are also almost similar. Tungsten is found as wolframite and scheelite in the nearby areas (Aery, 2000). The information about the effect of tungsten on seed germination and early seedling growth is lacking. A comparative study was carried out to explore the relative effect of tungsten and molybdenum on seed germination, early seedling growth, relative yield, vigour index, index of metal tolerance and grade of growth inhibition in wheat.

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

Materials and Method

The experiments were conducted in the month of January under laboratory conditions where the photoperiod was 8 hours day^{-1} with a light intensity of 3600 Lux. Twelve seeds of a certified variety Raj 4037 of *Triticum aestivum* L. were placed in each Petri plate with filter paper. Five concentrations of tungsten (3, 9, 27, 81 and 243 µg ml^{-1}) and molybdenum (0.1, 0.5, 2.5, 12.5 and 62.5 µg ml^{-1}) were applied as sodium tungstate (Na_2WO_4. $2H_2O$) (E. Merk) and sodium molybdate (Na_2MoO_4. $2H_2O$) (E. Merk). The concentrations were prepared separately by taking corresponding amount (calculated on the basis of molecular weight) of chemical per liter of water. No other supplement nutrients were added. Control constituted only distilled water. A fixed amount of solution was poured in each Petri plate to saturate the filter paper. Three replicates were used for each concentration. After the start of seed germination, the speed of seed germination was observed after every hour. Plants were harvested after seven days of treatment and root-shoot length was measured. The plant samples were dried at 80°C in an oven for 48 hours for the measurements of dry-weight.

Germination Relative Index: Germination relative index was computed after Sreevastava and Sareen (1972) by using the following formula: GRI = $\sum [X_n (h - n)]$ where X_n is the number of germinants at nth count; h, the total number of counts and n, the count number.

Tolerance Index: Tolerance index of seedlings were calculated by using the formula given by Turner and Marshall (1972).

Vigour Index: Vigour index was calculated by using the

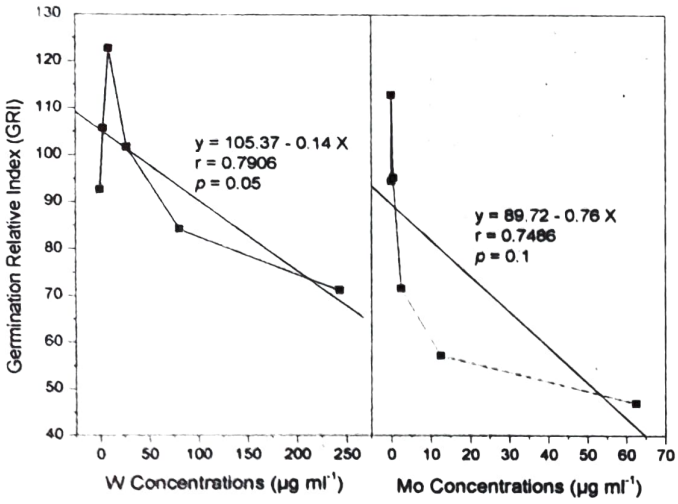

Fig: 1. Effect of various concentrations of W and Mo as sodium tungstate and sodium molybdate, respectively on the germination relative index (GRI) of *Triticum aestivum* L.

formula given by Dhindwal *et al.* (1991).

Grade of Growth Inhibition (GGI): Grade of growth inhibition was calculated by the following formula as described by Aery (2010): Grade of Growth Inhibition (GGI) = (Dry weight of control plant – Dry weight of treated plant/ Dry weight of control plant) × 100.

Results

Results on the effect of W and Mo on germination relative index are shown in Fig. 1. Both W and Mo showed stimulatory effect on the germination relative index which was observed to be maximum in lower applied doses of W (9 µg ml^{-1}) and Mo (0.5 µg ml^{-1}). Beyond the above levels, germination relative index decreased (Fig 1).

Tungsten and molybdenum showed promotory effect on root-shoot length of wheat. Maximum root-shoot length was observed in 9 µg ml^{-1} and 0.1 µg ml^{-1} treatments of W and Mo, respectively. The increments over the control respectively, for root and shoot length were 2.32% and 6.42% in tungsten and 15.42% and 5.41% in molybdenum treatment. Beyond the above level, root-shoot length of seedlings decreased regularly (Fig. 2). Minimum root and shoot length was observed at the highest applied doses of W (243 µg ml^{-1}) and Mo (62.5 µg ml^{-1}).

Lower applied doses of W (9 µg ml^{-1}) and Mo (0.1 µg ml^{-1}) resulted in an increment in relative yield. Maximum relative yield was observed at lower applied

Table 1: Effect of various concentrations of tungsten and molybdenum as sodium tungstate and sodium molybdate, respectively on relative yield of *T aestivum*

W	Relative yield	Mo	Relative yield
Control	100	Control	100
3	104	0.1	105
9	117	0.5	99
27	64	2.5	93
81	57	12.5	82
243	44	62.5	62

W=W conc. (µg ml^{-1}); Mo=Mo conc. (µg ml^{-1})

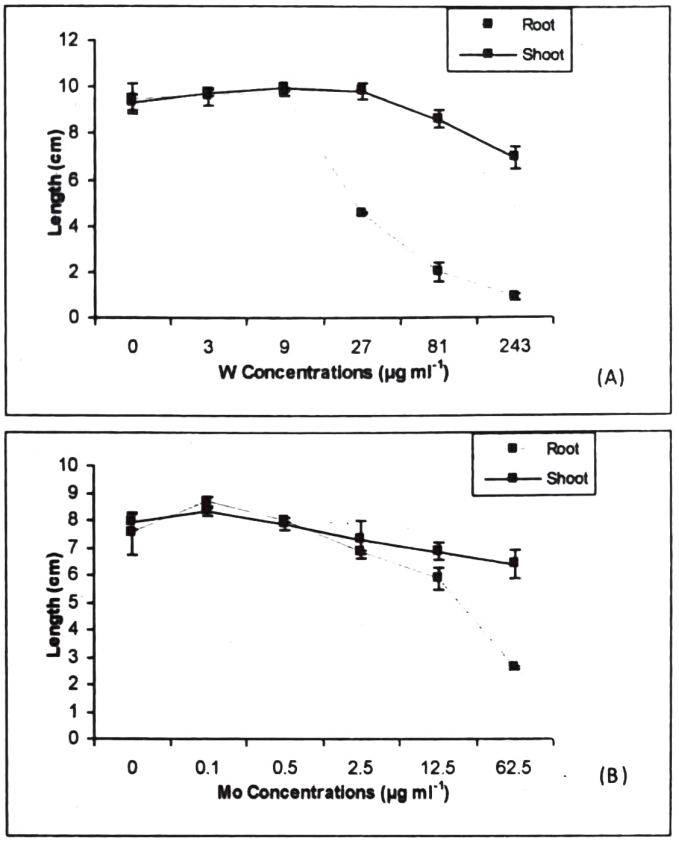

Fig: 2. Effect of various concentrations of W (A) and Mo (B) as sodium tungstate and sodium molybdate on the root-shoot length of *Triticum aestivum* L. (Error bars denote standard error)

doses of W (9 µg ml^{-1}) and Mo (0.1 µg ml^{-1}) and were 117 and 105, respectively. Higher applied doses of W and Mo showed a decrement in relative yield. Minimum relative yield was observed at the highest applied doses of W (243 µg ml^{-1}) and Mo (62.5 µg ml^{-1}) and were respectively, 44% and 62% lower, over the control (Table 1).

Vigor index of wheat increased in lower concentrations of tungsten and molybdenum. Higher concentrations of both these metals resulted in decreased vigour index. The decrement was more significant in Mo treatment (p = 0.001) than W treatment (p = 0.01) (Fig 3).

Lower applied doses of W (9 µg ml^{-1}) and Mo (0.1 µg ml^{-1}) positively influenced the index of metal tolerance. Beyond the above level index of metal tolerance concomitantly decreased. Minimum index of metal tolerance was observed at the highest applied doses of W and Mo (Fig 4).

Lower applied doses of W (9 µg ml^{-1}) and Mo (0.1 µg ml^{-1}) showed minimum grade of growth inhibition. It

Table 2: Relationship between applied metal concentrations and relative yield of *T. aestivum*

Parameter	Regression equation	Correlation coeff. (r)	Significance
RY (W)	y=95.9673-0.2473x	-0.7846	S*
RY (Mo)	y=98.0537-0.6059x	-0.9420	S**

RY=relative yield, y = parameter, x = applied metal concentrations, S* = significant at 0.05 probability level, S** = significant at 0.001 level

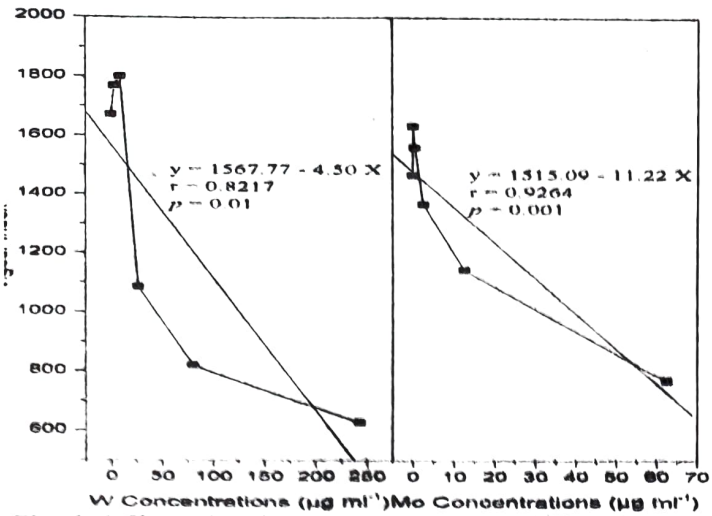

Fig: 3. Effect of various concentrations of W and Mo as sodium tungstate and sodium molybdate, respectively on the vigour index of *Triticum aestivum* L.

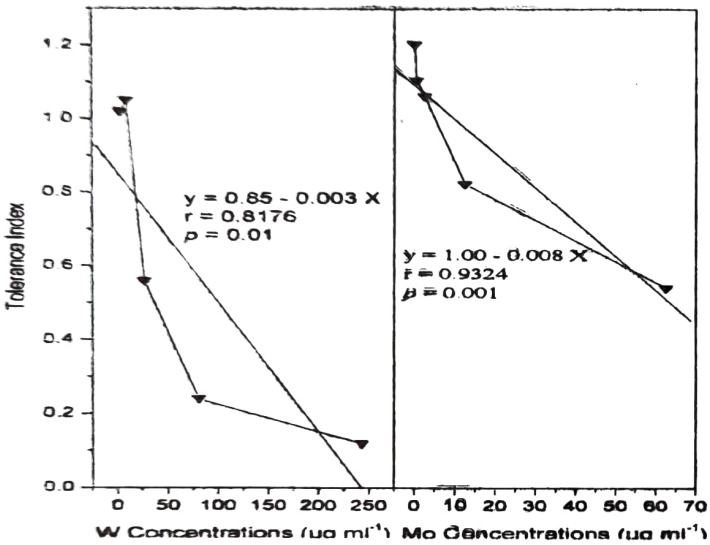

Fig: 4. Effect of various concentrations of W and Mo as sodium tungstate and sodium molybdate, respectively on the tolerance index of *Triticum aestivum* L.

was observed to be maximum at higher applied doses of W (243 µg ml^{-1}) and Mo (62.5 µg ml^{-1}).

Discussion

Tungsten and molybdenum positively influenced the seed germination and seedling growth. Both these metals showed a stimulatory effect on germination relative index at lower applied doses whereas higher applied doses adversely affected the seed germination and resulted in decreased germination relative index. Present results are in conformity to the findings of Rout and Das (2002) on rice who observed that seed germination rate declined at higher concentration (1.6 µM) of molybdenum. Similar findings have been reported on the effect of Li (Surana and Aery, 2005), Si (Mali, 2008), Ni (Jagetiya and Aery, 1994), W (Kumar and Aery, 2010a) and Mo (Chatterjee and Nautiyal, 2001). Results obtained from germination studies indicate that W enhances germination relative index more efficiently compared to Mo (Fig 1).

The increment in dry-matter with the application of Mo to plants is well established (Chatterjee and Nautiyal,

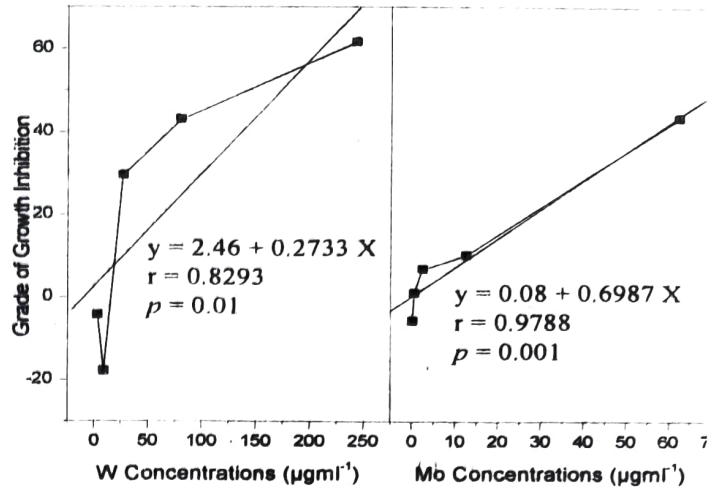

Fig: 5. Effect of various concentrations of W and Mo as sodium tungstate and sodium molybdate, respectively on the grade of growth inhibition of *Triticum aestivum* L.

2001 and Weng *et al.*, 2009). In the present study increased relative yield of wheat was also observed at lower applied doses of W and Mo. It has also been reported that sodium tungstate increases the growth rate and final yield of algae (Tyagi, 1974). Kumar and Aery (2010a, 2010b) reported an increment in dry matter and relative yield with the application of W in cowpea. Higher concentrations of both these metals resulted in decreased dry-matter. This might be due to higher accumulation of metals in cell which inhibits cell enlargement (Aery and Jagetiya, 2000; Mali and Aery, 2008) and/ or due to inhibition of cell division in the meristematic zone (Powell *et al.*, 1986). The decrement in relative yield of seedling was observed to be higher in W than Mo treatment (Table 1 and 2).

The root elongation method developed by Wilkins (1978) to quantify the inhibitory effect of metal ions on root growth has been used widely in ecological studies for testing of tolerance of plants to metals. Jayakumar *et al.* (2008) observed the increment at lower level and decrement at higher level in vigour index and tolerance index in response to cobalt. In the present study both tungsten and molybdenum were found to be inducer for vigour and tolerance indices. Vigour index was observed to be maximum at lower applied doses of tungsten (9 µg ml^{-1}) and molybdenum (0.1 µg ml^{-1}). The increment in vigour index indicates the positive effect of both these metals on the health of wheat. Minimum vigour index was observed at higher applied doses of W and Mo (Fig

Table 3: Relationship between applied metal concentrations and root-shoot length of *Triticum aestivum* L.

Parameter	Regression equation	Correlation coeff. (r)	Significance
Tungsten			
RL	y=8.17-.03x	-0.8207	S*
SL	y=9.42-.01x	-0.9090	S**
Molybdenum			
RL	y=7.68-.08x	-0.9531	S**
SL	y=7.76-.02x	-0.7994	S*

RL=root length, SL=shoot length, y = parameter, x = applied metal concentrations, S* = significant at 0.05 probability, level, S** = significant at 0.001 level

3). The magnitude of influence in vigour index of wheat varied in response to W and Mo. W cultured seedlings showed more increment in lower and decrement at higher levels, respectively, compared to Mo cultured seedlings.

A tolerance index of one means that the growth of root in the metal solution was the same as that in the reference solution (Freedman, 1995). In the present study lower applied doses of both tungsten (9 μg ml^{-1}) and molybdenum (0.1 μg ml^{-1}) showed maximum (more than one) tolerance index (Fig. 4). It indicates that lower applied doses of both W and Mo are favorable for plant growth. The increment in tolerance index is observed to be more remarkable in Mo treated plants compared to W treated plants. It reduced concomitantly at higher administration of both the metals. Jayakumar *et al.* (2008) and Pugalvendhan *et al.* (2009) have also observed the same results with the application of Co and Hg, respectively. An increase in the level of prolines has been observed (unpublished work of the authors) which may provide protection by chelating the metals in the cytoplasm and maintaining the water balance which is often disturbed by heavy metals (Xu *et al.*, 2009). Alternatively, plants may achieve metal tolerance by protecting the integrity of plasma membrane against metal damage by the use of heat shock proteins (Lewis *et al.*, 1999) or metallothioneins (Grennan, 2011).

Both W and Mo influence the grade of growth inhibition in the same fashion. A decrement in grade of growth inhibition in both W and Mo cultured seedlings was observed in lower concentrations. But the minimum grade of growth inhibition in tungsten treatment was three times than molybdenum treatment (Fig. 5). Higher concentrations of W and Mo showed toxic effect on seedling growth which resulted in increased grade of growth inhibition. Kumar and Aery (2010a) reported that application of W decreases the grade of growth inhibition at lower level and increases at higher level in cowpea.

Correlation coefficient between applied tungsten and molybdenum concentrations and relative yield and root-shoot length were also computed. In all the cases the negative value of correlation coefficient indicates the degree of toxic effect of both W and Mo on overall growth performance of wheat (Table 2 and 3).

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Designing primers to fish auto-inducer synthase gene(s) of the quorum sensing system in γ -proteobacteria and their *in-silico* PCR validation

Krishna Kant Yadav and Ranadhir Chakraborty*
Department of Biotechnology, University of North Bengal, Siliguri- 734 013, West Bengal, INDIA

Abstract
Quorum sensing is a well known phenomenon in bacteria that control diverse functions including colonization and formation of biofilm. The *luxI* gene, involved in quorum sensing of gram negative bacteria, codes for auto-inducer synthase/ acylhomoserine lactone synthase. As *luxI* homologues are sequence-diverse it is difficult to identify its loci by hybridization technique in different bacteria whose whole genome sequence(s) are unknown. We have used different bioinformatics tools taking the existing genome database into cognizance to design manually suitable degenerate primers for amplifying *luxI* gene homologues from diverse representatives of gamma-proteobacteria. Two primer pairs, Deg1F/Deg2R and Deg3F/Deg4R, were capable of in- silico PCR amplification from genome sequence(s) of *Halothiobacillus neapolitanus*, *Acinetobacter baumanii* ATCC 17978, *Acidithiobacillus ferrooxidans* ATCC 53993 and *Pseudomonas aeruginosa* PAO1 (with first primer pair); *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Erwinia tasmaneinsis*, *Serratia proteamaculans*, *Pectobacterium wasabiae*, *Pectobacterium carotovorum*, *Pectobacterium atrosepticum*, *Dickeya zeae* and *Yersinia pestis* (with second primer pair). The phylogenetic trees derived from sequences of *luxI* homologues and 16S rRNA gene sequences of the respective genomes were almost identical showing two distinct clusters. The degenerate primer pairs were also found to be cluster specific.

Keywords: Degenerate primer, *in silico* PCR, acylhomoserine lactone synthase, *LuxI*, Seaview4

Bacteria communicates with each other and can collectively form a group with properties not expressed when they are alone (Greenberg 2000). Quorum sensing is the term used to define and quantify such behavior. Actually the individuals in a bacterial population senses the quanta of certain chemical signals called autoinducer (AI) produced by themselves in course of growth (Shaw *et al* 1997). N-acylated derivatives of L-homoserine lactone (acyl-HSLs) which are identified as AIs are the products of acyl-homoserine lactone synthase (Shaw *et al*.,1997). With few exceptions, these enzymes constitute an evolutionarily conserved family of homologues known as the *LuxI* family of autoinducer synthase (Gray and Garey 2001). AI-based sensing mechanisms have been widely studied in gram negative bacteria (Greenberg 2000; Parsek and Greenberg 2000; Swift *et al*.,1999) which in turn control several functions like exoenzyme synthesis, conjugation, antibiotic production, luminescence, metal tolerance and biofilm formation (Sarkar and Chakraborty 2008).

Nucleotide sequence analyses with several alignment tools have revealed wide diversity with infrequent short stretches of similarity in *luxI* gene homologues. These stretches are too small to design a universal primer for conducting PCR amplification. Identifying *luxI* homologues from species (whose complete genome sequences are not available) by DNA-DNA hybridization using a single probe would also not be possible. The most viable solution to the problem is to design degenerate primer set for the amplification of such sequences. A PCR primer is called degenerate if some of its positions have several bases (Kwok *et al*., 1994.) For example in a degenerate primer like bchY fwd (5'-CCNCARACNATGTGYCCNGCNTTYGG-3') (Yutin *et al*., 2009) the R , Y and N are degenerate that means primer is a mixture in which one contain either A/G at position of R, similarly C/T at position Y and A/ T/G/C at position N.

As the whole genome sequences of many organisms are available in public domain it is possible to design gene specific primers for PCR amplification. A number of computer programs like Amplify (Jarmon 2004), simPCR (Rubin and Levy 1996), PCRAna (Nishigaki *et al*., 2000), PUNS (Boutros and Okey 2004), and Virtual PCR (Cao *et al*., 2005; Lexa *et al*., 2001) are available on-line which can predict the efficiency of PCR amplification with such designed primers. These programs however could not predict amplification with degenerate primer set (Cao *et al*., 2005).

In this work, we took advantage of certain output of SeaView version 4 program to manually design degenerate primers from the aligned sequences. SeaView is a multiplatform, graphical user interface for multiple sequence alignment and molecular phylogeny (Gouy *et al*., 2010) and is freely available at http:// pbil.univ-lyon1.fr/software/SeaView. Another program

**Corresponding author:*
E-mail: rcnbu2003@yahoo.com

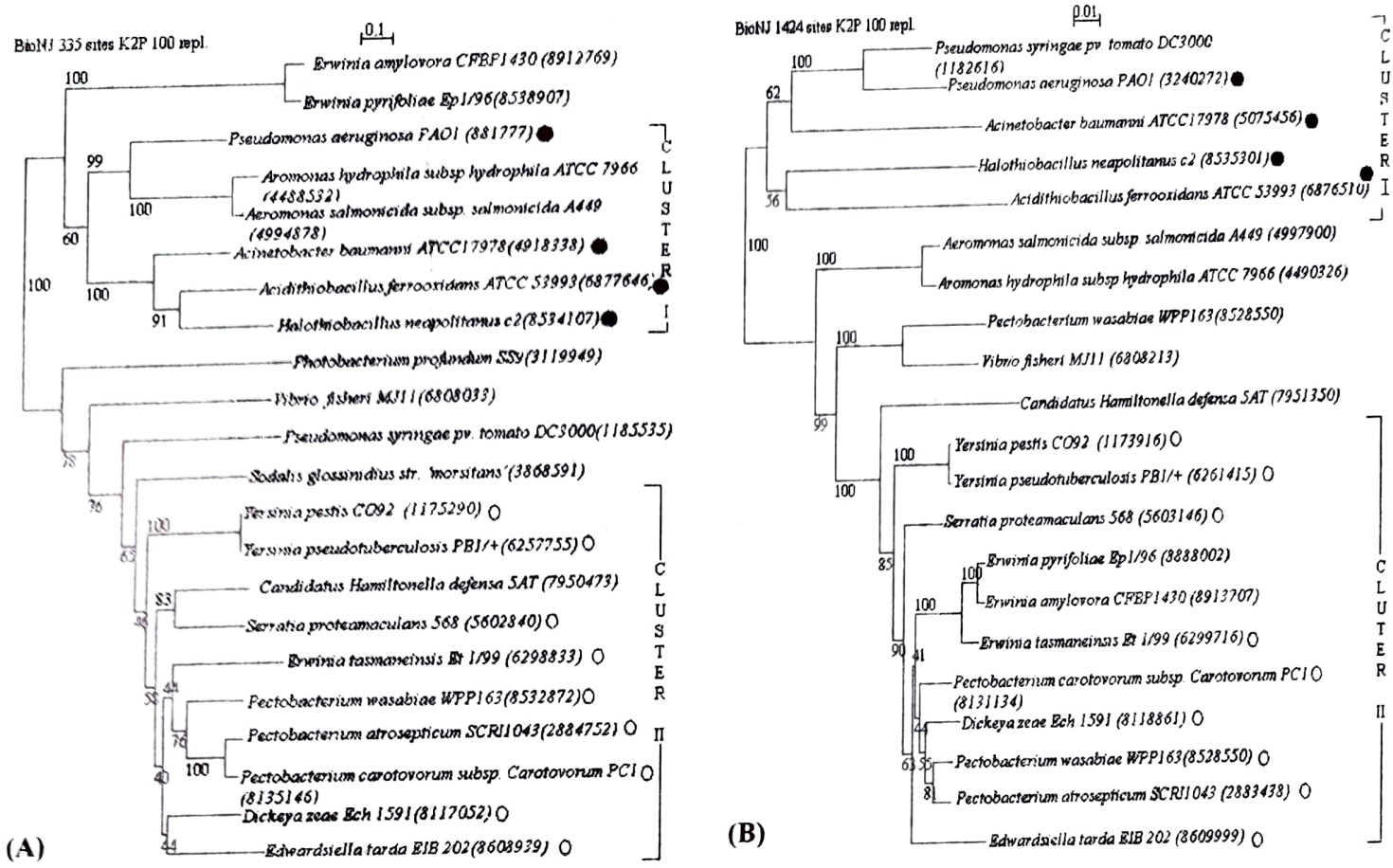

Figure. 1. Phylogenetic tree of (A) *luxI* homologue sequences, (B) 16S ribosomal RNA sequences from gamma proteobacteria. Dendogram generated in SeaView version 4 using distance method and BioNJ algorithm (Gascuel 1997). NCBI gene ID given in parenthesis. Nucleotide-level distances are observed divergence with Kimura's two-parameter. Bootstrapped to 100 replicates. (●) *luxI* homologue sequences amplified with degenerate primer pair Deg1F/Deg2R, (○) *luxI* homologue sequences amplified with degenerate primer pair Deg3F/Deg4R.

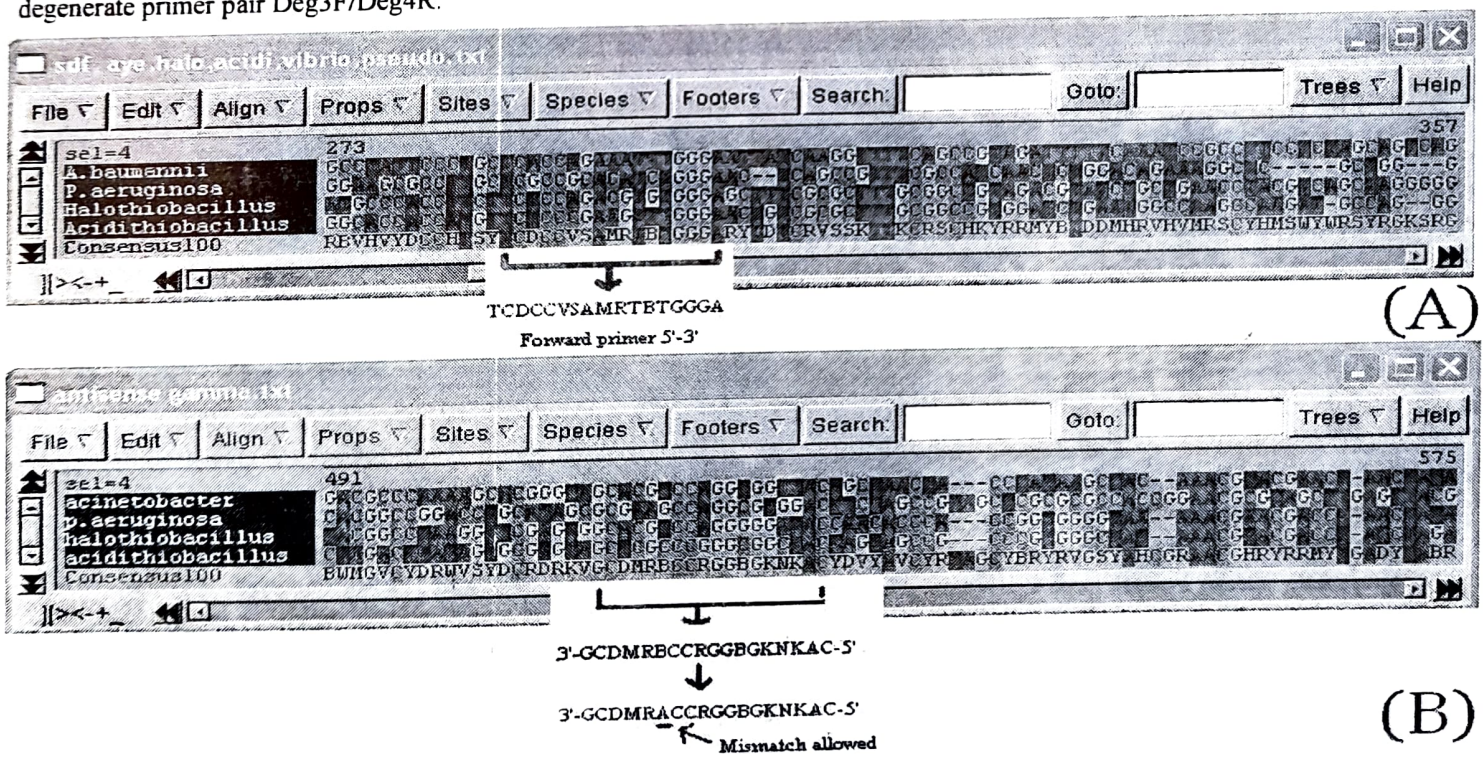

Figure. 2. Snapshot of SeaView window displaying ClustalW aligned *luxI* homologue sequences from gamma proteobacteria with degenerate letters just below the aligned sequences (A) forward primer, (B) reverse primer.

called *In silico* PCR (Bikandi *et al.*, 2004) (freely available at http://www.in-silico.com) was used to predict the possible *luxI* homologue amplification using degenerate primer pair(s) on template of whole genome sequences available in nucleotide databases.

Materials and Methods

Retrieval of luxI *homologous sequences:* Nucleotide sequences of *luxI* gene homologues in gamma-proteobacteria were retrieved from NCBI gene data bank (Table 1).

Table 1. List of gamma proteobacteria having *luxI* homologue sequences

Gamma proteobacteria		NCBI Gene ID[1]	NCBI Gene ID[2]
Aeromonadales	*Aeromonas salmonicida subsp. salmonicida* A449	4994878	4997900
	A. hydrophila subsp hydrophila ATCC 7966	4488532	4490326
Pseudomonadales	*Pseudomonas syringae pv. tomato* DC3000	1185535	1182616
	Pseudomonas aeruginosa PAO1	881777	3240272
	Acinetobacter baumanni ATCC17978	4918338	5075456
Enterobacteriales	*Edwardsiella tarda* EIB 202	8608939	8609999
	Yersinia pestis CO92	1175290	1173916
	Yersinia pseudotuberculosis PB1/+	6257755	6261415
	Erwinia amylovora CFBP1430	8912769	8913707
	Erwinia pyrifoliae Ep1/96	8538907	8888002
	Erwinia tasmaneinsis Et 1/99	6298833	6299716
	Serratia proteamaculans 568	5602840	5603146
	Candidatus Hamiltonella defensa 5AT (Acyrthosiphon pisum)	7950473	7951350
	Pectobacterium atrosepticum SCRI1043	2884752	2883438
	Pectobacterium carotovorum subsp. Carotovorum PC1	8135146	8131134
	Pectobacterium wasabiae WPP163	8532872	8528550
	Sodalis glossinidius str. 'morsitans'	3868591	3866445
	Dickeya zeae Ech 1591	8117052	8118861
Vibrionales	*Vibrio fisheri* MJ11	6808033	6808213
	Photobacterium profundum SS9	3119949	3120506
Acidithiobacillales	*Acidithiobacillus ferrooxidans* ATCC 53993	6877646	6876510
Chromatiales	*Halothiobacillus neapolitanus c2*	8534107	8535301

[1] *Coding for Acylhomoserine lactone/ Autoinducer synthase;* [2] *Coding for 16S Ribosomal RNA*

Table 2. Properties of designed degenerate primers

Degenerate primers 5' to 3'	Length	Degener-acy	TM in °C		%GC
			Oligo analysis[a]	Oligocalc[b]	
Deg1F-TCDCCVSAMRTBTGGGA	17	216	58.8	42-54(B),47-60(SA)	58.8
Deg2R-CAKNKGBGGRCCARMDCG	18	1152	64.4	46-64(B),51-70(SA)	64.4
Deg3F-AARGAYMGDCTNCAHTGG	18	288	56.8	41-55(B),47-61(SA)	56.8
Deg4R-AAYHYBCCADCCDGADC	17	972	57.1	40-57(B),45-62(SA)	57.1

a=freely available at www.operon.com/technical/toolkit.aspx, b=freely available at www.basic.northwestern.edu/biotools/oligocalc.html, B=Basic, SA= Salt Adjusted

Multiple Sequence Alignment: Sequences from the selected genera were aligned using ClustalW multiple alignment program in SeaView 4.

Designing Degenerate Primer: A phylogenetic tree of *luxI* sequences was obtained using distance method in SeaView 4 software (Figure 1A). This was used as the guide tree to select genera of closely related sequences for designing degenerate primers. Sequences of each cluster in the tree were separately aligned with ClustalW in SeaView 4. Most similar stretches of nucleotides having minimum 10 A+T+C+G in the multiple alignments were tentatively chosen to test the properties close to an ideal primer for PCR reaction (Fig.2A & B). Fast PCR (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) was used to get the complementary sequence for making the reverse primer. The primers were basically analyzed for melting point, %GC, hairpin, self complementarities and primer dimer check using OligoCalc (www.basic.northwester.edu/biotools/oligocalc.html) and Oligo Analysis Tool (www.operon.com/technical/toolkit.aspx). Attention was also paid to have 3□ GC clamps at 3□ end for increasing the specificity of the primer (Rouchcka *et al.*, 2005). Using 100% consensus, degenerate sequences (letter codes were followed according to the recommendations of Nomenclature Committee of the International Union of Biochemistry and Molecular Biology) were constructed from the aligned sequence.

In silico *PCR Amplification:* The designed degenerate primer pair was fed to the blank primer box followed by selection of bacterium (whose whole genome sequence is available in the database) in the interacting page of the online software, *In silico* PCR, for the output.

Results and Discussion

Phylogenetic analysis of the retrieved *luxI* homologue sequences from gamma proteobacteria has revealed two distinct clusters (Fig. 1A) which also correlates with the phylogenetic tree drawn from their 16S rRNA gene sequences (Fig. 1B).

Two degenerate primer pairs were designed using SeaView 4 and were designated as Deg1F/Deg2R and Deg3F/Deg4R and their properties are shown in Table 2. Using the *in silico* PCR tool (allowing one nucleotide mismatch but in one nucleotide at 3' end) it was found

Table 3. *In silico* PCR amplification using degenerate primer pair Deg1F/Deg2R (*) and Deg3F/Deg4R (#)

Organism	Amplicon	Sequence Name
Acinetobacter baumannii	238 bp*	Homoserine lactone synthase
Acidithiobacillus ferrooxidans	225 bp*	Autoindducer synthesis protein
Halothiobacillus neapolitanus c2	228 bp*	Acyl-homoserine-lactone synthase
Pseudomonas aeruginosa	213 bp*	Autoinducer synthesis protein *LasI*
Pectobacterium atrosepticum SCRI1043	390 bp#	Acylhomoserine lactone synthase
Erwinia tasmaniensis	390 bp#	Acylhomoserine lactone synthase
Pectobacterium carotovorum subsp. carotovorum PC1	390 bp#	Acyl-homoserine-lactone synthase
Pectobacterium wasabiae WPP163	390 bp#	Acyl-homoserine-lactone synthase
Edwardsiella tarda	393 bp#	AHL synthase
Edwardsiella ictaluri	393 bp#	Autoinducer synthase putative
Dickeya zeae	390 bp#	Acylhomoserine lactone synthase
Dickeya dadanti	390 bp#	Acylhomoserine lactone synthase
Serratia preteamaculans 568	390 bp#	Autoinducer synthesis protein
Yersinia pestis	390 bp#	N-acylhomoserine lactone synthase
Yersinia entercolitica	390 bp#	N-acylhomoserine lactone synthase
Yersinia pseudotuberculosis	390 bp#	N-acylhomoserine lactone synthase

All amplicons were of single band

that the degenerate primer pair, Deg1F/Deg2R was capable of amplifying *luxI* homologue sequences from *Acinetobacter baumanii, Pseudomonas aeruginosa, Halothiobacillus neapolitanus,* and *Acidithiobacillus ferrooxidans* and Deg3F/Deg4R enabled to amplify *luxI* homologue sequences from several bacterial species of the genera *Erwinia, Yersinia, Serratia, Pectobacterium, Edwardsiella* and *Dickeya* (Table 3).

The degenerate primer was manually designed using SeaView 4 since it allowed display of degenerate letters just below the aligned sequence (Fig 1A&B) and has an easy to use graphical interface (Gouy *et al* 2010). This has enabled us to select short nucleotide sequence containing of at least 10 A+T+G+C which is the prime requisite for in-silico PCR amplification. The *in silico* PCR software program linked with NCBI genome data bank was found to be important software tool which allowed amplification using degenerate primer. The output of this program showed the nucleotide positions of the genome region which have undergone amplification, length of the amplicon and a simulation of the electrophoretic mobility on agarose gel. Further information of each amplicon may be obtained following the corresponding links: DNA sequences, list of ORFs that are included in the amplicon, and a link to the NCBI site, which displays a map of the chromosome around the amplicon (Bikandi *et al.*, 2004).

Functional ability of the designed primers to amplify the target gene(s) could therefore be worked out in silico by accessing whole genome sequence (including plasmids when available) in the database. In all cases it would be mandatory to consider primers have been designed correctly, so that they do not form dimers, hairpin or any other aberrant structures preventing amplification.

Identification of two clusters in the phylogenetic tree constructed with *luxI* homologues has helped us to select the bacterial species for preparing the degenerate primers since the task has become easier because of lesser degeneracy among sequences of individual clusters. The applicability of the degenerate primers could be further enhanced if more mismatches are allowed under real wet lab conditions. To our observation in-silico PCR amplification with Deg1F/ Deg2R failed with *Aeromonas salmonicida* genome template (component of Cluster1, Figure 1A) as the present version of the software, *In silico* PCR, did not allow more than two mismatches.

Hence an exercise to design degenerate primers (when necessary in situations like *luxI* sequences) for amplification of gene homologue of interest with the help of a combination of SeaView 4 and *In Silico* PCR programs would be very useful for the researchers in real wet lab situations.

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Bacteriological quality of Mirik lake waters, Darjeeling district, West Bengal

Binod C Sharma* , Bimala Rai, Sujata Kalikotay, Basundhara Tamang, Rachana Rai
Microbiology Laboratory, Postgraduate Department of Botany, Darjeeling Government College, Darjeeling -734101, West Bengal, India

Abstract
A study was conducted on Mirik Lake to assess the bacteriological quality of the lake water during the period of March 2009 through February 2010. Water samples were collected from five different sites of the lake and analysed for bacteriological study to enumerate the seasonal distribution of total bacterial count, total coliform, faecal coliform bacteria and faecal streptococci. Total heterotrophic bacterial count per 100 ml (cfu/100 ml) was found to be $0.2x10^6$-$0.3x10^6$ in monsoon, 1.0×10^6- 6.7×10^6 in summer and 4.75×10^6 -10×10^6 in winter. The range of total coliform was observed to be 1100 - 1750 / 100 ml, 1100 – 2400/ 100 ml and 1750 – 2400 /100 ml in winter, summer and monsoon seasons, respectively. Faecal coliform was recorded to be 49 – 1245 / 100 ml in winter, 1320 – 2400 /100 in summer and 780 – 2400 / 100 ml in monsoon. The range of faecal streptococci was found to be 43-125 /100 ml in winter, 87 -1100 /100 ml in summer and 87 - 1340 /100 ml in monsoon.
The study indicated that the lake water was polluted by faecal contaminants of human origin to the extent that water was unsafe to be used for domestic as well as recreational purposes, also the total bacterial load exceeded the standard prescribed level (WHO, 1983) and both parameters showed variation according to the sampling sites and season.

Keywords: coliforms, faecal, streptococci

Mirik is a small town in the Darjeeling Hills, located at 26°54'N, 88°10'E/26.9°N,88.17°E with average elevation of 1495 metres (4904 feet) above msl. It has a pleasant climate all the year round with temperatures of maximum 30°C in summer and a minimum of 2°C in winter. Lake is man made, 1.25 km long, spread over an area of 110 ha. The lake gets water from several springs in its surrounding hilly catchment area. A dense coniferous forest lies on the southwest of the lake whereas the hills on the northern side experience extensive erosion. Sewage from the human settlements and tourist activities in its catchment directly enters the lake through several drains and solid wastes are also dumped into it. Washing and bathing activities too impinge upon the lake water quality.

Microorganisms are widely distributed in nature and their abundance and diversity may be used an indicator for the suitability of water (Okpokwasili and Akujobi, 1996). The use of bacteria as water quality indicators can be viewed in two ways; first, the presence of such bacteria can be taken as an indication of faecal contamination of the water and thus as a signal to determine why such contamination is present, how serious it is and what steps can be taken to eliminate it; second, their presence can be taken as an indication of the potential danger of health risks that faecal contamination posses (Baghel *et. al.*, 2005).The higher the level of indicator bacteria, the higher the level of faecal contamination and the greater the risk of water-borne diseases (Pipes, 1981). The faecal pollution indicator organisms can be used to a number of conditions related to the health of aquatic ecosystems and to the potential health effects among individuals using aquatic environments (McLellan *et. al.*, 2001). A wide range of pathogenic microorganisms can be transmitted to humans via water contaminated with faecal material. These include enteropathogenic agents such as *salmonellas, shigellas, enteroviruses,* and multicellular parasites as well as opportunistic pathogens like *Pseudomonas aeruginosa, Klebsiella, Vibrio parahaemolyticus and Aeromonas hydrophila* (Hodgekiss, 1988). The most widely used indicators are the coliform bacteria, which may be the total coliforms that got narrowed down to the faecal coliforms and the faecal streptococci (Kistemann *et. al.*, 2002, Pathak and Gopal, 2001; Harwood *et. al.*, 2001; Vaidya *et. al.*, 2001). The contamination of lakes and rivers by faecal material increases the risk to the populations due to water borne diseases (Rajakumar *et. al.*, 2006; Scott *et. al.*, 2003). Typhoid fever, cholera, infectious hepatitis, bacillary and amoebic dysenteries and many varieties of gastrointestinal diseases can all be transmitted by water (Rajakumar *et. al.*, 2006).

The study was undertaken to determine water quality of the lake water in terms of enumeration of total bacterial load, coliform bacteria, faecal coliforms, and faecal streptococci; their periodicity and pattern of distribution during March 2009 to February 2010.

**Corresponding author:*
E-mail: bcsdgc@rediffmail.com

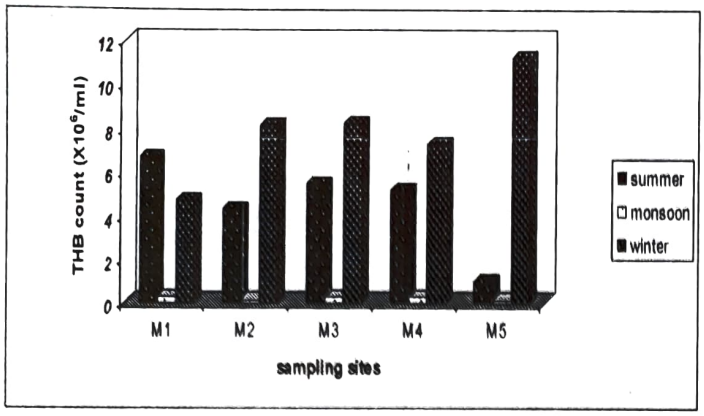


Figure 1 : Seasonal variation of Total Heterotrophic Bacteria (THB) count of Mirik Lake waters

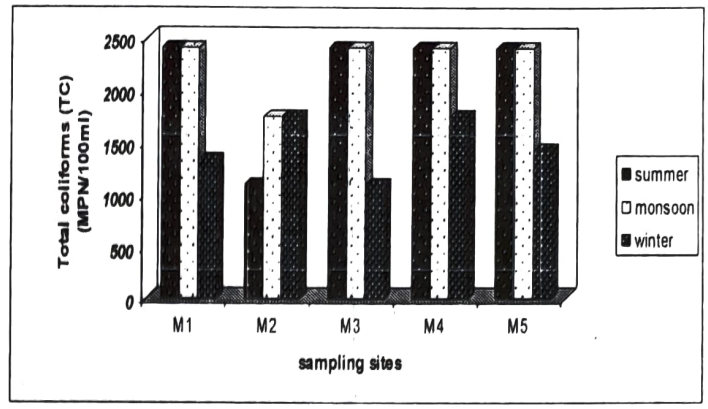


Figure 2: Seasonal variation of Total Coliform count of Mirik Lake waters (MPN /100 ml)

Materials and methods

Samples were collected from five different sites of the lake, sewage inlet (M1), area near human influence (M2), outlet (M3), boat house (M4) and the centre of the lake (M5), during three seasons i.e., summer, monsoon and winter, from March 2009 to February 2010. The samples were collected in pre sterilized stopper glass bottles and transported to the laboratory in an ice box and processed within 6 hours of collection.

The microbiological quality of water was determined by the standard most probable number (MPN) method and the data is represented as MPN/100ml. The total coliform and faecal coliform density were determined by inoculating the samples in Lauryl Sulphate Lactose Broth (LSLB) followed by their incubation at 37°C and 44°C respectively for 48 hours. Faecal streptococci density was determined by inoculation of samples in Glucose Azide Broth and incubated at 37°C for 24 - 48 hours (APHA, 1998).The total heterotrophic bacterial (THB) count was determined by serial dilution agar plate method on nutrient agar and represented as colony forming units per ml (cfu/ml).

All the culture media except otherwise stated were obtained from Hi Media Pvt. Ltd., Bombay, India.

Result and Discussion

THB count (THB/ml) were observed in the range of 5 x 10^6 to 11 x 10^6 in winter, 1 x 10^6 to 7 x 10^6 in summer and 0.2 x 10^6 to 0.3 x 10^6 in monsoon (Fig. 1).

An average of total coliforms in the five sites of the lake was found in the range of 1100 – 2400/100 ml in summer, 1750 – 2400/100ml in monsoon and 1100 – 1750/100ml in winter. The counts of faecal coliform observed during summer and monsoon and winter was in the range of, 1320 – 2400/100ml^{-1} and 780 – 2400/100 ml, 49 – 1245/100ml respectively. Faecal streptococci were in the range of 87 – 2400/100 ml in summer, 87 – 1340/100 ml in monsoon and 10 – 125/100 ml in winter (Table 1).

Total coliform count was the highest in sampling site M4 during all seasons i.e., 2400/100 ml in summer, 2400/100 ml in monsoon and 1750/100 ml in winter (Fig.2). The highest count of faecal coliform was also found in the site M4, i.e., 2400/100 ml, 2400/ 100 ml and 1245/100 ml in summer, monsoon and winter seasons respectively (Fig.3). The highest faecal streptococcal count was observed in the monsoon season in M3 showing the highest count of 1340/100 ml (Fig. 4).

Discussion

The study showed the variation in bacterial counts with season and the time of collection and sampling sites which was in agreement to other similar studies (Badge and Varma, 1982; 1991; Badge and Rangari, 1999; Rajakumar *et al.*, 2006). The bacteriological analysis revealed that water samples collected from five different sites of the lake were heavily loaded with coliforms, faecal coliforms and faecal streptococci. The highest and the lowest coliform population were observed in the monsoon and the winter season, respectively. The monsoon population was closely followed by the summer population; the pattern which was reported in earlier studies (Badge and Varma, 1982; Badge and Rangari, 1999, Rajakumar *et. al.*, 2006). The increase in

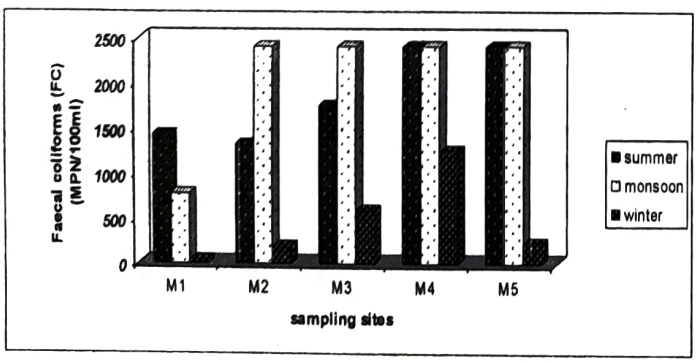
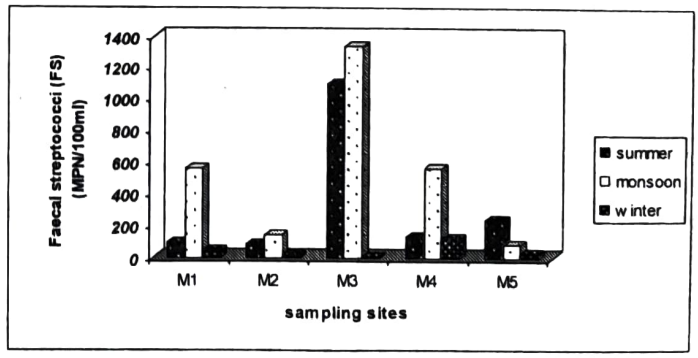


Figure 3: Seasonal variation of Faecal Coliform count of Mirik Lake waters (MPN/100 ml)



4: Seasonal variation of Faecal Streptococcal count of Mirik Lake waters (MPN/100ml)

Table 1: Range of total heterotrophic bacterial count, total coliforms, faecal coliforms and faecal streptococci in all the sampling sites of Mirik Lake during different sampling seasons

Indicator organisms	Sampling seasons (MPN/ 100 ml)		
	Summer	Monsoon	Winter
Total coliform	1100-1750	1100-2400	1750-2400
Faecal coliform	49-1245	1750-2400	780-2400
Faecal streptococci	10-125	87-1100	87-562
THB/ml ($x10^6$)	4.8 -8.3	01-6.8	0.2 -0.3

coliform population in monsoon may be due to the rain water that washed the faecal matter into the lake, as it was the major source of bacterial population in the lake water as observed by Quereshi and Dutka (1979). High population of coliforms during summer may be due to less available dilution (Badge and Varma, 1982) and also due to tourism activities in and around the lake. The lowest count during winter may be explained on the basis of lower multiplication rate and poor growth of bacteria due to low temperature during the season (Rajakumar *et. al.*, 2006). The fluctuations in the number of coliforms in different water samples can be attributed mainly to the intensity and age of pollution in addition to temperature and runoff waters (Badge and Varma, 1982). The highest faecal streptococci count was observed on the onset of .monsoon followed by summer and winter which may be due to runoff of human excreta as open air defecation is also common around the lake. Open air defecation and pouring of sewage pipes into the lake from the nearby vicinity around the site M3 may have contributed to the highest count of faecal streptococci at the site. Monsoon water samples showed highest population of faecal streptococci which may be due to drainage of sewerage pipes of the nearby habitation into the lake at the site. In the case of rainfall, the microbial loads of runoff water may suddenly increase and reach the lakes very quickly (Kistemann *et. al.*, 2002). Most strains of *E. coli* are harmless, but it is typically associated with more harmful bacteria that can cause illness. Therefore abundance of considerable population of faecal coliforms as well as faecal streptococci throughout the sampling periods indicates the continuous input of intestinal microorganisms of human origin warranting the population from outbreak of enteric diseases.

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Polyethylene glycol induced water stress in maize seedlings and evaluation of antioxidant defense mechanisms

R Lama and U Chakraborty*
Plant Biochemistry Laboratory, Department of Botany, University of North Bengal, Siliguri 734013, India

Abstract
Maize is one such crop, the production of which is highly challenged due to water shortage and soil water losses. The present study was undertaken on artificially induced water stress of maize *in vitro*, where stress was applied with PEG-6000 on one week old seedlings of four varieties BN 10, Dhanya, Kaveri-Super 244, and Swarna for 3, 5 and 7 days . The activity of antioxidative enzyme like peroxidase, catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase was assayed in the stressed and control plants. Peroxidase activity decreased on the 7th day in Dhanya and Swarna but in BN 101 and Super 244 the activity decreased slightly on the 5th day and increased again on the 7th day. Ascorbate peroxidase and superoxide dismutase showed a similar trend where the activity decreased after a certain period of stress. Similar trend was seen for GR activity too in case of Dhanya and Swarna. But in BN 101 and Super 244 there was an increase in the activity with the increase in the period of stress. Catalase activity declined during stress in Dhanya and Swarna while the other two varieties showed an increase during stress. Other than enzymatic activities, various biochemical analyses like proline, ascorbate, chlorophyll was also carried out. With the increase in intensity of drought there was an increase in both proline and ascorbate content in all. A significant increase in the ascorbate content was observed in BN 101 and super 244. H_2O_2 accumulation and lipid peroxidation showed an increase during stress in Dhanya and Swarna but no increase was seen in the other two varieties. Chlorophyll content showed a decline during the period of drought when compared to the control plants of all varieties. Enzymatic activity and biochemical tests show that Dhanya and Swarna are susceptible to drought stress than super 244 and BN 101 which are the tolerant varieties.

Keywords: maize, drought, antioxidant, proline, lipid peroxidation, H_2O_2.

Maize (*Zea mays* L.) is a major world crop and its productivity is greatly constrained by drought (Zinselmeier *et al.*, 2002). Water stress induces several physiological, biochem-ical and molecular responses in several crop plants, which would help them to adapt to such limiting environmental conditions (Bajaj *et al.*,1999; Arora *et al.*, 2002). Drought is a worldwide problem constraining plant production (Chinnusamy *et al.*, 2004) and is prone to acute periods due to little rainfall or an imbalanced distribution of rainfall in growing seasons as the environment deteriorates. Water deficit (commonly known as drought) can be defined as the absence of adequate moisture necessary for normal plant grow and to complete the life cycle (Zhu 2002). Plants can respond and adapt to water stress by altering their cellular metabolism and invoking various defense mechanisms (Zhu 2002, Boudsocq and Laurie`re 2005).The lack of adequate moisture leading to water stress is common occurrence in rain fed areas, brought about by infrequent rains and poor irrigation (Wang *et al.*, 2005).Proline and quaternary ammonium compounds, e.g. glycinebetaine, choline, prolinebetaine are key osmolytes contributing towards osmotic adjustment (Huang *et al.*, 2000 and Kavikishore *et al.*, 2005). One of the most important responses of plants to drought and other abiotic stresses is an overproduction of different types of compatible solutes (Ashraf and Harris, 2004; Serraj and Sinclair, 2002). Of these solutes, proline is widely distributed in plants and it accumulates in larger amounts than other amino acids in drought stressed plants (Ashraf, 2004; Irigoyen et al., 1992; Kohl *et al.*, 1991). Free proline and sugar contents significantly increased in *Vigna radiata* nodules under drought, but nodules had more proline than leaves (Hooda *et al.*, 1999).Great efforts have been made to decipher the molecular mechanisms of plant drought tolerance (Bartels and Nelson 1994 Bohnert 2000 Munns 2002 Ciais *et al.*, 2005 Mahajan and Tuteja 2005).It inhib-its the photosynthesis of plants, causes changes of chloro-phyll contents and components and damage to the photo-synthetic apparatus (Escuredo *et al.*, 1998). When plants are subjected to drought stress, a variety of active oxygen species are generated, such as superoxide, H_2O_2 and hydroxyl radicals, which cause damage in plants. They are toxic to living organisms and, unless removed rapidly, they destroy or inactivate various cellular components (Smirnoff 1993, Trippi *et al.*, 1989).

Considering the importance of maize cultivation in this region, the present study was undertaken to determine

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

how four varieties respond to water stress in terms of over expression of antioxidant enymes or other biomolecules.

Materials and Methods

Induction of water stress

Four maize varieties - BN 101, Kaveri-244 Super, Dhanya and Swarna were selected for experimental purposes. For induction of water stress, initially, seeds were soaked over night and surface sterilized with 0.1% $HgCl_2$ after which they were transferred to autoclaved petriplates in the laminar flow. The seeds were kept in petriplates and grown *in vitro* for a week. After a week the seedlings were subjected to drought stress by application of PEG 6000 and various biochemical tests were performed on the 3rd, 5th and 7th day of stress along with the control plants.

Preparation of enzyme extract

The leaves collected from treated and control plants were ground to fine powder with a mortar and pestle under liquid nitrogen in cold 50 mM sodium phosphate buffer, pH 7.5, containing 1% (w/v) polyvinylpolypyrrolidone. The homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was directly used as crude extract for enzyme assays.

Assay of activities

Peroxidase (POX: EC. 1.11.17)

Peroxidase activity was assayed spectrophotometrically in UV VIS spectrophotometer (Model 118 SYSTRONICS) at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et.al.*, 1993). Specific activity was expressed as ΔA_{460} mg protein $^{-1}$ min $^{-1}$.

Ascorbate peroxidase (APOX: EC.1.11.1.11)

Activity of ascorbate peroxidase was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada and Takahashi (1987) with some modification. Enzyme activity was expressed as ΔA_{290} mg protein $^{-1}$ min $^{-1}$.

Catalase (CAT: EC.1.11.1.6)

Catalase activity was assayed as described by Beers and Sizer (1952) by estimating the breakdown of H_2O_2 which was measured at 240 nm in a spectrophotometer. The enzyme activity was expressed as ΔA_{245} mg protein $^{-1}$ min $^{-1}$.

Superoxide dismutase (SOD: EC 1.15.1.1)

Activity was assayed by monitoring the inhibition of the photochemical reduction of NBT according to the method of Dhindsa *et al.*, (1981) with some modification. The absorbance of samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

Glutathione reductase (GR: EC 1.6.4.2)

Glutathione reductase activity was determined by the oxidation of NADPH at 340 nm as described by Lee and Lee (2000). Enzyme activity was expressed as μM NADPH oxidized mg protein^{-1} min^{-1}.

Protein contents in each case were determined by Lowry's method.

Lipid peroxidation

Lipid peroxidation was measured as MDA determined by the thiobarbituric acid (TBA) reaction. Cells (0.25 g) were homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at at 10,000 rpm for 10 min. To 0.5 ml of the aliquot of the supernatant, 2ml of 20% TCA containing 0.5% (w/v) TBA were added. The mixture was heated at 95 °C for

Table1: H_2O_2 accumulation and lipid peroxidation in maize seedling following water stress

Stress period (days)	Varieties	H_2O_2[a]	Lipid[b]
0	DHANYA	02.19	0.007
3		06.44	0.013
5		10.59	0.020
7		17.90	0.028
0	SWARNA	02.67	0.014
3		06.47	0.018
5		12.15	0.020
7		13.44	0.035
0	BN 101	01.87	0.008
3		04.19	0.006
5		06.24	0.012
7		05.25	0.006
0	SUPER244	02.89	0.009
3		06.20	0.011
5		07.55	0.011
7		05.43	0.009

[a]H_2O_2 content (μM/g tissue); [b]Lipid peroxidation (μM MDA/g tissue). All values are average of 3 replicates

Table 2: Proline content in leaf and root of four maize varieties following water stress

Stress period (days)	Varieties	Proline[a]	Proline[b]
0	DHANYA	0.09	0.44
3		0.28	0.28
5		0.38	0.56
7		0.84	0.84
0	SWARNA	0.23	0.23
3		0.25	0.25
5		0.30	0.34
7		0.70	0.60
0	BN 101	0.88	0.75
3		1.50	1.25
5		1.88	1.63
7		2.63	2.50
0	SUPER244	0.75	0.35
3		1.25	1.40
5		1.60	1.58
7		2.48	2.03

[a]Proline content (mg/g tissue) in leaf; [b]Proline content (mg/g tissue) in root. All values are average of 3 replicates

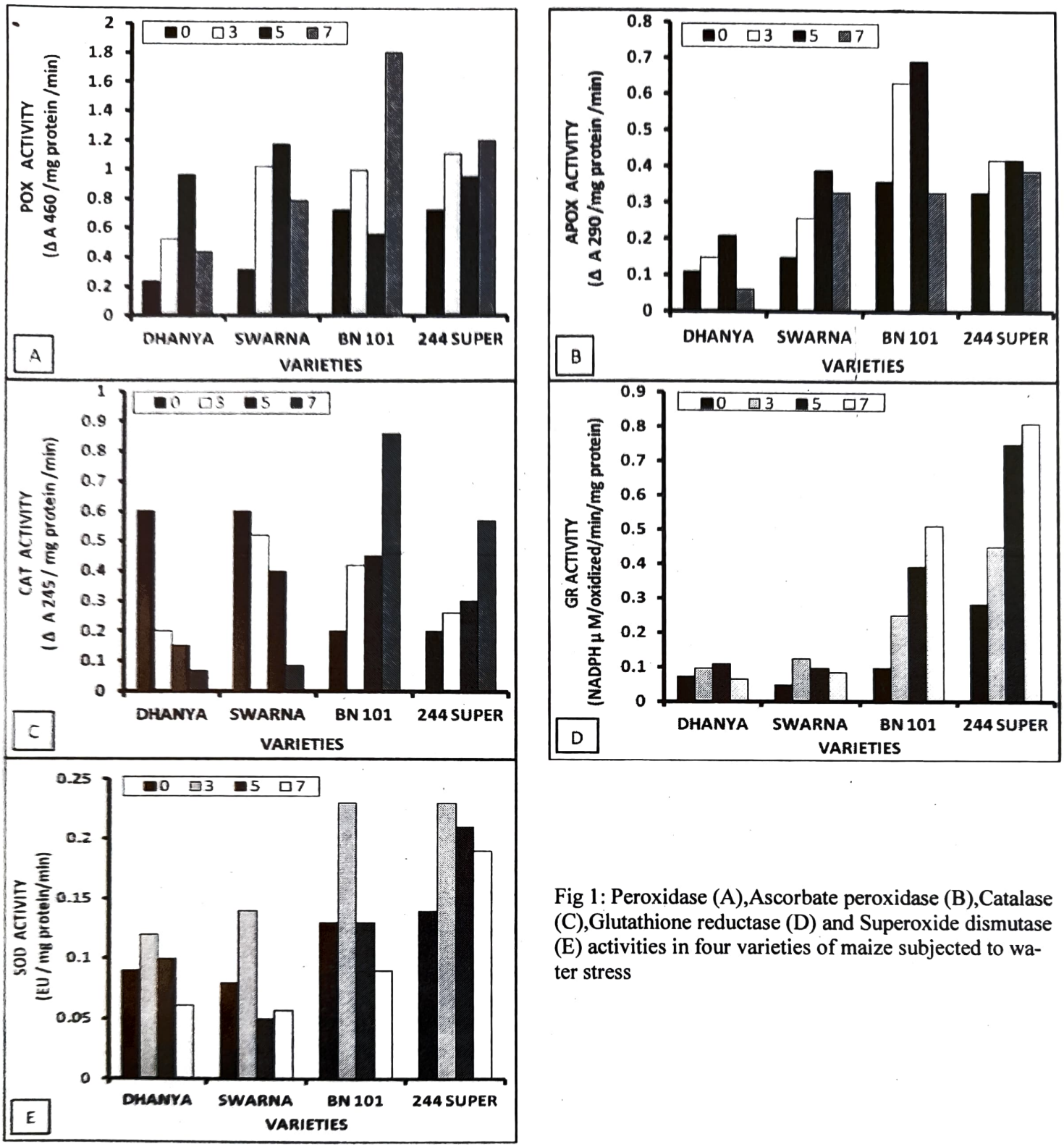

Fig 1: Peroxidase (A),Ascorbate peroxidase (B),Catalase (C),Glutathione reductase (D) and Superoxide dismutase (E) activities in four varieties of maize subjected to water stress

30 min and then quickly cooled on ice. The absorbance was measured at 532 nm and 600. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1}. (Heath and Packer, 1968).

Estimation of H_2O_2

H_2O_2 was extracted and quantified content was measured according to the method of Jena and Chowdhuri (1981).

Ascorbate

Ascorbate was extracted and estimated by following the method of Mukherjee and Choudhuri (1983). The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

Chlorophyll

Total chlorophyll content was estimated by following the method of Harborne (1973). Extraction was done in 80% acetone and the extract was filtered. Absorbance of the filtrate was noted at 663nm and 645nm in a VIS spectrophotometer and the chlorophyll content was calculated using the standard formula.

Results & Discussion

Four varieties of one week old seedlings of maize were subjected to drought stress *in vitro* by application of PEG-6000 (Polyethylene glycol).On the 3rd, 5th and 7th days of drought stress plants were sampled for various biochemical assays along with the controls. No significant morphological changes were observed in the test pants during initial stages of drought but slight

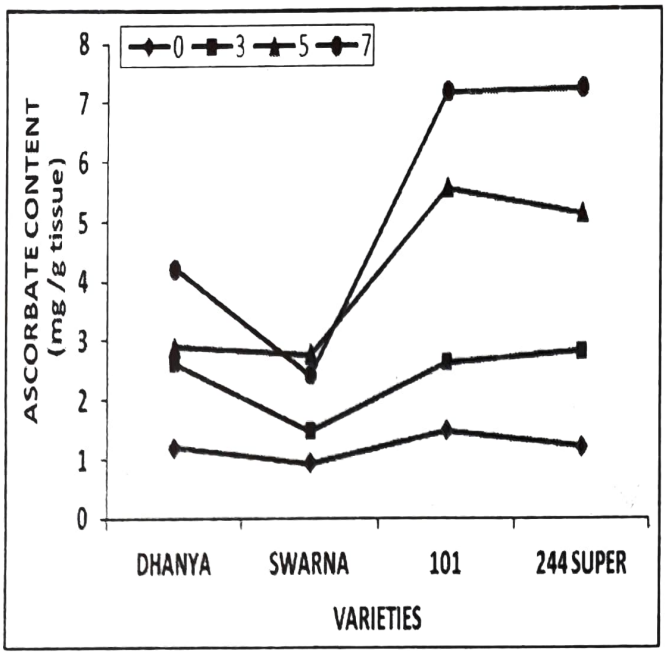

Fig. 2: Ascorbate content in four varieties of maize during water stress *invitro*

wilting was seen on the 7th day of stress. Assay of antioxidative enzyme activities showed that the POX activity increased in all varieties in the initial period of stress (Fig 1 A); in Dhanya and Swarna activity decreased on the 7th day but BN 101 and Super244 showed a different trend where there was a slight decrease in POX activity on the 5th day and again an increases on the 7th day. APOX (Fig 1 B) and SOD (Fig 1 E) in all the four varieties showed an increase in the initial period of stress but decreased on the later stages. In wheat, SOD activity increased or remained unchanged in the early phase of drought but decreased with prolonged water stress (Zhang and Kirkham 1995).CAT (Fig 1 C) activity decreased during drought in case of Dhanya and Swarna when compared to control but in the other two varieties there was a significant increase in the activity and the similar trend was also seen in GR activity too (Fig 1 D). When comparing antioxidative activity among the four varieties, BN 101 showed maximum increase in antioxidative activity during the period of drought stress. Maintaining a high level of antioxidative enzyme activities may contribute to drought tolerance by increasing the capacity of better protection mechanisms against oxidative damage (Sharma and Dubey, 2005; Tu" rkan et al., 2005). Among the four varieties Dhanya and Swarna showed greater accumulation of H_2O_2 during the stressed days and was maximum on the 7th day. But in BN 101 and Super 244 there was a slight decrease on the 7th day (Table1). Lipid peroxidation also increased in the same varieties (Table 1). During drought ascorbate content increased in all varieties but was maximum in BN 101 and Super 244 (Fig2). Ascorbate can act directly as a free radical scavenger (Bowler *et al.* 1992, Larson 1998). Results of present experiment showed that there was a decrease in chlorophyll content in all four varieties during drought stress (Fig 3). There are similar reports about decrease of chlorophyll in the drought stress conditions (Kuroda et al, 1990). Proline accumulation was more in case of drought stress than compared to the control in all the four varieties (Table 2). Maximum proline content was seen in BN 101 in both the leaves and root during drought condition. Accumulation of proline in plants under stress is a result of the reciprocal regulation of two pathways: increased expression of proline synthetic enzymes and repressed activity of proline degradation (Delauney *et al.*, 1993, Peng *et al.*, 1996). Accumulation of proline is an important indicator of drought stress tolerance in bacteria, algae, and higher plants. This amino acid has been reported to play multiple physiological functions in plants subjected to drought, such as osmoregulation, a sink for energy and nitrogen, and a signal of senescence (Aspinall and Paleg, 1981).

Results of the present study therefore indicate that water stress induces oxidative stress in all the four varieties but as antioxidative mechanism was much more pronounced in BN 101, this variety was the most tolerant to drought stress. Although laboratory conditions may not always reflect the behaviour of the plants exposed to water stress under field conditions, but such finding may help to understand the mechanism of drought stress

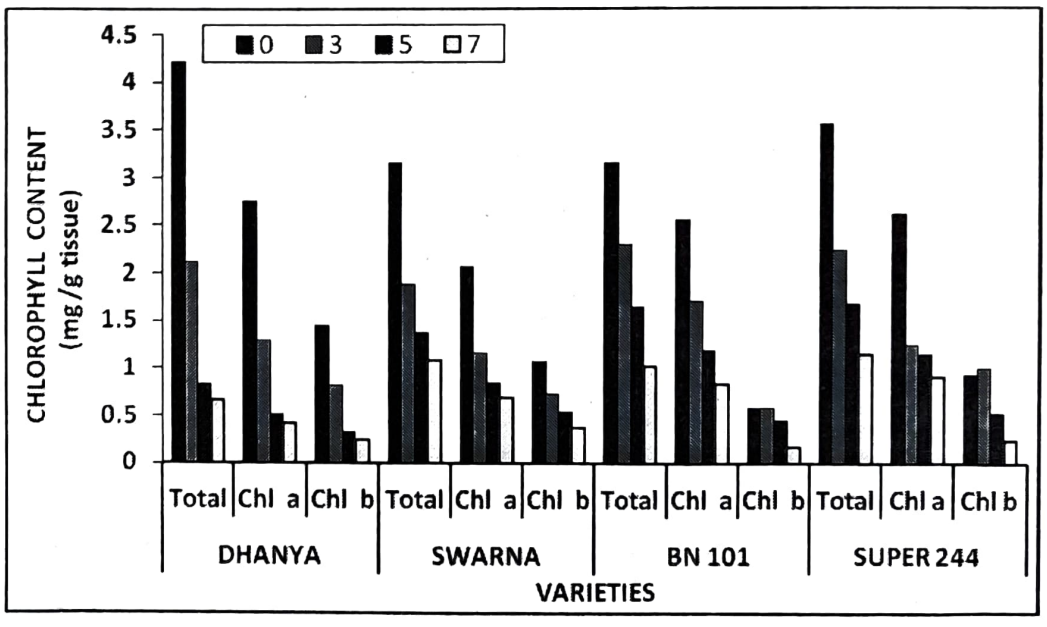

Fig.3: Changes in chlorophyll content of four varieties of maize following water stress

management and selection or development of maize genotypes resistant to drought stress.

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Root colonization of mandarin plants grown in orchards of Darjeeling hills and plains with arbuscular mycorrhizal fungi and their effects on plant growth

S Allay, UK De and BN Chakraborty*
Immuno-Phytopathlogy Laboratory, Department of Botany, University of North Bengal, Siliguri, 734013, Darjeeling, (W.B.), India

Abstract
Citrus reticulata is an ancient commercial crop being cultivated in Darjeeling-Sikkim hills. Many diseases are prevalent in mandarin plants, out of them, bacterial & fungal diseases are mostly dangerous. Arbuscular Mycorrhizal Fungi were screened from rhizosphere of mandarin plants from the four different regions using wet sieving and decanting method. Microscopical observation revealed the presence of different genus of AM fungi present in the root as hyphae, spores and sporocarp. *Glomus mosseae, G. fasciculatum, G. aggregatum, G. badium, G. constrictum, G. versiforme, Gigaspora gigantea, G. margarita, Acaulospora capsicula, A. bireticulata, Sclerocystis* and *Scutellospora rubra* were found to be dominant in all the soil samples of mandarin. Species of *Glomus* were found to be high in both hilly and foothill regions. *Glomus mosseae* and *G. fasciculatum* were selected for mass multiplication in maize plant in pots. Histopathological study of root showed the presence of vesicles and arbuscules. AMF infection and total number of spores per 100 gram of soil were recorded. Scanning Electron Microscopy (SEM) of AMF spores of mandarin revealed clear morphology, spore wall characters and hyphal attachment of spores. Total phosphate content of the soil, soil analysis and enzyme activities in roots and leaves of mandarin plant from the different regions were studied. Three major defense enzymes peroxidase, chitinase and β-1,3- glucanase showed enhanced activities and the total phosphate content also decreased in soil with respect to control. Present study evaluates the effect of AMF in plant growth and phosphate solubilization.

Keywords: *Citrus reticulata, Glomus mosseae, G. fasciculatum*

Mandarin orange (*Citrus reticulata* Blanco) the loose jacket orange which is a principal cash crop of India is cultivated to an alleviation of 1500 mm in Darjeeling – Sikkim hills. It belongs to the family Rutaceae under the order Sapindales. Mandarin is grown in the tropical / sub -tropical regions 35° N to 35° S of equator. There are four natural and one hybrid cultivars of mandarin in India grown in five different belts as a dominant cash crop. The total production is reckoned at about 760 thousand tonnes per year. Loose jacketed orange such as Nagpur Santra, Coorg orange, Kamala orange of Manipur, Khasi orange of Assam, Sikkim orange of Darjeeling and Kinnow of Punjab belong to the category of Mandarins in India.

Mandarin is widely consumed as fresh fruit and also used for producing canned segments, juice- concentrate, squash, beverages, jams as well as marmalades. The peel of Mandarin is the source of essential oils which are used in the cosmetic and pharmaceutical industries (Frazier and Westhote, 1978). It can also serve as a basic material for the production of cattle feed, candies and alcohol.

However, mandarin orange is susceptible to various pest and diseases which results to decline in production and productivity. The intensity sometimes is so severe that thousands of hectare cultivated areas are declined every year which is commonly referred as citrus decline or citrus dieback. The commons citrus diseases are growing, tristeza, cranker, foot/root rot, wilting etc. Root rot is an alarming problem of Darjeeling mandarin and one of its important causal organism is *Fusarium* spp.

AMF are an important group of soil–borne microorganisms that contribute sustainability to the establishment, productivity and longevity of natural or man-made ecosystems by the virtue of forming a symbiotic association with most terrestrial plants by forming an extensive network of external hyphae functioning as plant rootlets by spreading into a vast area underground and absorb nitrogen, phosphorus, potassium, calcium, sulfur, ferric, manganese, copper and zinc from the soil and then translocate these nutrients to the plants with those roots they are associated (Gerdemann, 1975). The symbiotic associations of AMF with most terrestrial plans are well documented but there are only few reports of symbiotic association between mandarin plant and AMF. The extrametrical fungal hyphae can extend several centimeters into the soil and absorb large amounts of nutrients for the host root (Khan *et al.*, 2000).

These extraradical hyphal networks and their hyphae help in improving the texture of the soil as they contain and release glomalin, which is a putative glycoprotein,

*Corresponding author:
E-mail: bncnbu@gmail.com

assayed from soil. Glomalin is a Glomalin-related soil protein (GRSP) that is correlated with aggregate water stability (Wright and Upadhyaya 1998, Rillig 2004; Rillig and Mummey 2006). Improved soil structure increases water infiltration and can reduce soil erosion (Tisdall and Oades 1982).Efforts are being undertaken to develop a bio formulations which can minimize the disease occurrence.

Considering the importance of association of AMF with mandarin orange the present investigation was made to assess the AMF population from three different locations of Darjeeling hills (Kalimpong, Mirik, Bijanbari). For the assessment of AMF population in plains, rhizosphere soil was collected from mandarin plants being grown in experimental garden of Immuno-Phytopathology Laboratory, Department of Botany, NBU.

Materials and method

Isolation of AMF spores

Arbuscular mycorrhizal fungal spores were screened from those soil samples of Mandarin rhizosphere by the wet sieving and decanting method (Gerdeman & Nicholson, 1963). Soil samples (100gm each of the representative root zone) were collected, suspended in water (1 l) in order to obtain a uniform suspension. Soil clusters are carefully dispersed in the water and is kept for 10 minutes to settle down the heavy particles. Aqueous suspension was passed through a set of sieves of different pore size (200, 170, 150, 80, 50μm) arranged one below the other. The spores were picked by the help of fine bristles / brushes and transferred to grooved slides or vials and observed under dissecting microscope. Few spores were stained with Melzar's reagent and studied under sterio-microscope. Healthy spores are separated by fine brush and are stored in autoclaved glass vials either in sterile distilled water or Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in one liter of boiled distilled water) at 4°C for further study and observation. It is evident from various studies that each plant has multiple AM fungi population. The soils of the collected samples were further analyzed to know the chemical composition of the soil, viz. moisture content, pH, amount of carbon, N_2 etc.

Plant material

Citrus reticulata seedlings were obtained from orchards of Kalimpong, Mirik, Bijanbari and experimental field of Immuno-phytopathology Laboratory of North Bengal University, Siliguri. They were maintained in 12" earthen pots with sterilized soil.

Identification of AMF spores

Spore samples were separated according to their morphology, size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachment etc. for the purpose of identification. The spores were identified up to species level with the help of standard keys (Walker 1981; Schneck and Perez 1987). Spores were critically examined with special reference to variation in vesicles (size, shape, wall thickness, wall layers, position and abundance), hyphal branching patterns, the diameter, structure (especially near entry points) and the staining intensity of hyphae.

Spore count

Rhizosphere soil (100g) was taken and suspended in 250 ml water. Wet sieving and decanting method was used for isolation of spores. Total number of spores were then counted and spore percentage of different genera was obtained.

Histo-pathological analysis

The root specimen were taken from field and washed with tap water. The root were cut into pieces, after washing treated with 10%KOH added, kept in water bath for 1h, then 1% HCL was added to neutralize the alkalinity. The root pieces were then washed with water (after 30 min) and staining was done by simmering the roots in cotton blue: lactophenol(1:4) for 3-4min with mild heating. Degree of contrast between fungal tissues and back ground plant cells was obtained according to the duration of storage of tissues. 1% HCl was added to acidify the tissues, as most histological stains are acidic. A little amendment in this process is noteworthy because it has been noticed that extraradical spore bearing hyphae and other extraradical fungal tissues with root segments are destroyed or dissolved when it is boiled in hot water bath at 90°C twice with 2% KOH followed by 0.05 cotton blue and lacto glycerol for staining the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxiliary cells etc. The total staining process can be done without heating but keeping the root fragments in 1-2% KOH for 24-48 hours in a Petri dish and another 12 to 18 hours in cotton blue and lactoglycerol with minimum movements of the samples yields remarkable result. In this method the spore bearing hyphal structures, auxiliary cells etc. are clearly visible and percent colonization can be determined with better accuracy. After preparing the roots the hyphal structures were viewed under dissecting stereomicroscope under 20X and 40X magnification. Percent root colonization was estimated by using slide method as described by Giovannetti and Mosse (1980).

Mass multiplication of AM spores

Glomus mosseae and *G. fasciculatum* were selected from among the mass of other AM fungi with the help of fine tweezers under dissecting microscope. The spores were washed several times with distilled water and Chloramin T to remove adhered debris. They were then inoculated in roots of 7-10 days old maize seedlings which were grown in petri plates. After inoculation they were transferred to black plastic pots (12 inch) having autoclaved soil to discard the presence of other fungal propagules. After 45 days the presence of spores of *G. mosseae* and *G. fasciculatum* were confirmed.

Artificial inoculation of mandarin roots

Healthy spores of *G. mosseae* and *G. fasciculatum* were collected from the maize plants and rinsed with sterile distilled water. Filter paper were cut into small circles of 5 mm diameter and about 5-6 spores were transferred to the filter paper. The paper was then adhered to the roots

Table 1: Physico-chemical factors and AM infection (%) in rhizosphere soil samples of mandarin

Factors	Kalim-pong	Mirik	Bijan-bari	Foot-hills
Soil type:	Clay	Sandy clay	Clay	Clay
Sand(%):	48	54	42	46
Silt(%):	10	04	16	14
Clay(%):	42	42	42	40
pH:	6.01	4.81	4.31	5.07
Moisture(%):	21.64	19.27	11.62	20.95
P_2O_5 ppm:	23.94	34.82	20.67	31.35
K_2O ppm:	31.34	197.67	84.89	48.97
Organic C(%):	0.48	1.11	1.31	0.75
Nitrogen(%):	0.05	0.11	0.14	0.08
Colonization (%):	98	79	86	84

of 1 month old seedlings of mandarin plants with the help of tweezers.

Extraction and quantification of soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper (Mehlich 1984). Ammonium molybdate-ascorbic acid method was followed for quantitative estimation of phosphate as described by Knudsen and Beegle (1988).

Assay of enzyme activities

Leaves and roots of mandarin seedlings grown in treated or control potted soil collected from different regions were used for all biochemical analyses. Samples were collected for assay 1 month after inoculation.

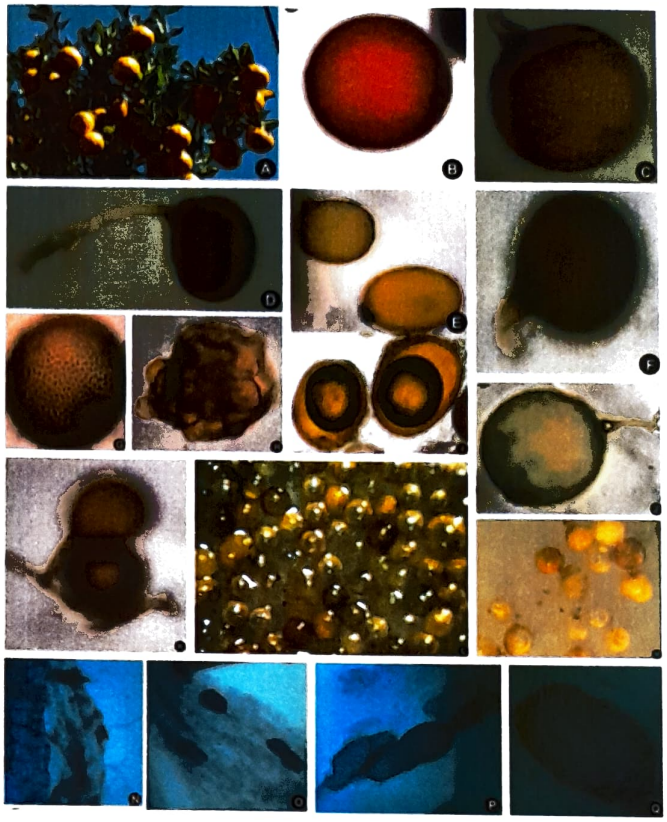

Figure 1 A: Fruit bearing mandarin plant; B: *Acaulospora capsicula*, C: *Giomus mosseae*, D: *G. mosseae* with long hyphal attachment, E: *G. badium*, F: *G. constrictum*, G: *Acaulospora bireticulata*, H: Sporocarp of *Glomus*, I: *Scutellospora rubra*, J: *Gigaspora margarica*, K: *Glomus fascicuiata*, L: Mass multiplied spores of *Gigaspora giganta*, M: Enlarged view of the same, N: Mycorrhizal hyphae in root tissue, O-Q: Vesicle, flattened vesicles and single enlarged view of vesicle

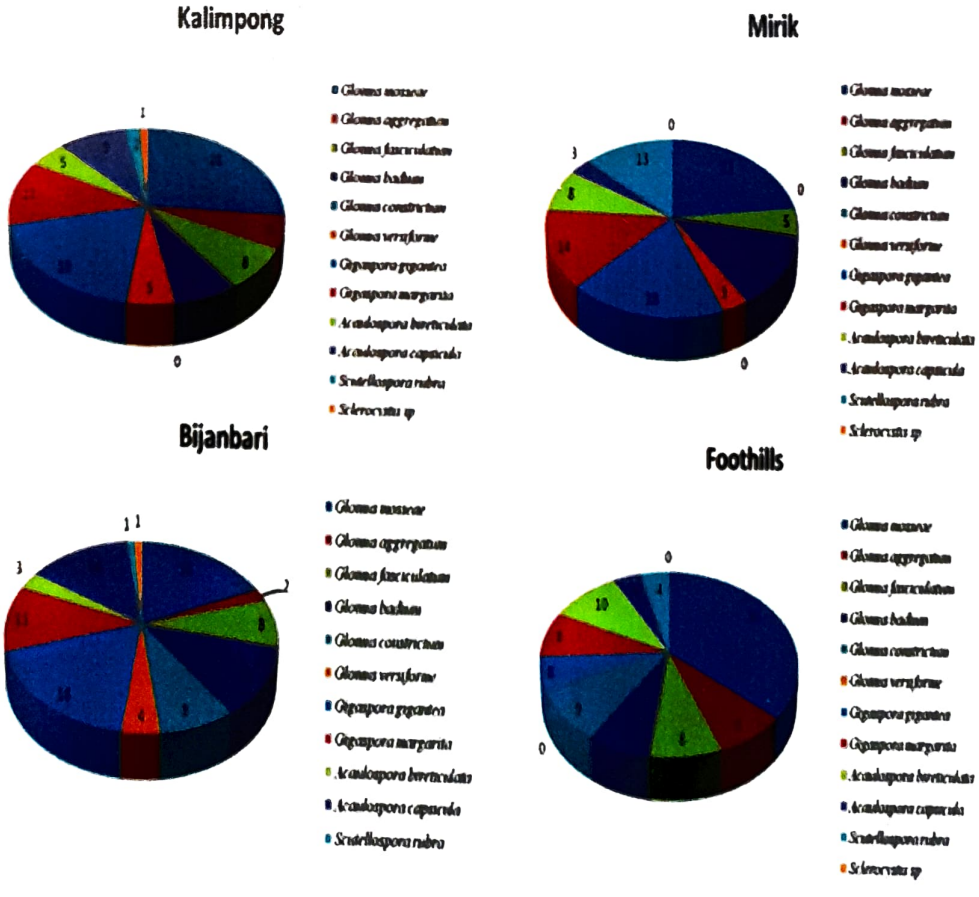

Figure 2: Percentage population of dominant AMF spores in mandarin soil

Table 2: Microscopic characters of AMF Spores associated with mandarin roots

Genus & species	Colour	Shape	Spore layer	Spore size (µm)	Other descriptions
Glomus fasciculatum	Pale yellow to bright brown	Globose to subglobose	3	70-120	Spore layer continuous
Glomus mosseae	Brown to orange -brown	Globose to subglobose	3	200	Hyphae are double layered
Glomus aggregatum	Pale yellow	Globose to oval	1-2	200-1800 x 200-1400	Sporocarps formed in loose clusters
Glomus badium	Reddish brown to dark brown to black	Globose, subglobose to ovoid	3	51-90 x 75-120	Subtending hypha of each spore is usually very short
Glomus constrictum	Brownish orange to dark brown	Globose to subglobose, sometimes ovoid	2	110-130 x 150-160	Subtending hyphae straight or curved, usually markedly constricted at the spore base
Glomus versiforme	Orange to red brown	Globose to subglobose, sometimes ovoid	2	60-160	Sporocarps are irregular, they arise from a basal pad of pale grayish yellow, loose mycelium with a few interspersed spores
Gigaspora gigantea	Greenish yellow	Globose to subglobose	2	250-270 x 265-370	Formed terminally or laterally on a bulbous sporogenous cell
Gigaspora margarita	Yellowish white to sunflower yellow	Globose to subglobose	2	300-340 x 360-380	Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell
Acaulospora capsicula	Orange red to capsicum red	Globose to subglobose	3	220-310 x 290-440	Sporiferous saccule pale yellow to brownish yellow which usually falls off when spores mature
Acaulospora bireticulata	Brownish	Globose	3	280-410	Surface ornamentation is prominent. Spores are borne laterally from the neck of a sporiferous saccule.
Scutellospora rubra	Dark orange-brown to red-brown	Globose to subglobose	3	140-220	Germinal walls are formed completely separate from the spore wall
Sclerocystis	Brown to blackish brown	Globose to subglobose		300-600 x 400-700	Chlamydospores arranged side by side in a single layer radially arranged on a central plexus of hyphae

Table 3: Soil phosphate (µg/g tissue) content in rhizosphere of mandarin plants after application of microorganisms

	Kalimpong	Mirik	Bijanbari	Foothills
Control	47.19± 0.625	49.13 ± 0.0144	48.39 ± 0.387	47.12 ± 0.071
Glomus mosseae	33.67 ± 0.287	32.45 ± 0.262	31.99 ±0.41	33.06 ±0.461
G. fasciculatum	31.75 ± 0.2165	31.64 ± 0.086	32.40 ± 0.318	32.03 ± 0.14
G. mosseae + G. fasciculatum	30.38 ± 0.198	30.23 ± 0.296	29.85 ± 0.13	29.58 ± 0.29±

Peroxidase (POX, EC1.11.1.7.). Extraction and assay of peroxidase was done following the method described by Chakraborty *et al* (1993). O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity expressed as the increase in Δ A 465/g tissue/ min.

Chitinase (CHT, EC 3.2.1.14). Chitinase was extracted and assayed following the method of Boller and Mauch (1988). The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released /min/ g fresh wt. tissue.

β- 1,3- glucanase (β-GLU, EC 3.2.1.38). β-1,3-glucanase was extracted and assayed from the samples following the method of Pan *et al* (1991). The amount of glucose liberated was determined spectrophotometrically using a standard curve. Activity was expressed as µg glucose released /min/g tissue.

Table 4: Activities of β-1, 3 glucanase in leaves and roots of mandarin following application of *G. mosseae* and *G. fasciculatum*

Glucanase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	212	113	200	105	198	123	215	118
Glomus mosseae	313	189	323	156	309	176	320	168
Glomus fasciculatum	252	179	263	163	261	175	250	173
G. mosseae + *G.fasciculatum*	302	191	346	170	304	169	321	198

Table 5: Peroxidase activity in leaves and roots of mandarin following application of *G. mosseae* and *G. fasciculatum*

Peroxidase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	89	29	78	30	88	36	76	32
Glomus mosseae	110	64	102	61	120	71	116	72
G. fasciculatum	101	40	105	45	106	42	98	50
G. mosseae + *G.fasciculatum*	135	68	129	70	131	72	140	73

Results and discussion

The benefits and wide host range of AM fungi has led to it being used as a bioinoculant to improve plant nutrition and growth. This study focussed on the use of AM fungi, its application and the quantification of the increased defence related enzymes responsible for disease resistance and subsequently improving plant health status. Arbuscular Mycorrhizal fungi from the selected places of hills (Kalimpong, Mirik, Bijanbari) and foothills were screened from the rhizosphere of mandarin plant. On observation it was found that *Glomus mosseae* dominated the AM population in all the soil samples followed by *G. fasciculatum.* Results are presented in Fig 2. Percentage of AM spores determined from different regions showed maximum of different *Glomus* sp., followed by *Gigaspora* sp., *Acaulospora* and *Scutellospora.* Histopathological study revealed the presence of vesicles and arbuscules in the root segments determining the fact that infection of the AM spores has taken place (Fig 1). Organisms of AMF have a bimodal pattern of differentiation (Morton, 1990). The vegetative thallus consists of arbuscules, intraradical vesicles (shared only by species in the suborder Glomineae), extraradical auxiliary cells (shared only by species in the suborder Gigasporineae), and intraradical and extraradical hyphae (Smith and Read, 1997; Morton and Benny, 1990). Arbuscules are finely branched structures in close contact with the cell plasma membrane, functioning in exchange of nutrients between host and fungal cells (Smith and Read, 1997). Hyphae are important in nutrient acquisition and as propagules to initiate new root colonization (Graham et al., 1982; Friese and Allen, 1991). Vesicles are globose structures arising from swelling of the hyphae and filled with glycogen granules and lipids and are considered to be storage structures (Bonfante-Fasolo, 1984; Brundrett, 1991).The different types of spores which were observed in the rhizosphere of mandarin soil have been identified and described as shown in Table 2. Spore colour, shape and size are the most prominent factors in their identification. The hyphae of each genus also differs in their morphology and number of wall layers.

Scanning Electron Microscopy (SEM) (Fig 3) of the spores revealed the spore wall morphology. *Glomus mosseae* has a rough outer surface. The outer layer is sloughed. Spores of *G. fasciculatum* are found in aggregates of 2-6. The surface is rough and shape is subglobose. The wall of *Gigaspora gigantea* is smooth walled with the typical bulbous suspensor. The polygonal reticulum on the spore wall surface is characteristic of *Acaulospora reticulata.*

Total phosphate content of soil had decreased due to application indicating that the plant could uptake phosphorus which had been solubilized by AMF (Table 3). Dual application of *G. mosseae* and *G. fasciculatum* was more effective in solubilising the insoluble phosphate present in the soil than when applied singly. This study aimed at improving the understanding of AM fungal interactions in the rhizosphere using a field trial which was to an extent successful. Application of *G. mosseae* in the rhizosphere of citrus plants led to an increase in the growth of seedlings in terms of increase in height and number of leaves. Joint inoculation with both the microorganisms (*G. mosseae* and *T. hamatum*) gave most significant results (Allay and Chakraborty, 2010). *B. pumilus* along with *G. mosseae* could improve seedling growth in terms of height and leaf number and also helped in solubilising phosphate, suggesting a synergistic effect (Chakraborty *et al,* 2011). Fresh shoot

Table 6: Chitinase activity in leaves and roots of mandarin following application of *G. mosseae* and *G.fasciculatum*

Chitinase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	88	80	86	75	89	77	92	81
Glomus mosseae	106	94	105	93	98	99	110	101
G. fasciculatum	89	83	93	91	101	82	102	85
G. mosseae + *G. fasciculatum*	133	97	124	95	126	98	145	100

biomass was also found to be increased when tomato plants were treated with *Glomus mosseae, Acaulospora laevis* and *Trichoderma harzianum* (Tanwar *et al,* 2010).

The pH of soil varied from 4.5 to 6. The pH of Kalimpong soil is around 6.0 which is ideal for citrus plants (Table 1). Not much difference was seen in the availability of carbon and nitrogen respectively in the 4 different regions. The moisture content of Kalimpong soil is also the highest. Citrus trees prefer draining sandy loam soil hence the amount of sand in all the soil was high.

Activities of 3 defense enzymes- chitinase, 1,3 - β glucanase and peroxidase were assayed in leaves and roots of mandarin seedlings subjected to various treatments- i.e., *G. mosseae*, *G. fasciculatum* and *G. mosseae* + *G. fasciculatum*. Activities of all 3 enzymes, in both leaves and roots, were significantly enhanced due to the various treatments (Table 4, 5 & 6). The most significant was in dual application of AMF in all the cases. In an earlier study, when mandarin seedlings were pre-treated either with *B. pumilus* or *G. mosseae* prior to challenge inoculation with the pathogen (*Fusarium oxysporum*), activities of all three defense enzymes increased significantly (Chakraborty *et al,* 2011).

The overall results of the present study have shown that *G. mosseae* and *G. fasciculatum* can promote growth of mandarin plants. However, *G. mosseae* was found to be the best colonizer as well as responsible for induced accumulation of defense enzymes in the host plant.

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In-vitro free radical scavenging activities of the leaves of *Malva verticillata* L.

Prashan Kumar Chhetri and Palash Mandal*

Plant Physiology and Pharmacognosy Research Laboratory, Department of Botany, University of North Bengal, Siliguri 734 013

Abstract

Antioxidants act as major defense against radical-mediated toxicity by protecting against the damages caused by free-radicals. Research on herbal products are increasingly focused on their effects on scavenging of different newly generated free-radical species and associated oxidative stress mediated complications on human health, but there are unexpectedly few studies evaluating the bioactivity of edible leafy vegetables of North Bengal, India. The purpose of the present study was to investigate the *in vitro* antioxidant potential of methanol extract and aqueous decoction of *Malva verticillata* L. [MV] leaves, consumed by local people of North Bengal. Extracts of leaves were analyzed for *in vitro* free radical scavenging capacity, the total phenol and flavonoid content and preliminary phytochemical analysis. The antioxidant property was estimated using reducing power, superoxide radical scavenging activity and DPPH· assays. Methanol extract of leaves were found to be effective in DPPH· and superoxide radical scavenging activity when compared with aqueous decoction of MV. Overall strong correlation between the mean values of total phenol content and IC_{50} values of DPPH and superoxide free radical scavenging capacity was observed. Principle Component Analysis (PCA) indicated that phenolic functional groups and reducing potential of methanol and aqueous extracts were mostly contributed for their antioxidant capacity. The present study revealed that methanol extract of the leaves of MV comprise effective source of natural antioxidants, which might be helpful in preventing the progress of various oxidative stress induced diseases.

Keywords: *Malva verticillata*, Antioxidant, DPPH, reducing power, Photochemical

The role of free radicals in many diseases has been well established. Several biochemical reactions in our body generate reactive oxygen species (ROS) that are capable of damaging crucial biomolecules (Kumaran & Karunakaran, 2006). ROS such as hydroxyl, superoxide and peroxyl radicals are formed in human tissues during metabolic operations which cause extensive oxidative damage that leads to age-related degenerative conditions, cancer, and a wide range of other human diseases (Aruoma, 1999; Reaven & Witztum, 1996). In recent years, one of the areas of therapeutic research which have been fascinated with great deal of attention is antioxidant, especially in the control of degenerative diseases in which oxidative damage has been implicated. Antioxidants play an important role in the protection of the human body against damage by free radicals and always maintain homeostatic balance with pro-oxidants inside cellular compartments (Lollinger, 1981).

Many plants contain substantial amount of antioxidants which can be utilized to scavenge the excess free radicals. The protection offered by different edible plants against oxidative stress in several diseases has been attributed to various antioxidants and vitamins. Potential antioxidant properties of the dietary phenolic compounds and flavonoids present in various fruits and vegetables have recently been recognized in a number of investigations (Mondal *et al.*, 2008; Bhaumik *et al.*, 2008). A great number of aromatic, spicy, medicinal and other plants contain chemical compounds, exhibiting antioxidant properties. Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt and Hudson, 1990). Many of these antioxidant compounds possess antiinflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Halliwell, 1994; Mitscher *et al.*, 1996; Owen *et al.*, 2000; Sala *et al.*, 2002).

Solvent extraction is most frequently used technique for isolation of herbal antioxidant compounds. However, the extractive yields and resulting antioxidant activities of the plant materials are strongly dependent on the nature of extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent. Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix. The most suitable of these solvents are (hot or cold) aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (Peschel *et al.*, 2006).

The plant *Malva verticillata* L. (Malvaceae) is

*Corresponding author:
E-mail: nbubotanypalash@rediffmail.com

commonly known as musk mellow or mellow in Korea and China. The plant has been traditionally used as stomach ailment, anticomplementary, hypoglycemic and postpartum fever in India, China and Korea. (Buragohain 2011; Tomado *et al.*, 1992; Jeong & Song 2011). In India, it is widely cultivated throughout temperate Himalayas from Punjab eastwards to Assam. In West Bengal, it is commonly known as 'Lapha' or 'Laffa' and its young tender twigs are cooked as vegetable (Bandyopadhyay and Mukherjee 2009).

A review of the literature did not throw ample light on the antioxidant and phytochemical study of this plant. The present study was therefore carried out to investigate the antioxidant potential of methanol extract and aqueous decoction from the leaves of MV in different *in vitro* models along with qualitative and quantitative analysis of active secondary metabolites for determining their relationship with antioxidants.

Materials and methods

Plant material

The plant material was purchased from the local markets of Shivmandir, West Bengal, India on 8th February 2011. Taxonomic position was authenticated by the Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The material has been deposited in the 'NBU Herbarium' and recorded against the accession number 9622 (Figure 1).

Figure 1: Herbarium specimen of *Malva verticillata* L

Chemicals

Methanol, (2, 2-diphenyl-1-picryl hydrazyl) DPPH˙, nicotinamide-adenine dinucleotide reduced form (NADH), phenazine methosulphate (PMS), potassium ferricyanide ($K_3[Fe(CN)_6]$), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), Folin-ciocalteu reagent, sodium carbonate (Na_2CO_3), nitro blue tetrazolium (NBT), sodium nitrite ($NaNO_2$), Griess reagent, aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), sodium nitroprusside, sodium sulphate (Na_2SO_4), lead acetate, 95% ethanol, 50% hydrochloric acid (HCl) and chloroform were either purchased from Himedia-BDH or Merck, Germany. All chemical and solvents used for experiments were of analytical grade.

Extraction

The leafy parts of MV were first separated from the young twigs. 20 g of leaves were separately crushed using mortar and pestle. After crushing, leaves were extracted separately with hot water and methanol in conical flask for 2 hours. The supernatants of refluxed samples were isolated from the residues by filtering through Whatman No. 1 filter paper. The excess solvent was removed by evaporation at 30° C and final concentration was taken as 500 mg/ml. 1ml of filtrates were dried *in vacuo* through lyophilization and their total extractive values were calculated on dry weight basis by the formula:

$$\text{yield}(\%) = \frac{\text{Wt. of dry extract}}{\text{Wt. taken for extraction}} X100$$

The samples were then kept in freeze for further use.

Extracts of each solvent were evaporated similarly under reduced pressure and final residues were used for assessment of antioxidant activity.

DPPH *free radical scavenging assay*

The free radical scavenging activity of the extracts was determined by Blois (1958). The leaves extracts were measured in terms of hydrogen donating or radical scavenging ability using a stable radical DPPH˙. 0.2ml of plant extracts prepared as various concentrations (5–500 mg/ml) were added to 2 ml of the methanol solution of 0.2mM DPPH, and the mixture was vortexed vigorously. The tubes were then incubated at room temperature for 30 minutes in dark, and the absorbance was taken at 517nm by UV-VIS spectrophotometer (Systronics, 2201). Ascorbic acid was used as reference. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH scavenging (\%)} = [(A_{control} - A_{sample})/ A_{control} \times 100]$$

Where; $A_{control}$ is the absorbance of DPPH radical + methanol; A_{sample} is the absorbance of DPPH radical + sample extract /standard

Linear regression analysis was used to calculate IC_{50} values wherever needed. IC_{50} value shows the amount of each extract needed for 50% inhibition of free radicals.

Table 1: Total phenol and flavonol content of MV leaf extracts

Sample	Concen-tration	TPC(mg/g FWT)	TFC(µg/g FWT)
Aqueous decoction	100mg/ml	0.53	25.3
Methanol	100mg/ml	1.73	7.2

TPC=Total phenol content, TFC=Total flavonoid content

Superoxide anions scavenging activity

The superoxide anions generated by PMS and nicotinamide-adenine dinucleotide reduced form (NADH), were detected by the reaction with 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-(3, 3′-dimethoxy-4, 4′-diphenylene) di-tetrazolium chloride (nitro blue tetrazolium-NBT). (Nishikimi, *et al.* 1972). Reaction mixture contained 1 ml samples (different concentration), 1 ml of NBT solution (312µM prepared in phosphate buffer, pH-7.4) and 1ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4). Finally, the reaction was accelerated by adding 100 µL PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixture was incubated at 25° C for 5 min and absorbance at 560 nm was measured against methanol as control. IC_{50} values were calculated by using the same analytical techniques mentioned above.

Reducing power assay

One milliliter of leaf extracts, 2.5 ml sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 ml K_3 [Fe (CN) $_6$] (1% w/v) were incubated at 50°C for 20 minutes. The tube was cooled on ice and 2.5 ml 10% TCA was added. The mixture was centrifuged at 3000 rpm for 10 minutes and the upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.25 ml of $FeCl_3$ (0.1% w/v). Finally, the absorbance was measured at 700nm. Phosphate buffer (pH=6.6) was used as blank solution (Aiyegoro and Okoh, 2009). Increased absorbance of the reaction mixture indicated superior reducing power.

Table 2: Phytochemical analysis of MV leaf extracts

Test	Aqueous decoction	Methanol
Steroid	++	+
Alkaloid	-	-
Cardiac glycoside	++	+
Flavonoid	++	+
Tannin	+	++
Glycosides	+	+
Saponin	+	+
Triterpenoid	-	-

+= Average, + =Minimum activity, - = No activity

Total phenol content

Total phenolic compounds of leaves extracts were determined by Folin-Ciocalteu method. (Folin and Ciocalteu, 1927). For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-Ciocalteu reagent (10 times diluted) and 4 ml Na_2CO_3 (75 g/L). The tubes were vortexed and allowed to stand for 30 min at 40 °C for colour development .The absorbance at 765 nm was measured after 1 hr. at 20 °C and the calibration curve was drawn. 1 ml plant extracts of various concentration was mixed to the same reagent and the mixture was incubated for one hour in room temperature. After 1 hour the absorbance was measured at 765nm.

Total Flavonoids Content

The total flavonoid content was determined using a spectrophotometric aluminium chloride method (Sultana *et al.*, 2009). 0.5 ml of the sample extracts (0-500 mg L^{-1}) (Quercetin in case of standard) were mixed separately with 4 ml of distilled water in a test tube, followed by the addition of 0.3 ml of 5% $NaNO_2$ solution. After 6 minutes, 0.3 ml of 10% $AlCl_3$ solution was added and the mixture was allowed to stand for 5 minutes before the addition of 2 ml of 1 M NaOH solution. About 2.4 ml of distilled water was finally added and the absorbance was measured immediately at 510 nm.

Phytochemical evaluation of the crude extracts

Test for steroid

0.5ml leaves extracts were evaporated and dissolved in 2ml chloroform. 2ml of conc. H_2SO_4 was introduced carefully by the side wall of the test tube. Formation of red colour ring confirmed the presence of steroid (Kumar *et al.*, 2009).

Test for triterpenoids

0.5 ml of leaves extracts were evaporated and dissolved in 1ml chloroform. 1ml acetic anhydride was then added and chilled. After cooling, conc.H_2SO_4 was added. If

Table 3: Correlation co-efficient matrix for different antioxidants and phytochemical parameters

	DPPHm	SOm	RPm	TPCm	TFCm	DPPHw	SOw	RPw	TPCw
SOm	0.727								
RPm	0.935*	0.885*							
TPCm	0.971**	0.835	0.943*						
TFCm	0.992**	0.713	0.913*	0.978**					
DPPHw	0.656	0.994**	0.829	0.786	0.648				
SOw	0.897*	0.948*	0.962**	0.964**	0.897*	0.917*			
RPw	0.874	0.957*	0.966**	0.943*	0.872	0.927*	0.996**		
TPCw	0.955*	0.832	0.930*	0.996**	0.972**	0.788	0.963**	0.945*	
TFCw	0.977**	0.794	0.931*	0.996**	0.989**	0.741	0.945*	0.923*	0.996**

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

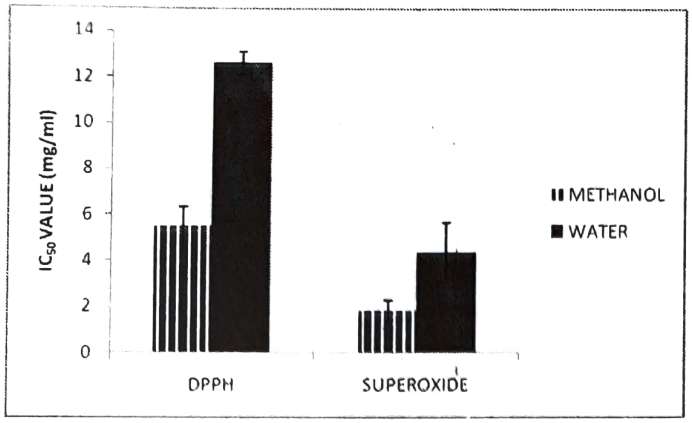

Figure 2: DPPH and Superoxide radical scavenging activity of MV leaves in aqueous decoction and methanol extract

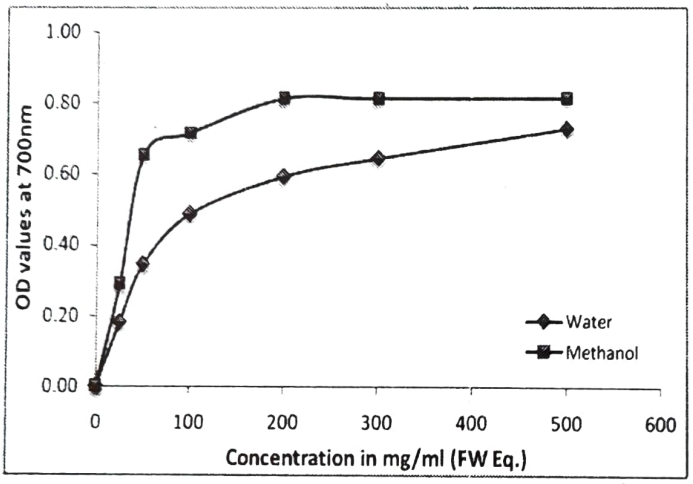

Figure 3: Reducing power activity of MV leaves in aqueous decoction and methanol extract

reddish violate colour appeared, the existence of triterpenoids was confirmed (Kumar *et al.*, 2009).

Test for cardiac glycoside

0.5 ml of leaves extracts were evaporated and dissolved in 1ml glacial acetic acid. One drop of 10% $FeCl_3$ was then added. 1ml of conc.H_2SO_4 was added by the side of the test tube. Appearance of brown colour ring at the interface indicated of presence of cardiac glycosides (Ngbede *et al.*, 2008).

Test for Flavonoids

To 1 ml of leaves extract, few drops of 10 % $FeCl_3$ solution were added. A green or blue colour indicated the presence of phenolic nucleus (Brain and Turner, 1975).

Test for tannin

0.5 ml leaves extract was added with 0.5 ml 1% lead acetate; a yellow colour precipitation indicated the presence of tannin (Kumar *et al.*, 2009).

Test for glycosides

0.5 ml leaves extract were added with 2ml of 50% HCl. The mixtures were hydrolyzed for 2 hrs on a water bath. After that 1ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% NaOH solution were added. Pink to red colour designated the presence of

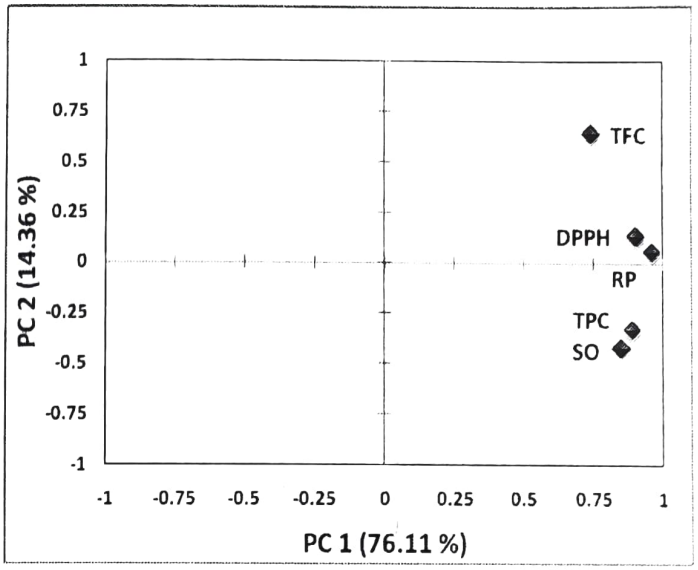

Figure 4: Principle component analysis with DPPH, SO, RP, TPC and TFC

glycosides (Kumar *et al.*, 2009).

Test for Saponins

2 ml of double distilled water was added with 1 ml of each leaves extract. Few drops of olive oil were added and agitated. Formation of soluble emulsion indicated the presence of saponin (Ngbede *et al.*, 2008).

Statistical analysis

The values are means of triplicate analysis of the samples (n=3) and were subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00) for drawing the relation between phytochemical properties and antioxidant attributes and MS Excel of Microsoft Office, 2007 was used for comparing the antioxidant attributes of methanolic and aqueous decoction extract of MV leaves. Smith's Statistical Package (Version 2.5) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE). In order to examine and visualize relationships between different phytochemicals and antioxidant traits, a principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1).

Results and Discussion

In this present study, MV leaves were evaluated for their antioxidant potential, considering the fact that antioxidant activity of the plant material is strongly dependent on the nature of extracting solvent due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent. The extractive values are useful to evaluate the chemical constituents present in the plant materials and also help in estimation of specific constituents soluble in a particular solvent (Ozarkar, 2005). According to Hsu *et al.*, 2006 the differences in the extractive values for the tested plant materials in different solvents might be due to the differences in the availability of extractable components, resulting from the varied chemical composition of plants. The extractive values obtained from aqueous

decoction of MV leaves and methanolic extract are 1.74% and 2.96% respectively. In our study, methanol solvent system was found to be more efficient in recovery of antioxidant compounds from MV leaves than aqueous decoction, thus offering higher extractive yield. Our findings suggest that higher extractive yield in methanol as compared to aqueous decoction may be due to more non-polar components in experimental plant sample. Similar result was found by Stanojevic *et al.* (2009) where the magnitude of extract yield of *Hieracium pilosella* leaf in methanol solvent system was higher than in water.

The methanolic and aqueous extracts were subjected to screening for their possible antioxidant activity. Four complementary test systems, namely DPPH free radical-scavenging, reducing power, and total flavonoid and phenol concentration, were used for the analysis.

DPPH has been extensively used as a free radical to evaluate reducing substances and is a useful reagent for investigating the free radical scavenging actions of compounds (Duan *et al.*, 2006). The scavenging capacity of aqueous decoction and methanol extracts of MV leaves were evaluated by measuring the decrease in DPPH radical absorption. The highest DPPH radical scavenging activity was detected in methanol extract (IC_{50} 5.4mg/ml) of MV leaves, when compared with aqueous decoction (IC_{50}12.6 mg/ml) (Figure 2). Methanol extract have greater antioxidant activity than the aqueous extracts may be due to the presence of the active less polar compounds in the leafy part of the vegetable that may dissolve only in the methanol, but not exactly in aqueous system. Similar result was found by Vadivelan *et al.*, (2009) where methanol extract of *Rubus ellipticus* root exhibited stronger radical scavenging activity than water, chloroform, ethyl acetate and petroleum ether.

In our study, the aqueous decoction and methanolic extracts of MV leaves were screened for their $O_2^{\bullet-}$ scavenging activity using PMS-NADH-NBT assay. In the PMS/NADH-NBT system, $O_2^{\bullet-}$ derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The effect of methanolic extracts of MV leaves was highest with IC_{50} value of 1.76 mg/ml than aqueous decoction with IC_{50} value of 4.33 mg/ml (Figure 2).

The reducing power was measured by the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power was increased by increasing the amount of extract and the reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al.*, 1995). The reducing power of the aqueous decoction and methanolic extracts of leaves was examined as a function of their concentration. The reducing capacity of aqueous decoction and methanolic extract is illustrated in Figure 3. The methanolic extract of MV leaves has more reducing power than aqueous decoction. The reducing ability of both extract was dose-dependent; the results indicated the presence of more hydrophobic phenolics in methanolic extracts, the values being directly correlated with reducing capacities.

The highest content of total phenol, total flavonoid and DPPH scavenging activities were found in methanol extracts of MV leaves. According to Ghasemzadeh *et al.*, (2010) it seems that the yield and efficiency of the phenolics extraction depends on the type and kind of the solvent as well as on the flavonoids, which is being isolated. For total phenolics and flavonoids extraction from ginger parts, methanol was found to be more efficient as compared to acetone and chloroform. Similar results were obtained from our study where methanol was found to be more effective for extracting phenolic compounds as these compounds are semipolar in nature and sometimes esterified with other conjugates. The higher total phenolic content of plants extracts resulted in higher antioxidant activity (Wong *et al.*, 2006). The total phenol contents of aqueous decoction and methanol extract of MV leaves were 0.53mg/g fresh weight tissue (FWT) and 1.73mg/g FWT respectively (Table 1).

Flavonoids are large family of polyphenolic components synthesized by plants. It was found that flavonoids functioned to reduce blood-lipid and glucose and to enhance human immunity (Atoui *et al.*, 2005). Flavonoids were also a kind of natural antioxidant capable of scavenging free superoxide radical, anti-aging and reducing the risk of cancer. The total flavonoid contents of aqueous decoction and methanol extract of MV leaves were 25.3µg/g FWT and 7.2µg/g FWT respectively. According to Winston (1999) , the leafy part of the vegetables contain the active component which consist of the flavonoid, terpenoid, lignan, sulphide, polyphenol, carotenoid, caumarin, saponin, curcumin and sterol. Aqueous decoction of MV leaves and methanolic extracts were also evaluated for semi-quantitative determination of major phyto-constituents i.e. steroid, alkaloid, cardiac glycoside, flavonoids, tannins, glycosides and saponin. The results of phytochemical analysis of MV leaves are presented in Table 2. Phytochemical screening of the MV leaves revealed some differences in the phytochemical constituents extracted in methanol and hot water solvent system. Phytochemical screening showed the presence of steroid, cardiac glycosides, flavonoid, tannin, glycosides and saponin in both aqueous decoction and methanolic extract of MV leaves whereas negative result was observed for alkaloid and triterpenoid test for both MV extracts.

Recently several authors have evaluated the relationship between antioxidant activity and defensive secondary metabolites like phenolics and flavonoids (Zhou *et al.*, 2006; Ninfali *et al.*, 2005; Aires *et al.*, 2011). In this study, Pearson's correlation coefficient was analyzed to determine the relationship between free-radical scavenging activity and metabolic components of MV. As shown in Table 3, overall strong correlation between the mean values of total phenol content and IC_{50} values of DPPH and superoxide free radical scavenging capacity was observed. Total flavonol content of both aqueous and methanolic extracts were also highly correlated with antioxidant activity. From Table 1,

reducing potential of both methanol and aqueous extracts were observed to be correlated with DPPH and superoxide radical scavengers. This could be explained from the basic concept that antioxidants are reducing agents and are capable of donating a single electron or hydrogen atom for reduction. However, not all reducing agents are antioxidants. Our investigation shows that reducing potential of MV were more associated with superoxide scavenging than DPPH'; indicating that compounds present in the polar extracts, capable of reducing DPPH radicals were also able to reduce ferric ions. A strong correlation between DPPH radical scavengers and reducing potential was determined with edible tropical plants (Wong *et al.*, 2006). Significant correlations were also found between total phenol content and ferric reducing antioxidant power in different genotypes of mulberry (Ozgen *et al.*, 2009). These results seem to confirm the idea that herbal polyphenols in fact are very important as free radical scavengers and extractable with polar solvents like methanol or water.

Principle component analysis (PCA) was performed in the classification of antioxidant activities of selected plant extracts (Wong *et al.*, 2006). In present investigation, PCA was performed to understand how the five parameters, namely, DPPH free radical scavenging ability (DPPH'), superoxide scavenging (SO), ferric ion reducing power (RP), total phenol (TPC) and flavonol content (TFC) contribute to antioxidant activity of plant extracts. The loadings plot (Figure 3) was used to gain an overview of the significance among antioxidant assays and the quantity of phytochemicals. The loadings of first and second principal components (PC1 and PC2) accounted for 76.11 and 14.36% of the variance, respectively. The most significant component, RP contributed the largest variation of approximately 24%, while the DPPH and TPC accounted for approximately 21% each of the total variation on PC1. Conversely, TFC was the most emphasized parameter (57% approximately) of PC2. But interestingly, superoxide did not contribute much on these two components, as it only indicated the ability of these extracts to control superoxide radicals under crisis condition, and is not directly responsible for antioxidant capacity in normalized system. Figure 3 shows that all five parameters were positively loaded on PC1 with significant quantity (squared cosine of variables are from 0.925 to 0.552), whereas in PC2 only TFC exhibited high quantum of positive loading. The results from PCA suggested that DPPH & RP may act with same components, as they are present in one cluster, while the other cluster having TPC & SO contributed in same fashion. From PCA score, it may also be proposed that TFC may be more useful marker of antioxidant activity in the leaves of MV, as the existence of one or more hydroxyl groups in A & B ring of flavonoids may directly involve in the transfer of hydrogen atom or an electron for detoxification of free radicals during oxidative stress.

Conclusion:

The results of this study revealed that the leaves of MV possess potential antioxidant activity. However, extracting solvent significantly affected antioxidant activity of MV leaves. Methanolic extracts of leaves exhibited stronger antioxidant activity in comparison to the aqueous decoction of MV leaves. It seems that yield and efficiency of the natural antioxidants depend on the type and kind of the solvent used. For total phenolics extraction from MV leaves, methanol was found to be more efficient compared to aqueous decoction but significantly for flavonoid extraction, aqueous decoction was more effective. The present data would certainly help to establish the effectiveness of the tested plant material as a potential source of natural antioxidants to be used for nutraceutical and functional food applications. Further research is required to identify individual components forming antioxidative system which could be exploited as cost effective food addictives for human health.

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Evaluation of genetic variation among three species of *Allium* on the basis of karyomorphology and SDS-PAGE profiling

Subhas Chandra Roy* and Tshering O Bhutia
Plant Genetics & Tissue Culture Laboratory Department of Botany, University of North Bengal, PO- NBU, Siliguri-734013 WB, India

Abstract
Determining the base number, ploidy level and type of ploidy are important aspects in the cytogenetic study of a species. Genetic variation among the three species of the genus *Allium* (*A. cepa*, *A. sativum* and *A. hookeri*) has been carried out on the bases of chromosomal karyotype and protein banding patterns on SDS-PAGE. Protein profiling was prepared from the observed bands on SDS-PAGE after electrophoresis and staining with coomassie brilliant blue. Shoot protein was extracted in phosphate buffer (pH 7.5) and run on gel for 3 hours at 20 mA constant current. Band numbers were varies from 31 to 39 in different position on the gel according to their *Rf* value. Bands were scored as binary data and computed in MVSP software for dendrogram analysis to examine their genetic relationship using Jaccard's coefficient. Three species were grouped into three distinct clusters in the dendrogram. Species *cepa* and *sativum* was grouped at the similarity coefficient value of 0.53 and species *hookeri* was placed in separate group at coefficient level of 0.40. Genetic variation was also examined on the basis of PCA analysis. The number of chromosome were found to be present 16 in *Allium cepa*,16 in *Allium sativum*, and 22 in *Allium hookeri*. The shortest chromosome in *Allium cepa* was 8.75(µm) and the longest one was 23.12(µm) with the ratio of shortest/longest chromosome of 0.37, the mean chromosome length of 15.23(µm) and a mean centromeric index of 0.16%. The shortest chromosome in *Allium sativum* was 10.62(µm) and the longest one was 30.0 (µm) with the ratio of shortest/longest chromosome of 0.35, the mean chromosome length of 17.41(µm) and a mean centromeric index of 0.60%. The shortest chromosome in *Allium hookeri* was 5.62 (µm) with the ratio of shortest/longest chromosome of 0.21, the mean chromosome length of 16.58(µm) and the mean centromeric index of 0.46%. *Allium hookeri* is considered to be much more advanced than the rest of the species (*Allium sativum*,and *Allium cepa*) because karyotype of the *A. hookeri* was ranges from submedian to telocentric chromosome, which is evolutionarily advance characters.

Keywords: *Allium* sp, chromosome karyotype, ideogram, SDS-PAGE, Dendrogram, PCA analysis

The genus *Allium* is widely distributed over the region from the dry sub- tropic to the boreal region. Evolution of the genus has been accompanied by ecological diversification. The majority of the species grow in open, sunny, rather dry sites in arid and moderately humid climates. The onion is a variety of plants in the genus *Allium*, specifically *Allium cepa*. *Allium cepa* is also known as the "garden onion" or "bulb" onion. *Allium cepa* is known only in cultivation but related with other species occurs in Central Asia. The most closely *Allium* related species include *Allium vavilovii* (Popov and Vved) and *Allium asarense* (R.M.Fritsch and Matin) from Iran. The name *Allium sativum* is derived from the Celtic word 'all' meaning burning or stinging, and the Latin "*sativum*" meaning planted or cultivated. *Allium hookeri*, an important member of family Alliaceae subgenus Amerallium, is reported in the wild from Ceylon, Greece, Yunnan, Southern China, Bhutan, Sri Lanka and India (Hooker 1892). Cytological study has been carried out by some other workers using different Alliums as plant material (Sharma *et al.*, 2011 and Sen, 1974). In India, plants of this species are commonly found in forest margins and meadows of the Khasi hills in Northeastern Himalaya and upper gangetic plains (Pandey *et al.*, 2008). Because of characteristic flavor of genus Allium and some therapeutic properties, these plants are used by the locals, Khasi tribals in particular, for seasoning dishes, treating cough and cold, healing burn injuries and wounds (Kala, 2005). Since leaves and fleshy roots of this species are also consumed as vegetables, it has good marketability in areas of its common occurrence (Pandey *et al.*, 2008). Estimates of genetic relatedness are important in designing plant improvement programmes. Information on genetic diversity is also valued for the management of germplasm and for designing conservation strategies. Molecular markers are the best tools for determining genetic relationships between different species of the same genus.

The main objective of this present investigation was to study the genetic diversity among the three species of *Allium* on the basis of plant protein profiling through SDS-PAGE polymorphisms in combination with karyomorphology.

*Corresponding author:
E-mail: subhascr@rediffmail.com

Table 1 :Karyomorphological data of *Allium cepa*

Chromosome number	Long arm (μm)	Short arm(μm)	Chromosome length(μm)	F%	Classi-fication	Centromery index	Mean arm ratio
1	11.25	6.87	18.12	37.91	nsm	0.61	1.63
2	11.25	6.87	18.12	37.91	nsm	0.61	1.63
3	12.5	7.50	20.0	37.5	nsm	0.60	1.66
4	12.5	7.50	20.0	37.5	nsm	0.60	1.66
5	5.0	3.75	8.75	42.85	nm	0.75	1.33
6	11.25	7.50	18.75	40.0	nm	0.66	1.50
7	8.125	2.5o	1o.62	23.52	nsm	0.30	3.25
8	5.0	3.75	8.75	42.85	nm	0.75	1.33
9	5.0	3.75	8.75	42.85	nm	0.75	1.33
10	5.0	3.75	8.75	42.85	nm	0.75	1.33
11	11.25	7.50	18.75	40.o	nm	0.66	1.50
12	10.0	8.75	18.75	46.66	nm	0.87	1.14
13	10.0	8.75	18.75	46.66	nm	0.87	1.14
14	8.75	2.50	11.25	22.22	nsm	0.28	3.5o
15	16.25	6.25	22.5	27.77	nsm	0.38	2.6o
16	16.25	6.87	23.12	29.71	nsm	0.42	2.36
Total	159.375	94.36	253.73			0.61	1.82
		Mean=	15.85				

Table 2:Karyomorphological data of *Allium sativum*

Chromosome number	Long arm (μm)	Short arm(μm)	Chromosome length (μm)	F %	Classification	Centromery index	Arm ratio
1	13.75	6.87	20.62	33.31	nsm	0.49	2.0
2	9.37	5.62	14.99	37.49	nsm	0.59	1.66
3	9.37	5.62	14.99	37.49	nsm	0.59	1.66
4	13.75	12.5	26.25	47.61	nm	0.90	1.10
5	13.75	12.5	26.25	47.61	nm	0.90	1.10
6	17.5	12.5	30.0	41.66	nm	0.71	1.40
7	17.5	11.87	29.37	40.41	nm	0.67	1.47
8	13.75	6.87	20.62	33.31	nsm	0.49	2.0
9	7.5	3.12	10.62	29.37	nsm	0.41	2.41
10	8.12	5.0	13.12	38.10	nsm	0.61	1.62
11	8.12	3.75	11.87	31.59	nsm	0.46	2.16
12	9.37	3.75	13.12	28.58	nsm	0.40	2.49
13	8.12	4.37	12.49	34.98	nsm	0.53	1.85
14	7.5	3.12	10.62	29.37	nsm	0.41	2.40
15	6.87	5.0	11.87	42.12	nm	0.72	1.37
16	6.87	5.0	11.87	42.12	nm	0.72	1.37
Total	171.21	107.46	278.67			o.60	1.75
		Mean=	17.29				

Table 3: Karyomorphological data of *Allium hookeri*

Material and Methods

Plant materials

Two species of *Alllium* were collected (*Allium cepa,* and *Allium sativum*) from different places of the NBU campus and *Allium hookeri* from Medicinal plant gardens of the Department of Botany, NBU.

Root tips for Karyomorphology

Root tips of *Allium cepa*, *Allium hookeri* and *Allium sativum* were pre-treated in saturated aqueous solution of p-dichlorobenzene at 4°c for 5 minutes, and then transferring it at 12-14°c for about 3-4 hours, and followed by fixation in 3:1 (v/v) solution of absolute ethanol: glacial acetic acid for overnight at room temperature. After that root tips were kept in 45 % acetic acid for 10-15 minutes followed by staining with 2% acetic-orcein and HCl(1N) mixture at the ratio of 9:1 and kept for 30 minutes for staining after heated at 60-65° C for 30-40 seconds. The stained root tips were macerated in 45 % (v/v) acetic acid on a grease free new slide. The well spread metaphase plates of each taxon were analysed. The camera-lucida drawing was done for karyotype and ideogram analysis.

Shoot protein for SDS-PAGE electrophoresis

SDS-PAGE was performed according to Laemmli (1970). Shoot tissues were used for the protein profiling purposes. One gram shoot tissue was taken from three samples *Allium cepa, Allium hookeri* and *Allium sativum.* Shoot tissues were crushed separately with 3 ml phosphate buffer (7.5 pH) in ice cold condition. The

Table 3: Karyomorphological data of *Allium hookeri*

Chromosome number	Long arm(μm)	Short arm (μ m)	Total length (μ m)	F%	Classification	Centromery ratio	Arm ratio
1	18.12	3.75	21.87	17.14	nst	0.20	4.85
2	18.12	3.75	21.87	17.14	nst	0.20	4.85
3	18.75	7.5	26.25	28.57	nsm	0.4	2.5
4	8.12	7.5	15.62	48.33	nm	0.92	1.08
5	6.25	5.0	11.25	44.44	nm	0.8	1.25
6	7.5	4.37	11.87	36.81	nsm	0.58	1.71
7	8.12	7.5	15.62	48.01	nm	0.92	1.08
8	3.75	1.87	5.62	33.27	nsm	0.49	2.00
9	9.37	1.87	11.24	16.63	nst	0.19	5.01
10	8.75	1.87	10.62	17.60	nst	0.21	4.67
11	3.75	1.87	5.62	33.27	nsm	0.49	2.00
12	4.37	3.12	7.49	41.65	nm	0.71	1.4.
13	18.75	1.8	20.55	8.75	st	0.09	10.41
14	18.75	7.5	26.25	28.57	nsm	0.4	2.5
15	7.5	4.37	11.87	36.81	nsm	0.58	1.71
16	8.75	1.87	10.62	17.60	nst	0.21	4.67
17	6.25	3.12	9.37	33.29	nsm	0.49	2.00
18	4.37	3.12	7.49	41.65	nm	0.71	1.40
19	6.25	5.0	11.25	44.44	nm	0.8	1.25
20	6.25	3.12	9.37	33.29	nsm	0.49	2.00
21	18.75	1.8	20.55	8.75	st	0.09	10.41
22	9.37	1,87	11.24	16.63	nst	0.19	5.01
Total	219.96	83.54	303.5			0.46	4.20
		Mean= 13.79					

Table 4: Comparison of chromosome properties of *Allium taxa*

Taxa	2n	S	L	S/L	Mean length	MCI	MAR	TF%	D.I	HCL
A. cepa	16	8.75	23.12	0.37	15.23	0.61	1.82	37.18	45.08	127.49
A. sativum	16	10.62	30.0	0.35	17.41	0.60	1.75	38.56	47.71	140.59
A. hookeri	22	5.62	26.25	0.21	16.58	0.46	4.20	27.52	64.63	151.75

2n=Chromosome number; S=short chromosome length; L=long chromosome length; MCI=Mean Centromery Index; MAR=mean arm ratio; all measurement are in μm

crude extract was filtered with cotton cloth. The crushed materials were then centrifuged at 10000 rpm for 10 minutes. Supernatant was collected in 1.5ml eppendorf tube. 25 μl 1X SDS sample buffer was mixed with 25 μl protein sample in the ratio 1:1. Then sample were heated for 3 minutes in water bath. 15 μl each of the samples were loaded into the well of the gel by micropipette. Electrophoresis was performed at 60-70 volt, 20-30 mA current for a period of about 3 hours till the tracking dye reach the bottom of the gel. Gel was stained overnight with 0.2% Comassie Brillant Blue. The gel was kept in destainer solution until clear blue bands were appeared on the gel. The gel was viewed over the illuminator, photographed for band analysis purposes.

Results and Discussion

Shoot protein profiling for genetic diversity analysis

Data Analysis

The presence (1) and absence (0) of each band was considered in each species of *Allium*. The binary data matrix was used to calculate Jaccard's similarity index for genetic diversity analysis. The genetic relationship among 3 species were analyzed, and viewed in Dendrogram using Multi Variate Stastical Package MVSP version 3.2 and Unweighted Pair Group Method of Arithematic Means (UPGMA). The numbers of band varied from 25-34 among the species, that of the *Allium sativum* was the maximum bands whereas the *Allium hookeri* was the minimum band numbers. Electrophoregram showing protein banding pattern of different species of *Allium* were given in Figure-1.

Cluster Analysis

According to the statistical analysis data of the present and absent of each band was used to construct Dendrogram based on Jaccard's similarity index. Dendrogram showed single cluster showing highest similarity between *A. cepa* and *A. sativa* at 0.53 coefficient value (Figure 2). The *Allium hookeri* was placed in the same cluster but at different claude at 0.40 coefficient value. Principal component analysis (PCA) also showing the same genetic diversity in compare to dendrogram in a graphical mode (Figure 3). The protein analysis through SDS-PAGE showed the presence of 34 bands out of which 7 bands were common (in case of *Allium cepa shoot, Allium sativum Shoot, Allium hookeri shoot*) while the other 27 were polymorphic.

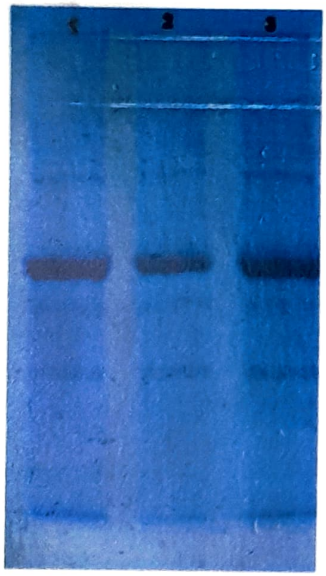

Figure 1: Electrophoregram showing protein banding pattern of different species of *Allium* genotypes (Lane 1;*A. cepa*, lane 2:*A; sativum* and lane 3 :*A. hookeri*)

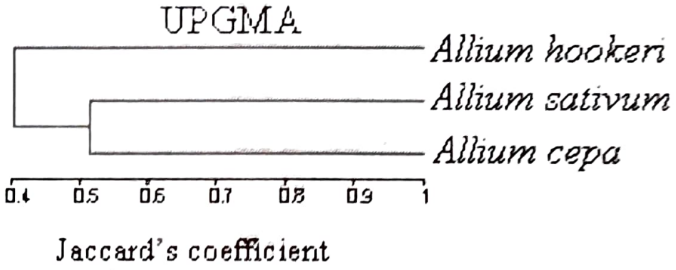

Figure 2. Dendrogram showing the genetic relationships among the 3 species of *Allium* on the bases of SDS-PAGE protein profiling using MVSP software in UPGMA algorithm

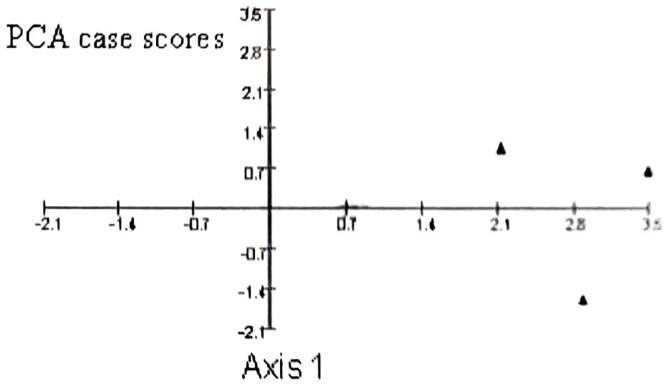

Figure 3. Principal component analysis (PCA) showing graphical representation of the genetic variation among the three species of *Allium* based on SDS-PAGE protein profiling

Dendrogram revealed that the *Allium hookeri* differ from *Allium cepa*, and *Allium sativum* at about coefficient value 0.40. Nearly 50% similarity was there between the *Allium cepa* and *Allium sativum*.

Karyomorphological analysis

The number of chromosome were found to be 16 in *Allium cepa*,16 in *Allium sativum*, and 22 in *Allium hookeri* (Figure 4 & 5). Chromosomes of all the investigated types were nearly submedian, nearly median, nearly sub-terminal, and terminal. Form percentage (F %) was recorded maximum (48.0) in *Allium hookeri*. Minimum form percentage (8.75) was recorded in *Allium hookeri*. The shortest chromosome in

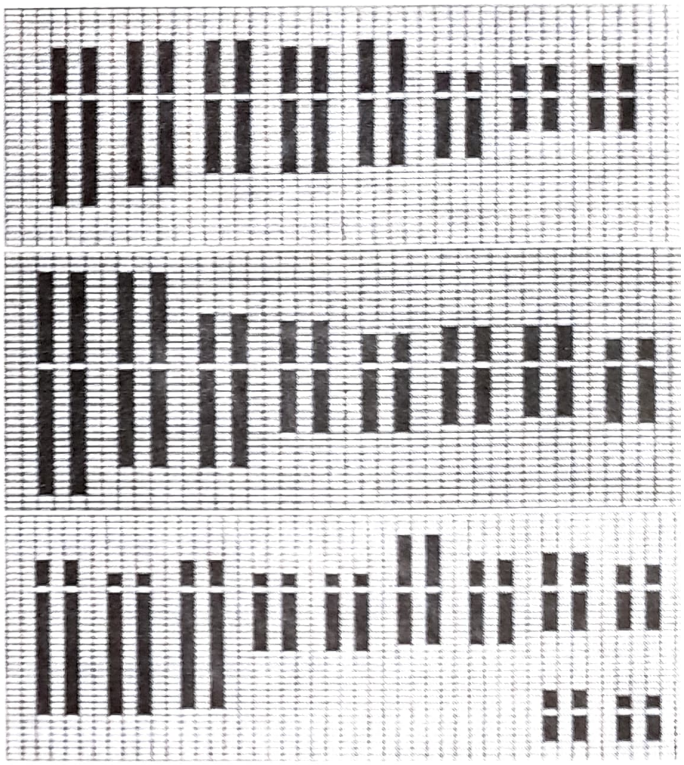

Figure 4: Ideogram of *Allium* taxa, top=*A. cepa*; middle= *A. sativum*; bottom= *A. hookeri*

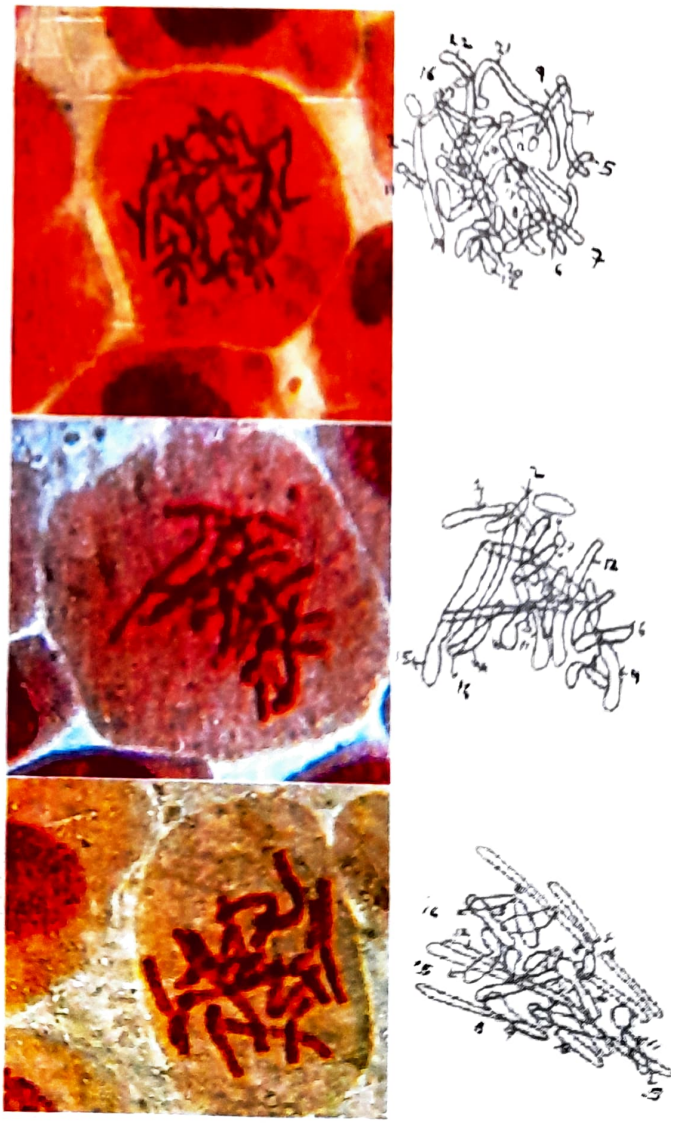

Figure 5: Showing the light micrograph of metaphase chromosome complement and free hand cameralucida drawing of three *Allium* sp. karyotype; top=*A. cepa*; middle= *A. sativum*; bottom= *A. hookeri*

Allium cepa was 8.75(μm) and the longest one was 23.12(μm) with the ratio of shortest/longest chromosome of 0.37, the mean chromosome length of 15.23(μm) and a mean centromeric index of 0.16%. The shortest chromosome in *Allium sativum* was 10.62 (μm) and the longest one was 30.0 (μm) with the ratio of shortest/longest chromosome of o.35, the mean chromosome length of 17.41(μm) and a mean centromeric index of 0.60%. The shortest chromosome in *Allium hookeri* was 5.62(μm) with the ratio of shortest/longest chromosome of 0.21, the mean chromosome length of 16.58(μm) and the mean centromeric index of 0.46% (Table 1, 2, 3 & 4).

Nomenclature for the centromeric positions of chromosome follows Levan *et al.* (1964) and the karyotype classification follows Stebbins (1971). The number of chromosome were found to be 2n = 16 in *Allium cepa*, 2n = 16 in *Allium sativum* and 2n= 22 in *Allium hookeri*. The total length of chromatin were found to be 253.73μm in *Allium cepa*, 278.67μm in case of *Allium sativum* and 303.5μm in case of *Allium hookeri*. Chromosome of all investigated types were nearly submedian, nearly median, nearly subterminal and terminal. Form percentage (F%) was recorded maximum (48) in *Allium hookeri*. Minimum form percentage (F%) was recorded in *Allium hookeri*. *Allium hookeri* has 2 sub-terminal chromosome and 6 nearly sub-terminal chromosomes and according to Stebbins species having greater number of submedian and telocentric chromosomes should be treated more evolved than those in which there are lesser number of sub metacentric and telocentric chromosome.

The above finding in the assessment of evolutionary tendencies suggested that *Allium hookeri* was much advanced than the rest of species, however *Allium sativum, Allium cepa* were much more primitive than *Allium hookeri* as their chromosome were nearly median and submedian type.

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ROS production, H_2O_2 detection and biochemical characterization of water stressed wheat (*Triticum aestivum* L.) varieties

B Pradhan and U Chakraborty*
Plant Biochemistry Laboratory, Department of Botany, University of North Bengal, Siliguri 734013, India

Abstract
One month old plant of four varieties (MW, KD, GY and GN) of wheat (*Triticum aestivum* L.) was taken and subjected to water stress for 3, 6 and 9 days. RWC was found to be higher in case of GY and KD when compared to MW and GY. There was an initial enhancement in the activities of all five tested antioxidative enzymes- peroxidase, ascorbate peroxidase, catalase, glutathione reductase and superoxide dismutase in K and GN varieties, while in MW and GY, the activity of catalase and superoxide dismutase showed a decline at all periods of water stress. Peroxidase and glutathione reductase activities increased even on 9th day of stress in K and GN, but all other activities showed a decline after 3 days of stress. The accumulation of H_2O_2 showed an increase with increasing days of water stress but in K and GN there was a decline during prolonged water stress. Lipid peroxidation increased significantly which was higher in case of MW and GY. With increase in the duration of water stress proline, phenol and ascorbate content increased. Higher values of MSI and total antioxidant were observed in the cultivar KD and GY with increase in the severity of water stress than in MW and GY. After an initial enhancement the content of carotenoid increased followed by a decline. Total chlorophylls showed a general decline during water stress, but the ratio of chla/b showed an initial increase in the 3rd day of water stress which declined during the latter stages of water stress. Results of the present study indicate that two of the varieties- MW and GY are susceptible to water stress, while the other two-K and GN is tolerant.

Keywords: antioxidative enzymes, Lipid peroxidation, phenol, ascorbate, MSI, carotenoid

Since plants have limited mechanisms of drought stress avoidance, they require flexible means of adaptation to changing drought conditions (Zhang et al 2004). Tolerance to this abiotic stress is a complex phenomenon, comprising a number of physio-biochemical processes at both cellular and whole organism levels activated at different stages of plant development. Both enzymatic and non-enzymatic antioxidants provide protection against oxidative damage (Munne-Bosch and Alegre, 2000). Various tolerance mechanisms have been suggested on the basis of the biochemical and physiological changes related to drought (Quartacci *et al.* 1994, Quartacci *et al.* 1995, Sgheeri *et al.* 2000).

Reactive oxygen species (ROS) are known as toxic metabolic products in plants and other aerobic organisms. An elaborate and highly redundant plant ROS network, composed of antioxidant enzymes, antioxidants and ROS-producing enzymes, is responsible for maintaining ROS levels under tight control (Gechev et al 2006). ROS, resulting from excitation or incomplete reduction of molecular oxygen, are unwelcome harmful by-products of normal cellular metabolism in aerobic organisms (Halliwell, 2006) Plants, facing an even greater burden of excess ROS, initially developed various protective mechanisms, such as small antioxidant molecules and antioxidant enzymes, to keep ROS levels under control (Van, 2001).

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

It is generally believed that maintaining a high reduced to oxidized ratio of ascorbic acid and glutathione is essential for the proper scavenging of ROS in cells. The ratio is mainly maintained by glutathione reductase (GR, EC 1.6.4.2) (Noctor and Foyer 1998; Asada 1999).

Moreover, it has been noted that the balance between ROS-scavenging enzymes is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide (Bowler *et al.* 1991). All cellular compartments are well-equipped with antioxidant enzymes and antioxidants. Therefore, ROS are normally scavenged immediately at the sites of their production by the locally present antioxidants. However, when this local antioxidant capacity cannot cope with ROS production (for example, during stress or temporarily reduced antioxidant levels due to developmental signals), H_2O_2 can leak into the cytosol and diffuse to other compartments. Plants can also deal with excess H_2O_2 by transporting it into vacuoles

for detoxification (Bienert, 2006 and Gould , 2002). Decline in the efficacy of the H_2O_2 decomposing system is probably responsible for the oxidative damage occurring in water stressed leaf (Baisak R et al,1994) This deficit has an evident effect on plant growth that depends on both severity and duration of the stress (Araus *et al.* 2002; Bartels & Souer 2004).

Considering the importance of water stress in crop production, the present work was undertaken to evaluate for wheat varieties on their tolerance and susceptibility

to the above stress in terms of defense responses.

Materials and Methods

Plant Material

Seeds of four varieties of wheat (*Triticum aestivum* L.) – Kedar (KD), MW, GY and GN were selected were initially surface sterilized with 0.1% (w/v) $HgCl_2$ for 3-4 minutes, washed with sterile distilled water and were then transferred to petriplates under aseptic conditions. These seeds were allowed to germinate in the petriplates for one week and then the seedlings were transferred to earthen pots. Plants were maintained in growth chamber at a temperature of 20-25^{0}C, Relative Humidity (RH) 65 -70%, 16 h photoperiod and irradiance of 400 μmol $m^{-2}s^{-1}$. One month old plants (taken as control or zero day treated) were subjected to water stress by withholding water completely for specific period and the sampling was done after 3, 6 and 9 days and after each period of water stress. Morphological changes were noted and relative water content (RWC) of leaves was determined as described by Farooqui *et al.* (2000) calculated by the following formula:-

Various other biochemical assays were then performed as given below.

$$RWC(\%) = \frac{\text{fresh wt.} - \text{dry wt.}}{\text{fully turgid wt.} - \text{dry wt.}} X100$$

Antioxidant enzyme extraction and assays

Extraction for enzymatic and isozymatic analysis

Leaves of wheat seedlings were homogenized in 5mL of ice-cold 50 mM sodium phosphate buffer, pH 7.2, containing 1% (w/v) polyvinylpolypyrrolidone using liquid nitrogen in a chilled mortar and pestle. The homogenate was then centrifuged at 6708 g for 20 min at 4°C and the supernatant was directly used as crude extract for enzyme assays. Protein contents in extracts were quantified following the method of Lowry *et al.* (1951), using BSA as standard.

Assay

Peroxidase (POX: EC. 1.11.17) activity was assayed in 4802 UV VIS spectrophotometer (Cole Parmer, USA) at 460 nm by monitoring the oxidation of o-dianisidine in presence of H_2O_2 (Chakraborty *et al.* 1993). Specific activity was expressed as mmol O-dianisidine mg $protein^{-1}$ min^{-1}.

Ascorbate peroxidase (APOX: EC.1.11.1.11) activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada and Takahashi (1987) with some modification. Enzyme activity was expressed as mmol ascorbate mg $protein^{-1}$ min^{-1}.

Catalase (CAT: EC.1.11.1.6) activity was assayed as described by Beers and Sizer (1952) by estimating the breakdown of H_2O_2 which was measured at 240 nm. The enzyme activity was expressed as as μmol H_2O_2 mg $protein^{-1}$ min^{-1}.

Glutathione reductase (GR: EC 1.6.4.2) activity was determined by the oxidation of NADPH at 340 nm as described by Lee and Lee (2000). Enzyme activity was expressed as μM NADPH oxidized mg $protein^{-1}$ min^{-1}.

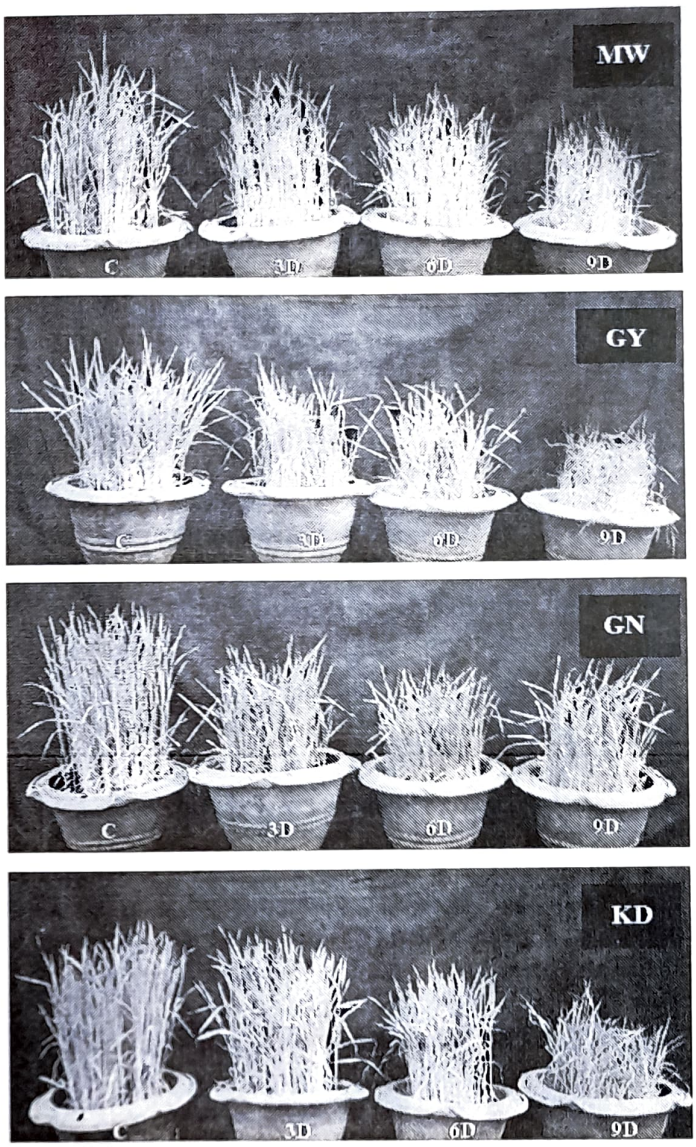

Fig 1. Different varieties of wheat seedlings subjected to water stress for 3, 6 and 9 days

Superoxide dismutase (SOD: EC 1.15.1.1) was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Dhindsa *et al.* (1981) with some modification. The absorbance of the samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

Isozymatic analysis of Peroxidase and Catalase

POX isoenzymes were separated on 7.5% non-denaturating acrylamide gel run at 10 mA for 4 h at 4°C following the method of Davis (1964). Isoforms of POX were visualized as bright blue bands by staining the gel in 50 ml solution containing 1.04% of Benzidine, 9% of acetic acid and 3% H_2O_2 as described by Reddy and Gasber (1971). After development of the bands the reaction was terminated by arresting the reaction by immersing the gel into a large volume of 7% acetic acid for 10minutes.

Quantification of H_2O_2 and in-situ detection of H_2O_2

Table 1. Effect of water stress on APOX, POX, GR, CAT & SOD in different wheat varieties

Varieties	Days of treatment	CAT	GR	SOD	APOX	PER
MW	0d	1.67 ±0.07[a]	0.14±0.02[a]	0.086±0.008[a]	0.116±0.007	0.020±0.006
	3d	1.43 ±0.02[b]	0.40±0.03[b]	0.073±0.006[a]	0.730±0.010	0.029±0.013
	6d	1.19 ±0.01[b]	0.33±0.01[b]	0.055±0.005[b]	0.263±0.006	0.038±0.015
	9d	1.09 ±0.02[b]	0.23±0.01[b]	0.041±0.004[b]	0.168±0.004	0.026±0.009
GY	0d	1.77 ±0.02[a]	0.18±0.03[a]	0.059±0.006[a]	0.089±0.005	0.023±0.005
	3d	1.46 ±0.04[b]	0.22±0.01[a]	0.055±0.005[a]	1.017±0.003	0.048±0.007
	6d	1.41 ±0.02[b]	0.15±0.01[a]	0.036±0.003[b]	0.385±0.004	0.051±0.006
	9d	1.27 ±0.01[b]	0.11±0.01[a]	0.027±0.002[b]	0.244±0.006	0.044±0.008
KD	0d	1.77 ±0.03[a]	0.13±0.01[a]	0.043±0.003[a]	0.189±0.011	0.045±0.007
	3d	2.76 ±0.06[b]	0.28±0.02[b]	0.068±0.002[b]	0.633±0.005	0.062±0.006
	6d	1.25 ±0.03[b]	0.76±0.04[b]	0.020±0.001[b]	0.067±0.009	0.095±0.007
	9d	1.13 ±0.02[b]	1.53±0.08[b]	0.015±0.002[b]	0.044±0.004	0.193±0.007
GN	0d	1.18 ±0.01[a]	0.17±0.01[a]	0.036±0.003[a]	0.065±0.004	0.035±0.010
	3d	2.19 ±0.03[b]	0.32±0.02[b]	0.061±0.003[b]	0.166±0.003	0.042±0.011
	6d	1.73 ±0.02[b]	0.51±0.03[b]	0.022±0.002[b]	0.107±0.004	0.072±0.009
	9d	1.50 ±0.04[b]	0.97±0.04[b]	0.013±0.001[b]	0.068±0.008	0.126±0.004

Enzyme activities is expressed as APOX= m moles ascorbate mg protein^{-1} min^{-1}, POX= mmol o-dianisidine mg protein^{-1}min^{-1}, GR= µmoles NADPH oxidized mg protein^{-1} min^{-1}, SOD= EU mg protein^{-1} and CAT= μmole H_2O_2 mg protein^{-1} min^{-1}; ± = S.E.; Different superscripts indicate values differ significantly at 1% in t test

The quantification of H_2O_2 levels in the leaves were done according to the method given by Jena and Choudhuri (1981). The intensity of yellow color was measured at 410 nm in the spectrophotometer and the H_2O_2 levels were calculated using extinction coefficient 0.28 µmol^{-1} cm^{-1}. *In-situ* detection of H_2O_2 was done following the method of Thordal-Christensen et al (1997) with minor modifications using diaminobenzidine (DAB). H_2O_2 was visualized as reddish-brown colour at the site of DAB polymerization. DAB polymerizes instantly and locally at sites of peroxidase activity into a reddish-brown polymer.

Determination of lipid peroxidation

MDA content, a measure of lipid peroxidation was determined by the thiobarbituric acid (TBA) reaction. The absorbance was measured at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Extraction and estimation of non-enzymatic antioxidants and DPPH based free radical scavenging activity

The extraction and estimation of carotenoids were following the method described by Lichtenthaler (1987). Extraction was done in methanol and the extract was filtered and the absorbance of the filtrate was measured at 480, 663 and 645 nm in a VIS spectrophotometer. The carotenoid content was calculated using a standard formula. Ascorbic acid was extracted and estimated following the method described by Mukherjee and Chaudhuri (1983).

Total antioxidant activity or DPPH based free radical scavenging activity was measured by following the method of (Blois 1958) and expressed as percent (%) inhibition of DPPH absorbance which was measured at 515 nm. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition % = (Absorbance at T_0 – Absorbance at T_{30}) / A T_0 X 100

Where, A T_0 was the absorbance of DPPH at time zero, and A T_{30} was the absorbance of DPPH after 30 min of incubation. Total antioxidant activity was thus measured as free radical scavenging ability in terms of inhibition of absorbance by DPPH.

Membrane stability index (MSI)

The leaf membrane stability index (MSI) was determined according to the method of Premchandra *et al.* (1990) as modified by Sairam (1994). The MSI was calculated as given by the following equation:

Membrane stability index (MSI) = [1 – (C1/C2)] x 100

Results and Discussion

Morphological symptoms of plants and RWC during stress

Severe wilting symptoms were observed in the four tested varieties only during the 9th day of stress and the plants showed little or no wilting symptoms morphologically during the initial stages of stress (Fig 1). The relative water content in the leaf of the plants declined significantly with induction and duration of water stress. It was noted that the decline in RWC (Fig 2) after 9 days in relation to control (0 day treatment) was lesser in KD and GN (35.7 and 36% respectively) compared to MW and GY (53 and 53.4% respectively). It has also been reported in previous studies that drought resistant cultivars maintain higher RWC during water stress, while in susceptible cultivars RWC shows greater decline (Farooqui *et al.* 2000, Chakraborty *et al.* 2002,

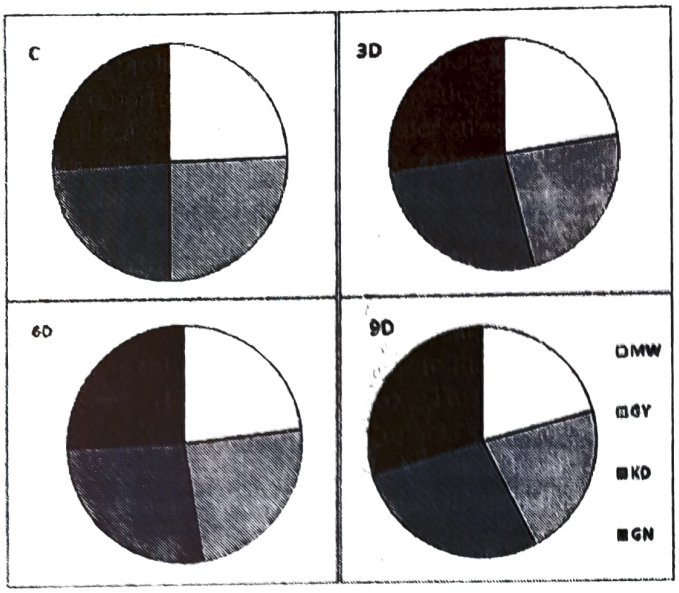

Fig 2. Relative water content of four varieties of wheat subjected to water stress. Results are expressed as the mean of three replicates (10 plants each). C-0 day; 3, 6 and 9 D, days after withholding water.

Iqbal and Bano 2009).

Effect of water stress on antioxidative enzymes and isozymatic patters in wheat varieties

After 3 days of water stress the activities of APOX, POX and GR (Table 1) showed an initial enhancement in all four varieties. With increase in the duration of stress, the activities of POX, APOX and GR showed a decrease in the case of MW and GY whereas POX and GR increased with prolonged water stress in KD and GN. However, the activity of APOX decline after 3 days in these two varieties. It was observed that at all periods of water stress activities of CAT and SOD (Table 1) declined in MW and GY, whereas it showed an initial increase followed by a decline in case of KD and GN. The data of the present study thus reveal that in KD and GN, which are more tolerant than the other two, activities of all antioxidant enzymes increased initially and activities of POX and GR continued to increase, indicating their involvement in tolerance, whereas APOX, CAT and SOD did not contribute directly to tolerance. Previous workers have also reported differential responses of genotypes to water stress with respect to antioxidant enzymes (Dhanda *et al.* 2004, Nair *et al.* 2008). It is evident from results of the present study that POD has a greater role in tolerance than CAT during prolonged water stress. Chakraborty *et al.* (2002) also reported that peroxidase activities increased initially in all tea cultivars following water stress, but in tolerant cultivars it increased even with prolonged periods. Iqbal and Bano (2009) obtained greater increase in activities of POD and CAT in wheat accessions which were tolerant to water stress than those which were less tolerant. Enhancement of GR activity in tolerant varieties indicated that tolerant plants exhibit a more active ascorbate- glutathione cycle than the less tolerant cultivars (Chai *et al.* 2005). This cycle has been implicated in mitigating the effects of reactive oxygen species (Molina *et al.* 2002; Mandhania *et al.* 2006). Under drought stress, enhanced SOD activity was found in pea, tobacco and bean (Moran *et al.* 1994, Van Rensburg *et al.* 1994, Zlatev *et al.* 2006), decreased SOD activity in sunflower seedlings and banana (Quartacci and Navaro 1992, Chai *et al.* 2005) and unaffected SOD activity in maize (Luna *et al.* 1985). In wheat, SOD activity increased or remained unchanged in the early phase of drought but decreased with prolonged water stress (Zhang and Kirkham 1995), as was also obtained in the present study.

Isozyme analysis of POX and CAT (Fig 3) revealed that water stress induced the over expression of isozymes in both tolerant and less tolerant varieties. Hence assay of activity and isozyme analysis could not be directly correlated.

In-situ detection of *accumulation of H_2O_2 and MDA content in the leaf after water stress*

In situ detection of H_2O_2 in leaf tissues and microscopic observations revealed darker staining in tissues subjected to prolonged drought stress, especially in the less-tolerant varieties (Fig 4). A decline in CAT activity following water stress was correlated with increased accumulation of H_2O_2 as well as increased lipid peroxidation in all varieties. However, both H_2O_2 accumulation and lipid peroxidation (Fig. 5) were significantly higher in susceptible varieties in comparison to tolerant ones. The present results are in conformity with those of several previous workers (Chai *et al.* 2005, Zlatev *et al.* 2006). Increased concentrations of H_2O_2, a strong oxidant causes localized oxidative damage, disruption of metabolic functions and lipid peroxidation (Foyer *et al.* 1997, Velikova *et al.* 2000, Zlatev *et al.* 2006). However, besides being an ROS, H_2O_2 is also a signal molecule which is involved in signal transduction mechanisms for several processes in plants such as stomatal closure, root growth and responses to pathogen challenge (Neill *et al.* 2002, Laloi

Table 2.Effect of water stress on cell membrane stability expressed as percent (%) relative injury and free radical scavenging activity (total antioxidant activity) expressed as percent (%) inhibition of DPPH absorbance

Var.	Days of treatment	Relative Injury (%)	Total atioxi-dant activity
MW	0d	64.86	8.43
	3D	58.18	4.49
	6D	51.61	10.53
	9D	20.27	3.33
KD	0d	91.12	8.81
	3D	88.70	21.43
	6D	89.21	29.71
	9D	37.75	56.37
GN	0d	87.42	4.83
	3D	91.51	23.61
	6D	64.09	26.83
	9D	49.78	36.02
GY	0d	95.12	4.56
	3D	80.37	5.47
	6D	64.68	9.54
	9D	49.78	4.11

For cell membrane stability K=0.946, cell constant=1, solution condition=84μS,coefficient-1 at 25°C

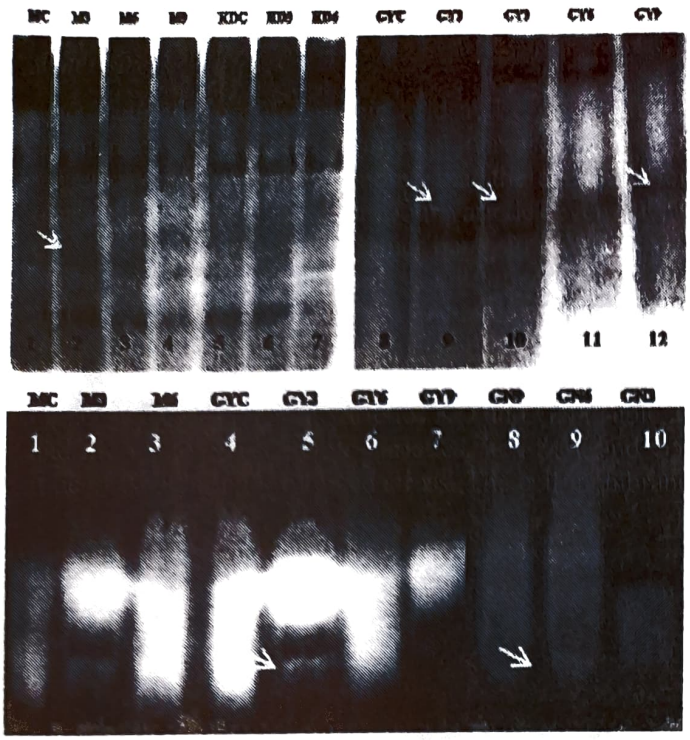

Fig 3. Peroxidase isozyme analysis (upper) of MW, KD and GY during water stress; ln1, 5, 8: Control (0 day), ln 2, 6, 9, 10: 3rd of day, ln3, 7, 11: 6th day, ln4, 12: 9th day and catalase isozyme analysis (lower) of MW, GY and GN during water stress; ln1, 4: Control (0 day), ln2, 5, 10: 3rd day, ln3, 6, 9: 6th day, ln 7, 8: 9th day

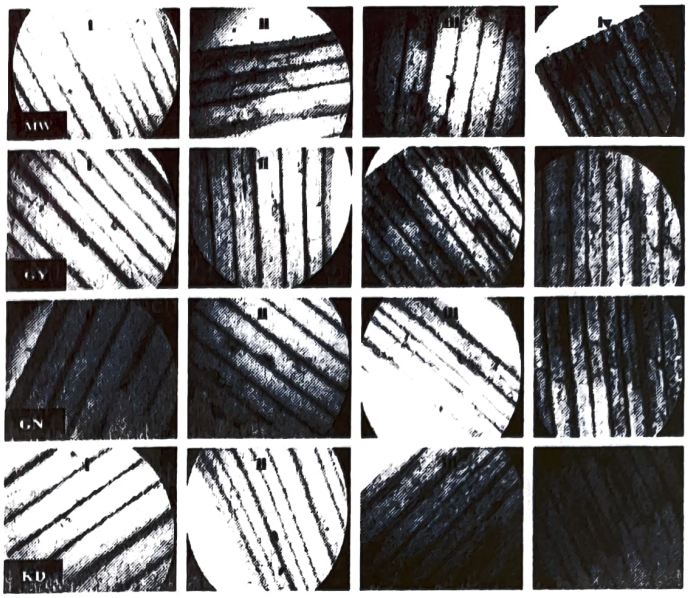

Fig 4. *In situ* detection of H_2O_2 in mid-portions of leaves of four wheat varieties (1st, 2nd, 3rd & 4th row = variety of MW, GY, GN & KD respectively) of wheat following drought, i-0d, ii-3d, iii-6d & iv-9d

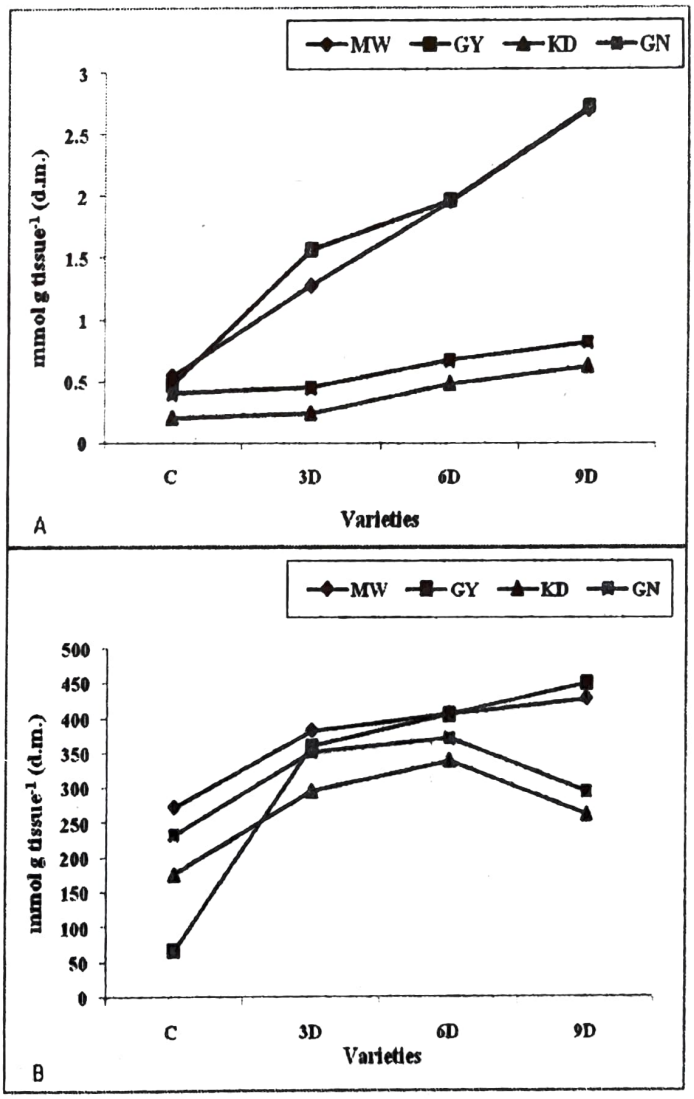

Fig 5. Effect of water stress on the accumulation of MDA (A) and H_2O_2 (B) in four varieties of wheat C= 0 day treatment

et al. 2004, Desikan *et al.* 2005). Thus levels of H_2O_2 are efficiently controlled to maintain balance between production and breakdown. In the present study, though H_2O_2 accumulation increased during water stress, in tolerant varieties, after a period of prolonged drought there was a decrease in H_2O_2 levels indicating greater antioxidant activity.

The peroxidation of lipids in the cell membrane is one of the most damaging cellular responses observed in response to water stress (Thankamani *et al.* 2003) and the amount of lipid peroxidation is considered as one of the determinants which indicate the extremity of stress experienced by a plant. It was observed that during water stress, MDA content which is a measure of lipid peroxidation, increased in all varieties. After 9 days of stress the MDA content in susceptible varieties was more than 3 times that of tolerant varieties.

Change in the content of non-enzymatic antioxidants, total antioxidants and relative injury of the leaf during water stress

The accumulation of ascorbic acid and carotenoid (Fig 6) in plants showed a significant increase in all four varieties. However, it was noted that the accumulation of ascorbate was enhanced all four varieties even after 9 days of water stress; on the other hand, in MW and GY carotenoids declined after 3 days and after 6 days in K and GN. The data was in accordance with the accumulation of total antioxidants in the plants under stress. The value total antioxidant was found to be higher in case of tolerant varieties KD and GN (Table 2) and in the more susceptible varieties, i.e., MW and GY it decreased during prolonged stress. The cell membrane stability expressed as percent relative injury was lower in case of varieties which were susceptible and the tolerant varieties showed higher percentage (Table 2). In an earler study, Nair *et al.* (2008) reported that ascorbic acid contents in cowpea decreased with severity of water

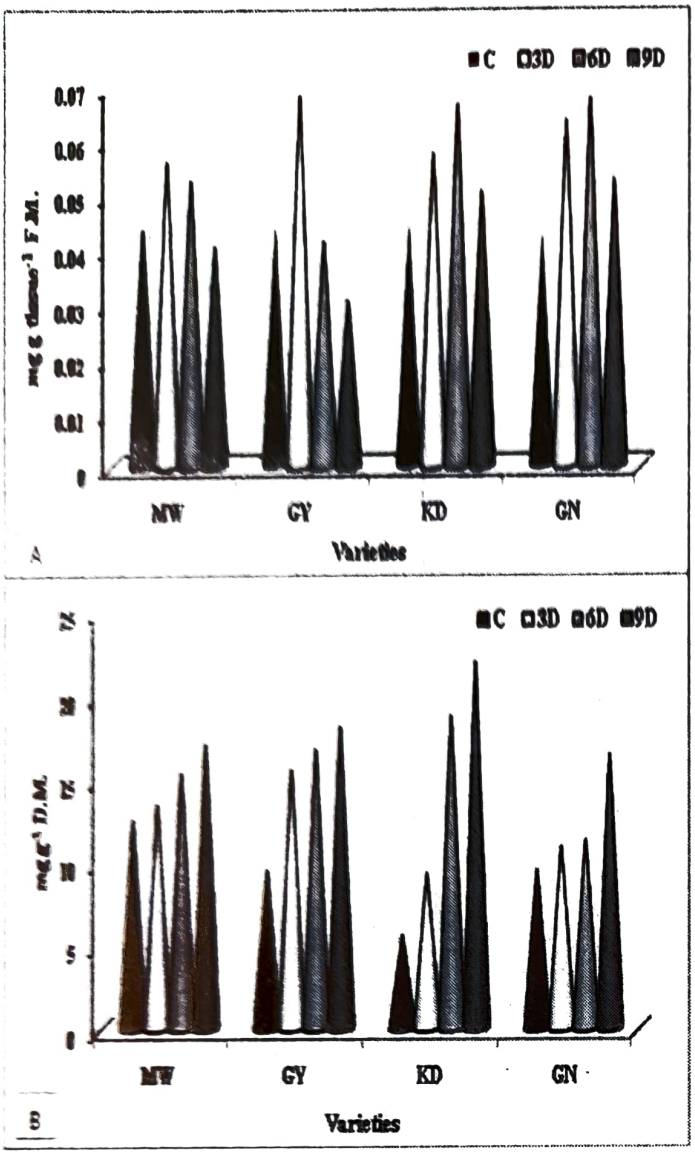

Fig 6. Carotenoid (A) and ascorbate (B) content in the leaves of four wheat varieties subjected to water stress. Means ± S.E., n=10, C= 0 day treatment

stress but tolerant cultivars had higher ascorbic acid contents during severe stress in comparison to susceptible cultivars. Jaleel (2009) reported enhanced accumulation of ascorbic acid during water stress in *Withania somnifera*. L-ascorbic acid is a strong antioxidant but also performs several other functions in the plant (Noctor and Foyer, 1998). In the present study, increase in ascorbate, along with glutathione reductase, indicates the involvement of ascorbate-glutathione cycle as a predominant mechanism of oxidative stress detoxification.

Results of the present study clearly indicate that while water stress induced oxidative damage in wheat varieties as evidenced by decrease in RWC, increased lipid peroxidation, accumulation of H_2O_2, antioxidative mechanisms including enhanced activities of antioxidative enzymes, accumulation of other antioxidants. Antioxidative mechanisms were much more pronounced in two of the varieties- Kedar (KD) and Gandhari (GN), and hence, these were protected from oxidative damage to a great extent. Taking into consideration all the available data, it is concluded that while Kedar and Gandhari could be considered as tolerant, Mohan Wonder and Gayetri were susceptible to water stress.

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Responses of Zn and Cd treatment in soybean and fenugreek

NC Aery* and Sunanda Sarkar
Laboratory of Geobotany and Biogeochemistry, Department of Botany, University College of Science, Mohanlal Sukhadia University, Udaipur-313001, Rajasthan, India

Abstract
The effect of various doses of zinc and cadmium on morpho-anatomical and physiological parameters of soybean and fenugreek was studied. High concentrations of both Zn as well as Cd resulted in chlorosis, necrosis, retardation of growth and reduction in leaflet size especially in soybean. A characteristic symptom was the formation of a red-brown pigment in the leaves, stem and roots. An increase in the phenolic contents was also observed with increasing Cd doses. The reasons for the formation of pigment have been explored.

Keywords: Cadmium, zinc, toxicity, soybean, fenugreek, anatomy

Zinc and cadmium are major environmental contaminants (Cakmak *et al.*, 2000). They form aerosol which disperse and precipitate out with dust (Friberg *et al.*, 1971) and may be absorbed by leaves and subsequently transported to other organs. Zinc is well known as an essential mineral nutrient for normal growth of plants. The small difference between adequate and toxic zinc is shown in the report of Roberg (1932) that 0.001 mg zinc per 100 ml solution stimulated whereas 0.005 mg retarded the growth of *Chlorella*. Zinc toxicosis has been reported in several plants in green house studies when excessive levels of zinc were added (Chaney, 1973).

Cadmium is not an essential element for plant growth but in spite of this it is accumulated in quite high amounts by different plant species (Aery and Tiagi, 1988). In view of the importance of plants in most food chain (both natural and agricultural) a number of studies have been directed towards cadmium accumulation and its effects on plants. The gross effects of cadmium toxicity have been shown to be chlorosis, necrosis and reduction in growth (Haghiri, 1973; Sandalio *et al.*, 2001; Clemens, 2006; Radha *et al.*, 2010; Wang *et al.*, 2011; Khatamipour *et al.*, 2011; Houshmandfar and Moraghebi, 2011).

Though lot of work has been done on the phytotoxicity of zinc and cadmium little work has been attempted especially on the localisation of these metals in various plant tissues (cf. Aery and Sarkar, 1988) and the concerned anatomical variations and that too in cultivated plants. In order to know the critical and toxic levels of zinc and cadmium for soybean and fenugreek the effect of these two metals added to soil in different doses was studied. The main object was to study the response of soybean and fenugreek in respect of various morpho-anatomical and physiological parameters towards these environ-mental contaminants.

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

Materials and Methods

The seeds of *Glycine max* (L.) Merr. var. Gaurav (Soybean) and *Trigonella foenum-graecum* L. (fenugreek) strain UM-34 were used for the study. Garden soil of average fertility was used for the study. Four kilogram of soil was filled in each earthen pot of 30 centimeter height and 25 cm diameter. Nine concentrations of zinc and seven concentrations of cadmium were prepared separately by taking corresponding amounts (calculated on the basis of their molecular weights) of the chemicals per kilogram of air dried soil. The chemicals were mixed thoroughly in the soil. For zinc, zinc sulphate ($ZnSO_4.7H_2O$; E. Merck, GR) and for cadmium, cadmium chloride ($CdC1_2.2H_2O$; Analar, BDH, England) were used. The zinc was applied in the doses of 10, 25, 50, 100, 500, 1000, 2500, 5000 and 7500 µg/g while for cadmium the doses were 5, 10, 50, 100, 250, 500 and 1000 µg of cadmium per gram of air dried soil. Pots without any added metal constituted the control. Ten seeds of each crop were sown equidistantly at 2 centimeter depth in each pot. Before sowing, soybean seeds were inoculated with its *Rhizobium* strain obtained from the microbiological division of IARI, New Delhi.

Watering was done on alternate days for fenugreek and every day for soybean as recommended in agricultural practices. After establishment, seedlings were thinned to five in number for soybean and three for fenugreek in each pot.

Four sets in duplicate were prepared to record observations for each crop in all the four stages of their life span, i.e., seedling, vegetative, flowering and fruiting for both the metals separately for shoot and root length nodule number and weight, leaf area, dry weight, grain yield and morpho-anatomical features. The material was dried in an oven, digested in acid and Zn and Cd contents were determined with the help of an AAS. Total phenol contents were estimated by Folin-Ciocalteau method (Aery, 2010). Results on the effects on morpho-anatomical features are being discussed here.

Results and Discussion

Visual Toxicity Symptoms:

Effect of zinc on soybean:

Chlorosis, thickening of leaf and necrosis started appearing in the lower pair of leaflets at zinc doses of 2500, 5000 and 7500 µg/g even in the seedling stage. Chlorosis first started appearing on the margins and on the adaxial surface of leaf which extended towards the midrib. Base and tips of leaves became necrotic. The above symptoms increased with concentration of zinc and age of the plant. In the fruiting stage at a zinc level of 1000 µg/g, lower 3-4 leaflet pairs became completely chlorotic. Zinc doses of 5000 to 7500 µg/g also decreased stem elongation which resulted in stunted growth.

Another symptom of zinc toxicity in soybean was a characteristic deep red-brown pigment formation in the roots especially in the plants treated with high zinc (5000 µg/g and 7500 µg/g) doses.

Effect of cadmium on soybean:

At higher doses of cadmium, i.e., at 250 to 1000 µg/g, there was a retardation of growth, reduction in leaflet size and shortening of stem internodes. Additional stems with abnormal small-sized trifoliate leaves developed in plants at 250, 500 and 1000 µg/g cadmium doses. Further, 10 to 1000 µg/g cadmium levels showed an increasing degree of red-brown pigmentation in the roots of the plants (Figure 1A).

Formation of red-brown pigment was also observed in leaves which formed in the veins at 250 to 1000 µg/g cadmium dose in seedling stage. With the increasing age, red-brown pigment deposition spread along the major veins and petioles of primary leaves and was more apparent on the adaxial surface where it appeared earlier. Necrotic spots could also be observed on the leaves. The extent of pigment formation was always higher at higher cadmium treatments than at lower concentrations.

Table 1 Cadmium toxicity symptoms

Cd dose	Symptoms
0 & 5 µg/g	No visual toxicity symptoms.
10 µg/g	Red colouration at petiole base and base of primary leaf and lower trifoliate leaves.
50 µg/g	Red colouration at petiole base of all leaves; red colouration extended slightly from base to the margin or tip of the primary as well as trifoliate leaves.
100 µg/g	Symptoms present in the 50 µg/g Cd dose; primary as well as lower 3-4 trifoliate leaflets showed signs of redness.
250 & 500 µg/g	Leaves smaller than control, curved, chlorotic with slight to prominent reddish tinge in veins.
1000 µg/g	Leaves comparatively more small, thick, somewhat leathery, completely yellowish with red veins; leaf curvature more prominent

Table: 2 Total phenol contents in soybean under the influence of different cadmium concentrations

Cadmium Concentrations (µg/g)	Total phenols (mg/g)
Control	2.45
5	2.30 (-6.12)
10	2.70 (+10.20)
50	2.95 (+20.40)
100	3.25 (+32.65)
250	3.45 (+40.81)
500	3.85 (+57.14)
1000	3.85 (+57.14)

Leaflets also showed intraveinal chlorosis which increased with age and applied cadmium concentrations. At acute toxicity levels (1000 µg/g) the first and second leaves appeared pale-yellow and largely devoid of chlorophyll (Figure 1A). The chlorosis was accompanied by a severe laminar deformation followed by abscission in some cases.

Effect of zinc and cadmium on fenugreek:

Higher concentration of zinc and cadmium affected both root as well as top growth. Plants showed stunted growth, with less expansion of internodes, increased diameter of stem and thick leathery leaves. Cadmium was found inhibitorier to the growth than zinc. Roots showed red-brown pigmentation. Here plants could not survive even after 100 µg/g in fruiting stage which showed early lethality as compared to zinc. Leaves were not retained on the plant and were shed due to early leaf fall.

Anatomical Toxicity Symptoms:

Anatomical studies were carried out by cutting free hand sections to study the deposition pattern of red-brown pigment in the leaf, stem as well as root and to observe any histological changes under the influence of toxic doses of cadmium.

In a T.S. of the root of soybean in control (Figure 1B) no traces of any pigmentation were visible. The pith region was intact and no disorganization has occurred.

In comparison to control (Figure 1D) a T.S. of the root of cadmium treated (1000 μgg^{-1}) plant showed (Figure 1C) a completely disorganised pith and disorganization in patches in the cortex. A heavy deposition of red-brown pigment in patches in the epidermis, cortical, endodermal, pericycle and pith cells adjoining metaxylem elements was observed. The epidermal and cortical cells showed the maximum pigment deposition. Though the pigment deposition led to the filling of some of the parenchyma cells completely (Figure 1C), the deposition was primarily in the intercellular spaces and cell walls of the parenchyma cells. Often the cell wall and the deposits protruded some distance into the cell cavity. No deposition was observed in the xylem vessel lumens.

Similarly, in comparison to control (Figure 1F) a T.S. of the stem of the cadmium treated (1000 µg/g) plant also showed a completely disorganised pith and heavy deposition of red-brown pigment in the epidermal,

Table: 3 Showing relation among the soil applied cadmium concentrations and Cd contents in different plant parts at maturity in soybean and fenugreek

Cadmium conc. (µg/g)	Cadmium concentrations (µg/g) in plant parts					
	Soybean			Fenugreek		
	Shoot	Leaf	Root	Shoot	Leaf	Root
Control	0.48	0.58	0.75	0.28	0.38	0.68
5	3.15	3.58	6.30	10.37	11.35	12.17
10	6.30	11.89	54-59	12.69	12.70	12.83
50	18.48	30.09	99.73	21.19	31.70	40.97
100	28.51	35.82	191.52	57.69	64.78	80.67
250	44.42	47.28	355.62	-	-	-
500	45.85	60.18	696.43	-	-	-
1000	117.50	141.86	829.70	-	-	-

cortical, endodermal and margins of disorganised pith regions. The red-brown pigment completely masked the green colour of chlorenchymatous cortical cells. No such deposition was observed in the pericycle, phloem and xylem regions (Figure 1E). Moreover, the, pattern of secondary vascular tissue formation was found to be somewhat irregular. At some places the amount of secondary xylem was much more than at other places (Figure 1E).

In cadmium treated (1000 µg/g) plants a T. S. of the pulvinus showed a red-brown pigment deposition mainly in the epidermal region and that too in patches (Figure 1G). The epidermal hairs, epidermis, as well as 1-2 cortical layers adjoining epidermis got heavy but patched deposition of the pigment. Pigment deposition was also observed in patches in the cortical zone at the place of departure of leaf trace and endodermal cells. No such deposition was visible in vascular zone as well as pith which remained intact (Figure 1G).

In the T.S. of petiole, (Figure 2A) in the control, the cells are neither disorganised nor showed any pigmentation. In cadmium treated (1000 µg/g) plants a heavy deposition of red-brown pigment in the epidermis, upper cortical layers, as well as pith region was observed (Figure 2B). The xylem and phloem bundles were free from pigmentation. Moreover, a slight to marked elongation of cells both in the cortical and pith region was also observed (Figure 2B & C).

A T.S. of the primary leaf of cadmium treated (1000 µg/g) plant showed heavy deposition of red-brown pigment in the epidermal and adjoining mesophyll cells both on the adaxial, as well as the abaxial side (Figure 2 E). This deposition was highly prominent in the midrib region. Even the hairs present both on abaxial and adaxial sides in this region got impregnated with the pigment (Figure 2 E). This gave the red-brown colour to the veins of primary leaf and trifoliate leaflet as visual symptoms. A slight disorganization of tissues, both below and above the vascular bundle occurred. In contrast to control (Figure 2D) the cells above and below the vascular bundle in the T.S. the leaf of cadmium treated plants gave a storied appearance (Figure 2F). Xylem and phloem parenchyma also showed slight deposition of red -brown pigment. No pigment was present in the lumen of xylem vessels.

Several studies have shown the effect of heavy metals on plants resulting in reduced growth and phytomass accumulation (Marques *et al.*, 2000; Sandalio *et al.*, 2001). However, few studies are available in the literature on visual symptoms of zinc and Cd toxicity. John *et al.* (1972) observed chlorosis of leaves and stunted growth in radish plants due to addition to 50 mg of Cd to 500 g of silt loam soil. However, no such symptoms could be observed for lettuce. Rauser (1973) reported colouring of leaf veins in white bean (*Phaseolus vulgaris*) plants grown under conditions of zinc toxicity. Root *et al.* (1975) has reported the presence of a Cd induced inter-veinal chlorosis and correlated it with a decrease in chlorophyll content in corn. Haghiri (1973) has shown in soybean a progressive browning of the veins which ended in chlorosis and correlated it with increased Cd treatment in soybean. Boggess and Koeppe (1977) and Boggess *et al.* (1978) studied a number of soybean varieties grown on soil amended with $CdCl_2$ or sewage sludge. Cd toxicity symptoms appeared from slight effects such as a red to red-brown colouration at the junction of the leaf blade and petiole to severe leaf curling and extensive reddening of the leaf veins, chlorosis and finally a brittle condition which was followed by abscission (Boggess and Koeppe, 1977). Brisson *et al.* (1977) reported toxicity symptoms not only due to heavy metals but also to infection by certain pathogens. Visual symptoms developed by plants in response to heavy metals have also been reported by Fontes and Cox (1998). Srighar *et al.* (2005) and Vollenweider *et al.* (2006) have studied the effects of Cd on the leaf anatomy of *Brassica juncea* and *Salix viminalis*, respectively. Gomes *et al.* (2011) studied the effect of heavy metals on biomass production, photosynthetic capacity, and anatomical changes in roots and leaves of *Brachiaria decumbens*. Reduction in size and number of conducting elements of the xylem in response to heavy metals has been reported by Sandalio *et al.* (2001). Chandra *et al.* (2010) has studied the effects of Cd and Cr on the structure of root and stem as well as accumulation of Cd and Cr in the plant body of *Vigna unguiculata* and *Vigna radiata*. Cvetanovska *et al.* (2010) has studied the anatomical and physiological disorders in tobacco (*Nicotiana tabacum* L.) after intoxication with copper, cadmium and lead. Hussain (2010) has reported the presence of safranin stained masses in the xylem vessels of stem of *Bacopa monnieri* (L.) Pennell under the effect of $HgCl_2$ contaminated water.

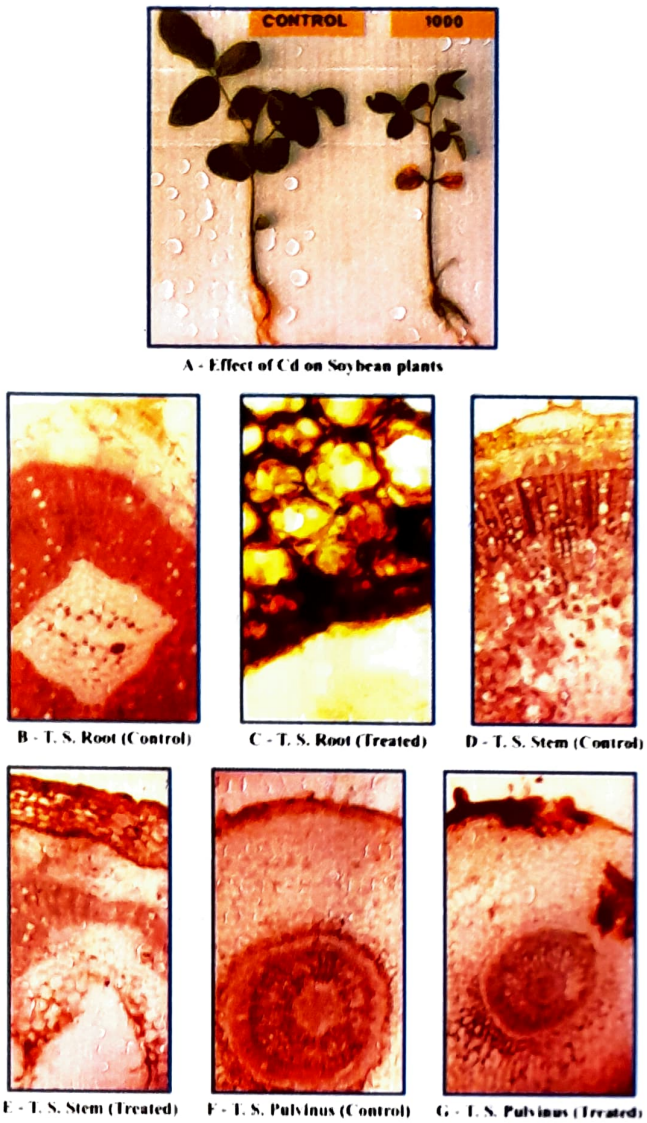

Figure 1 See text for details

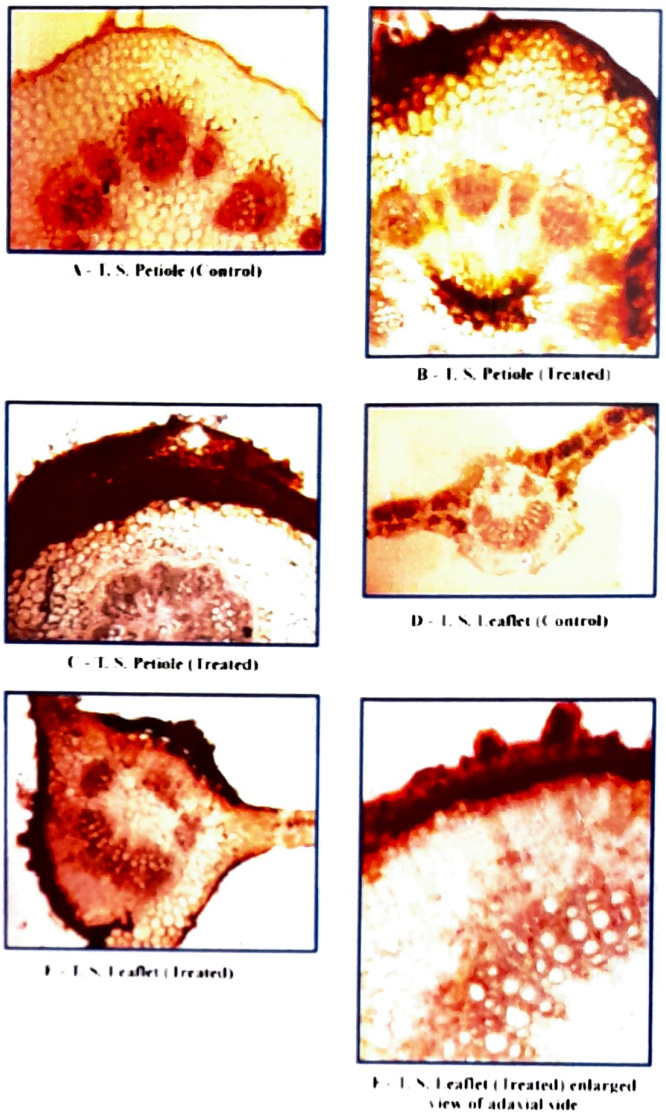

Figure 2 See text for details

In the present studies no characteristic toxicity symptoms could be observed in fenugreek. High zinc doses caused stunted growth, less expansion of internodes, increased diameter of stem and thick leathery leaves. Higher doses of Cd caused the development of red-brown pigmentation in roots and early senescence.

In soybean, though higher doses of zinc (2500-7500 µg/g) led to necrosis and stunted growth and chlorosis which started appearing at the adaxial surface of leaves, no red-brown pigment formation in the leaves as reported by Rauser (1973) for *Phaseolus vulgaris* and soybean could be observed in the present study.

An interesting observation not appreciated till date was the characteristics deep red- brown pigment formation in the roots of soybean plants treated with higher doses of zinc and Cd (Figure 1A).

Cadmium toxicity symptoms in soybean were much more marked than zinc. Though the symptoms were in a continuous series it could be possible to know the Cd toxic levels only by observing the symptoms (Figure 1& 2). The symptoms are being presented in Table-1.

The red-brown pigment has been found to be readily solubilized by extraction with 0.1 % methanolic HCl (Cunningham *et al.*, 1975). It could be a polyphenolic substance and probably a mixture of cyanidin glycosides (Harborne, 1967).

In the present studies, a steady increase in the amount total phenols was observed (Table-2) with increasing Cd doses. This further corroborates that the compound is a phenolic one.

It appears that the formation of pigment in certain cells is a response to higher metal contents. High cadmium levels have been observed in the leaves of Cd-treated plants (Table-3). Whether Cd is a part of this phenolic compound is not known although Jaakola and Ylaranta (1976) reported that cadmium is concentrated in the veins of radish and Cunningham *et al.* (1975) found that in soybean the movement of Cd was restricted to the major veinal areas which coincided with the deposition of anthocyanin pigment. Further, in X-ray films, localized plugging of vascular tissue along major veins in the leaf and at the stem nodes has been observed and tissues beyond blocked veins accumulated less cadmium than regions below the blockade points (Cunningham *et al.*, 1975).

Aery and Sarkar (1988) localized the accumulated zinc in soybean and fenugreek with the help of dithiozone and found it to accumulate along the cell wall suggesting an apoplastic movement. An apoplastic movement of

cadmium also has been observed by Cunningham *et al.* (1975). Occurrence of metals such as Cd has been verified in the apoplast (cell walls and intercellular spaces) of the cortical parenchyma of roots (Wójcik *et al.*, 2005).

Cadmium toxicity caused cell degeneration in root tissues. The cortex has wide intercellular spaces suggesting that greater metal deposition occur in the roots (Table-3) and lower transfer to the shoot part (Gomes *et al.*, 2011). Leaf curling may be a strategy to reduce the transpiration area on the surface (Turner and Jones, 1980).

Gomes *et al.* (2011) observed thickening of walls of xylem elements and cortical parenchyma of roots in *Brachiaria decumbens*. This may help to maintain the hydraulic safety of the root which is essential for its activity and constitutes a barrier to water loss by reflux (Gomes *et al.*, 2011). Further, the binding of heavy metals in the cell wall reduces the amount of cytosolic heavy metals and thus has a protective action (Vázquez *et al.*, 1992; Wójcik *et al.*, 2005).

Chlorosis observed in leaves may be related to lower chlorophyll contents. The effect of metal ions on pigment production has been investigated in several species (Chugh and Sawhney, 1999; Horváth et al., 1996).

Aery (1994) have shown leaf iron as well as chlorophyll contents to be negatively correlated with leaf cadmium and leaf Zn. The negative correlation between leaf Fe x leaf Zn, leaf Fe x leaf Cd and positive correlation between leaf Fe x chlorophyll contents suggest that chlorosis arises due to reduction in the adsorption and the translocation of Fe resulting in Fe deficiency at the site of chlorophyll synthesis in leaves (Ambler *et al.*, 1970).

Caststelfranco and Beale (1983) have suggested that during chlorophyll synthesis, the conversion of Mg-Proto-Me to Protochlorophyllide (Pchlide) requires iron, as Mg-Proto (-Me) accumulates in Fe deficient plants (Spiller *et al.*, 1982). Abnormally high Mg-Proto/ Pchlide has been found in etiolated tissues treated with compounds such as α,α1, dipyridyl that decrease the activity of Fe by forming bidentate complex (Vicek and Gassman, 1979).

Moreover, Labbe and Hubbard (1960) have described an enzyme from rat liver which is considered to be responsible for the insertion of iron into the protoporphyrin chelate to give haem. This mechanism might be of universal significance and if so could be involved in chlorophyll synthesis if ferrous protoporphyrin is a precursor of the magnesium compound. Metal toxicity might result from competition between the metal and ferrous ions at the enzyme site.

Cunningham *et al.* (1975) have indicated the possibility of clogging of phloem element which results in restricted transport of nutrients from cotyledons which ultimately resulted in growth reduction, chlorosis and inhibition of lateral root formation. This would result in unusual accumulation of sugars at the point of blockage which would induce formation of anthocyanin as indicated by Harborne (1967). The deposition of red-brown pigments in the stem and leaves might be due to the above reason.

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Serological and molecular detection of *Macrophomina phaseolina* causing root rot of *Citrus reticulata*

BN Chakraborty*, U Chakraborty, K Rai, K Sunar and PL Dey
Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal Siliguri 734013, West Bengal, INDIA

Abstract
Polyclonal antibodies (PAbs) were raised against mycelial antigens of *Macrophomina phaseolina* a causal organism of root rot disease of mandarin plants. IgG was purified and further packaged into immunological formats such as immuno diffusion, Plate trapped antigen (PTA)-ELISA, dot immunobinding assay, Western blot analysis and indirect immunofluorescence for quick and accurate detection of pathogen from soil. Indirect staining of mycelia and sclerotia of *M. phaseolina* with homologous PAb and labeling with goat antirabbit IgG conjugated with FITC developed strong fluorescence in young hyphal tips and sclerotia of *M. phaseolina*. Genomic DNA prepared from mycelia of *M. phaseolina* was purified and PCR amplification of 18S rDNA was done using ITS region specific primer pair. The amplified DNA was sequenced and aligned against ex-type strain sequences from NCBI GenBank using BLAST and phylogenetic analysis was obtained using MEGA4 software. Amplification of ITS1 region of the rDNA can be considered as a rapid technique for identifying pathogens successfully in all cases.

Keywords: *Citrus reticulata, Macrophomina phaseolina*, PTA-ELISA, DIBA, immunofluorescence, rDNA, ITS region

The most widely cultivated cultivar in India is the mandarin (*Citrus reticulata* Blanco) followed by sweet orange (*Citrus sinensis* Osbeck) and acid lime (*Citrus aurantifolia* Swingle), sharing 65, 25 and 10 percent of total production respectively but still remains unexplored for systemic collection, evaluation and characterization to shortlist them in order to identify active germplasm. There are four different strains of mandarin cultivated in India viz. Khasi mandarin grown in north-eastern states, Darjeeling mandarin grown in the hills of Darjeeling (Figure.1A) and Sikkim, Nagpur mandarin grown in Maharashtra, Coorg mandarin grown in south India (Allay and Chakraborty, 2010).

The mandarin cultivation in Darjeeling has a massive decline due to various pathological, entomological and nutritional stresses. *Macrophomina phaseolina, Fusarium solani* and *Fusarium oxysporum* infects roots of both nursery grown and field grown mandarin plants. The use of immunological assays for both detection and diagnosis of plant diseases have increased rapidly (Chakraborty and Chakraborty, 2003). It has long been known that most plant pathogens possess as part of their structures, specific antigenic determinants or recognition factors in the form of proteins, glycoproteins, complex carbohydrate polymers or other complex molecules (Chakraborty, 1988).

Since it is difficult to detect *M. phaseolina* in root tissue or in soil until it is too late, the present study was undertaken for early and accurate detection of the pathogen by serological techniques. It is expected that such an early detection would prove useful for better management practices

The development of serological techniques has produced a number of highly sensitive methods for identifying microorganisms in diseased plant tissue (Chakraborty *et al.* 1995). These rely on the recognition of the solid or solid antigenic materials by antibodies raised against the organisms and the subsequent use of an enzyme labeling system. The purpose of the present study was rapid identification of *Macrophomina phaseolina*, a root rot pathogen of *Citrus reticulata* (Blanco) in Darjeeling hills based on the sequence analysis of ITS regions of the rDNA gene and development of rDNA markers for analysis of genetic variability.

Materials and Methods

Fungal culture

Fungal pathogen (*Macrophomina phaseolina*) was isolated from samples of diseased roots of mandarin (*Citrus reticulata*) plants grown in Mirik busty by culturing pieces of internal tissues. Infected root tissues were thoroughly washed in sterile water, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to potato dextrose agar (PDA) slants and incubated at 28°C for two weeks. The isolated organism was examined under microscope. Healthy seedlings of mandarin (*Citrus reticulata*) plants (1-year-old) were further inoculated with this isolated organism and incubated for a period of 4 weeks for completion of Koch's postulate. Subsequently, the infected roots were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 2-3 minutes,

*Corresponding author:
E-mail: bncnbu@gmail.com

rewashed with sterile distilled water, transferred to PDA slants and incubated at 28°C. At the end of two weeks, the reisolated organism was examined, compared with the original stock culture and its identity was confirmed following microscopic observations as *Macrophomina phaseolina*. Mycelia – septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelia mat even and appressed. Sclerotia – black, moderate size (34-78 μ in diameter), round or irregular uniformly reticulate with no difference in internal structure. The culture was maintained on PDA slants and stored under three different conditions (5°C, 20°C and 30°C (room temperature)) in sterile liquid paraffin. The culture was examined at a regular interval to test its viability and pathogenicity of the fungus.

Inoculation techniques and disease assessment

M. phaseolina was grown in sand maize meal medium supplemented with citrus root pieces, for inoculation of healthy mandarin seedlings as it increases the survival capacity and viability of the pathogen in the soil. Initially, *M. phaseolina* was grown on PDA in Erlenmeyer flasks (250ml) for 2 days. Subsequently 30 sterilized mandarin root pieces (one inch long) were transferred to each flask and incubated for 15 days. Sand maize meal medium (50g) containing five such pieces covered with the mycelia and sclerotia were inserted in the rhizosphere of each plant. The inoculated plants were examined at an interval of 7 days up to a period of 28 days. Each time, the plants were uprooted, washed and symptoms noted. Finally roots were dried at 60°C for 96h and weighed. Root rot index was calculated on the basis of percentage root area affected and they were graded into 6 groups and a value was assigned to each group (viz. no. root rot = 0; up to 10% root area affected = 0.10; 11-25% = 0.25; 26-50% = 0.50; 51-75% = 0.75; 76-100% = 1.0). The root rot index in each case was the quotient of the total values of the replicate roots and the number of roots (i.e. number of plants).

Preparation of antigen

Antigens were prepared from mycelia of *M. phaseolina*, healthy and artificially inoculated root tissue of mandarin plants following the methods as described by Chakraborty and Purkayastha (1983). They were stored at –20°C and used as mycelial and root antigens.

Production and purification of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against mycelial antigens of *M. phaseolina* following the method of Chakraborty and Purkayastha (1983). Normal sera were collected from the rabbit by ears vein puncture before immunization. The antigen emulsified with an equal volume of Freund's complete/incomplete adjuvant was injected subcutaneously at weekly interval for six consecutive weeks. The blood samples were collected after six weeks following injection and kept for 1h at 30°C. The clots were loosened and stored at 4°C. The antisera were then clarified by centrifugation and stored at –20°C until required. IgGs were purified by DEAE-Sephadex column chromatography following the protocol of Clausen (1988).

Immunodiffusion

Agar gel double diffusion tests were performed using PAb raised against *M. phaseolina* following the method of Ouchterlony (1967).

PTA-ELISA

Optimization of ELISA was done using purified IgGs of known concentration which was predetermined using the referred formula. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10000) and p-nitrophenyl phosphate (100 mg ml^{-1}) were used for PTA-ELISA as enzyme substrate (pNPP), reaction was terminated after 60 min and the absorbance values were recorded as mean of five adjacent wells measured at 405 nm essentially as described by Chakraborty and Sharma (2007). Antigens from fungal pathogen was diluted with coating buffer and IgGs were diluted to 1:125 with PBS-Tween containing 0.5% BSA. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate and 4-nitrophenyl phosphate (pNPP) as enzyme-substrate, were used for ELISA tests. Absorbance values were measured at 405 nm in an ELISA reader (Multiskan EX, Labsystems). Absorbance values in wells not coated with antigens were considered as blanks.

Dot immunobinding assay

Mycelial antigens prepared *M. phaseolina* were loaded on nitrocellulose membrane filters using Bio-Dot apparatus (Bio-Rad). Dot immunobinding assay was performed using PAb of *M. phaseolina* as outlined by Lange *et al.* (1989).

Western Blotting

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electrotransferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of *M. phaseolina* following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-choloro-3-indolylphosphate (NBT-BCIP) as substrate. Immuno-reactivity of the proteins was visualized as violet coloured bands on the NCM.

Immunofluorescence

PAbs of *M. phaseolina* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC) were used for indirect immunofluorescence study to detect the pathogen. Observations were made using a Biomed microscope (Leitz) equipped with an I3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Leica Wild MPS 48 camera on Kodak 800 ASA film.

Genomic DNA extraction

Genomic DNA was isolated from 4 day old fungal mycelia *M. phaseolina* by a modified method of Raeder and Broda 1985. Fungal mycelia from 3-4 days old cultures grown on potato dextrose broths was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA

(pH8.0), 100 mM NaCl and 2% SDS, for 4 h at 65°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min, and further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and chloroform (in the ratio of 1:4 v/v) was added followed by 0.5M Na-acetate (in the ratio of 1:10 v/v). Next, isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12000 rpm for 15 min followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Qualitative and quantitative estimation of DNA

The extraction of total genomic DNA as per the above procedure was followed by RNAase treatment. Genomic DNA was re-suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

PCR amplification of ITS region and sequencing

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq polymerase buffer, 1 µl of 1U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers. For amplification of the ITS regions of the ribosomal DNA primer pairs, ITS1 and ITS4 were used. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

PCR product (10 µl) was mixed with loading buffer (5ml) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 1.5% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis. Nucleotide base pairs of the amplicons were determined on the basis of its migration and conformation relative to the molecular size marker (1000 base pair, wide range DNA ladder, Genie,Bangalore) PCR products were sent for sequencing to Chromous biotech, Bangalore, India.

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm included in the Megalign module (DNASTAR Inc.) Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters were used for the pair wise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the UPGMA method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained (Felsenstein 1985). An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny. There were a total of 138 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4 as described by Tamura *et al.* (2007).

Results and Discussion

Charcoal root rot incidence of Citrus reticulata

Symptoms of Charcoal root rot caused by *M. phaseolina* were characterized by a gradual decay of the root tips, lateral roots and root crown. This gradual destruction of the root system causes the seedlings to become stunted and chlorotic, and finally to die. In some cases, the margin of the infected area breaks away from the healthy area and may curl back. Lesions can eventually girdle the entire tree trunk leading to the death of the tree. Mandarin seedlings were collected from eight different locations, viz. Rangali Rangliot, Bijanbari, Sukhia Pokhari, Kurseong, Mirik, Kalimpong Block I, Kalimpong Block II and Gorubahan and maintained in

the Glass house conditions. Charcoal root rot pathogen (*M. phaseolina*) isolated from mandarin orchards of Darjeeling hill was used for present study, after completion of Koch's postulate. Mycelia – septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelia mat was even and appressed. Sclerotia – black, moderate size (34-78 u in diameter), round or irregular (Figure 1 G) uniformly reticulate with no difference in internal structure.

Healthy seedlings of mandarin (*Citrus reticulata*) plants (1-year-old) grown in earthenware pots were inoculated with this isolated organism and incubated for a period of 4 weeks. Pathogenicity of *M. phaseolina* was tested on twenty mandarin plants each of eight different locations. The inoculated plants were examined after 4 weeks. Colour of root, root rot index and percentage loss in dry weight of roots were noted. Young seedlings showed light brown discolouration of the root at the soil line initially which gradually turned dark brown to blackish brown and finally to black. In advanced stage of disease symptoms also appeared at ground level. Lower leaves turned yellow and remained attached, sometimes

showed wilting symptoms. In advanced stage defoliation of lower leaves were evident (Figure 1 H). When epidermis was removed, small, black bodies (sclerotia) were discerned. These propagating bodies were abundant enough to impart a grayish black colour like charcoal to the tissues. The root rot index as well as percentage loss in dry weight of roots were very low at the initial stage of infection, which increased significantly with time in compatible interaction. Mandarin seedlings of three locations (Mirik, Kalmpong Block-I and Sukia Pokhri) were found to be highly susceptible (Table 1).

Culture filtrate of the pathogen (*M. phaseolina*) following two weeks growth in Richards' media at 28°C, was collected and the young seedlings of mandarin of three different locations ((Mirik, Kalmpong Block-I and Sukia Pokhri) which showed susceptible reaction, were further tested in *in vitro* conditions in comparison with sterile distilled water control (Figure 1 B-E). It is interesting to note that seedlings showed same symptoms in this case also. Wilting followed by chlorosis and browning reaction of green leaves were evident in seedlings grown in culture filtrate of the pathogen. Wilting symptoms first appeared 7 days after treatment. However within two weeks leaves of all the seedlings turned into brown colour.

Cultural conditions affecting growth of the pathogen

Macrophomina phaseolina infect mandarin plant roots and their interactions affect the development of root rot disease. Initially it was considered worthwhile to study the effects of some major factors such as incubation time, temperature and pH of substrate on growth of the pathogen *in vitro*.

Effect of incubation time.

The effect of incubation time on the growth of *M. phaseolina* was studied *in vitro*. *M. phaseolina* was grown in Richard's media for a period of 24 days at 28°C. Mycelial growth of the fungus was recorded after 2,4,8,12,16,20, and 24 days. The results are embodied in Table 2. Maximum growth of *M. phaseolina* (755 mg) was observed after 12 days of incubation and then the rate of growth declined. Mycelial growth increased by 24% from 8 to 12 days of incubation and decreased by 5% from 12 to 16 days (Table 2).

Effect of pH on growth.

It is well known that the pH of the medium usually plays an important role in the growth of microorganisms. The utilization of nutrients depends partially upon the pH of the culture medium. Therefore, it was considered imperative to use a buffer system to stabilize the pH of the culture medium during incubation. In the present study, buffer solutions with pH values ranging from 4-8 (4.0, 5.0, 6.0, 7.0 and 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH of the medium was adjusted using N/10 NaOH or N/10 HCl to obtain the corresponding range of pH values (4.0-8.0). Both the medium and the phosphate buffer were sterilized. Equal parts of the buffer solution and medium were mixed before use. Each flask containing 50ml of the medium was inoculated with fungus and incubated for 12 days at 28°C. The results are given in Table 3. It appears that *M. phaseolina* grew well over a range of pH (4.0-8.0) and optimum growth was recorded at pH 5.5. It is necessary to mention that mycelia growth increased up to pH 5.5 and then gradually declined.

Table 1: Pathogenicity test of *Macrophomina phaseolina* on different root samples of *Citrus reticulata*

Locality of saplings	* Root rot index	**Colour intensity
Rangli Rangliot	0.10	+
Bijanbari	0.25	++
Sukhia Pokhari	0.75	++++
Kurseong	0.25	++
Mirik	0.75	++++
Kalimpong Block I	0.75	++++
Kalimpong Block II	0.50	+++
Gorubathan	0.50	+++

* On the basis of root area affected; 0-10% (0.10); 11-25% (0.25); 26-50% (0.50); 51-75% (0.75); 76-100% (1.0). ** + Light brown, ++ Deep brown , +++ Blackish brown, ++++ Black

Table 2: Effect of incubation time on growth of *M. phaseolina*

Incubation Time (days)	Average dry weight of mycelia (mg)
2	97.20 ± 2.24
4	214.35 ± 4.44
8	618.00 ± 3.72
12	755.00 ± 1.81
16	717.52 ± 1.58
20	689.32± 2.86
24	653.62± 2.43

Average of 3 replicates/treatments; Temperature 28°C; pH of medium – 5.4

Effect of temperature on growth

Temperature is also a major factor affecting growth of a pathogen. Therefore, the effects of different temperatures (15, 20, 25, 28, 30, 35, 40°C) on growth of *M. phaseolina* was studied *in vitro*. Maximum mycelial growth was noted at 30°C with a decline at 40°C (Table 4).

Immunological assays

Serological assays were performed using Polyclonal antibodies (PAb) raised against mycelial protein of *M. phaseolina*. Effectiveness of antigen in raising antibodies were checked initially using agar gel double diffusion technique. Strong precipitin reactions were noticed in homologous reaction with antigen and antibody raised against *M. phaseolina* (Figure 1). Dot immunobinding assay using mycelia antigen and PAb of *M. phaseolina* was also carried out. For this, soluble protein obtained from seven-day-old mycelia of *M. phaseolina* were reacted on nitrocellulose paper with

PAb of the pathogen (*M. phaseolina*). Results shows development of deep violet colour indicating a positive reactions suggestive of effectiveness of mycelial antigen in raising PAb against the pathogen (Figure 1 J). Optimization of ELISA was done by considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity (Figure 2).

Root antigens were prepared from healthy as well as artificially inoculated plants of *C. reticulata*. Three days and seven days following inoculation with *M. phaseolina*, root antigens were prepared along with healthy root antigens and reacted with PAbs of *M. phaseolina* for comparison. Absorbance values were higher in those root samples which showed susceptible reaction when tested against root pathogens. Following inoculation with the pathogens absorbance values were always higher in artificially inoculated plant roots in comparison with healthy root antigens when tested against PAbs of the respective pathogens (Table 5).

Western blot analysis using PAb of *M. phaseolina* was also performed to develop strategies for rapid detection of the pathogen. For this total soluble protein of young mycelia was used as antigen source and SDS-PAGE was performed as described previously followed by probing of the localized antigen with alkaline phosphatase conjugate. The bands on nitrocellulose membrane was compared with corresponding protein bands on the SDS-PAGE. Bands of varying intensities was observed ranging from 14 KDa to 95 KDa (Figure 1. K&L). Bands of lower molecular weight were more in number. Hence the result suggests that Western blot formats could be used as one of a refined tool for detection of pathogen. Indirect immunoflorescence of hyphae and young sclerotia of *M. phaseolina* were conducted with homologus antibody (PAb of *M. phaseolina*) and reacted with fluorescein isothiocyanate (FITC) labeled antibodies of goat specific for rabbit globulin. Strong apple green fluorescence were evident in both mycelia and scletoria which confirmed the detection of the pathogen (Figure 1. M&N).

Identification of M. Phaseolina using rDNA sequence

Genomic DNA of *M. phaseolina*, the isolate obtained from mandarin root tissue collected from Mirik orchard, the causal organism of root rot of *Citrus reticulata* was amplified. Main focus was on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *M. phaseolina*. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 for sequencing of its 18S rDNA region. PCR products produced sequences and chromatogram and 18S rDNA sequence of *M. phaseolina* that could be aligned and showed satisfactory homology with ex-type strain of *M. phaseolina* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 1 region. ITS1 showed the highest number of nucleotide substitutions, and it was used for the phylogenetic study.

Phylogenetic analysis

Studies involving isolates of *M. phaseolina* revealed that the partial sequence of ITS1- 5.8S-ITS4 rRNA gene is as variable as rDNA regions. The sequence information was then analysed through BLASTn program which indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *M. phaseolina* with 100% similarity. This sequence has been deposited to NCBI genebank to get accession number.

Identified *M. phaseolina* rDNA gene sequences obtained from NCBI genebank of various host plants were selected for comparison with the rDNA gene sequence of *M. phaseolina* isolate of mandarin plant. The sequence alignment of the isolate of *M. phaseolina* shows variation in this gene. These available sequences of *M. phaseolina* from NCBI were used in the pair wise and multiple sequence alignment using Bioedit software (Figure 3) for determining the conserved regions of rDNA gene. This partial sequence was deposited to NCBI database (Acc. No.JN241996) (Table 6).

Multiple sequence alignment revealed that there were quite a number of gaps introduced in the alignment within the ITS region which were closely related. Similar sequence indicated that the isolates were closely related. From the sequence alignment, variations were observed between other *M. phaseolina* isolates in species level. Multiple and pair wise sequence alignment were generated and used to calculate evolutionary distances and percent of sequence similarity values (Table 7) and to construct a phylogenetic tree.

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.56368608 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 294 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Figure 4).

PAbs raised against *M. phaseolina* was tested with homologous and heterologous antigens of mandarin roots. Strong precipitation reactions occurred in homologous reactions in immunodiffusion test. Among the root antigens of mandarin plants of eight different locations tested against PAbs of *M. phaseolina*, strong and positive reactions were noticed in root antigens of four specific locations. Previous studies have also suggested that common antigens may be indicators of plant host-parasite compatibility (Chakraborty, 1988). Optimization of ELISA was done by considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity. ELISA values increased with a concomitant increase of antigens concentrations. Concentration as low as 25 µg/L, could

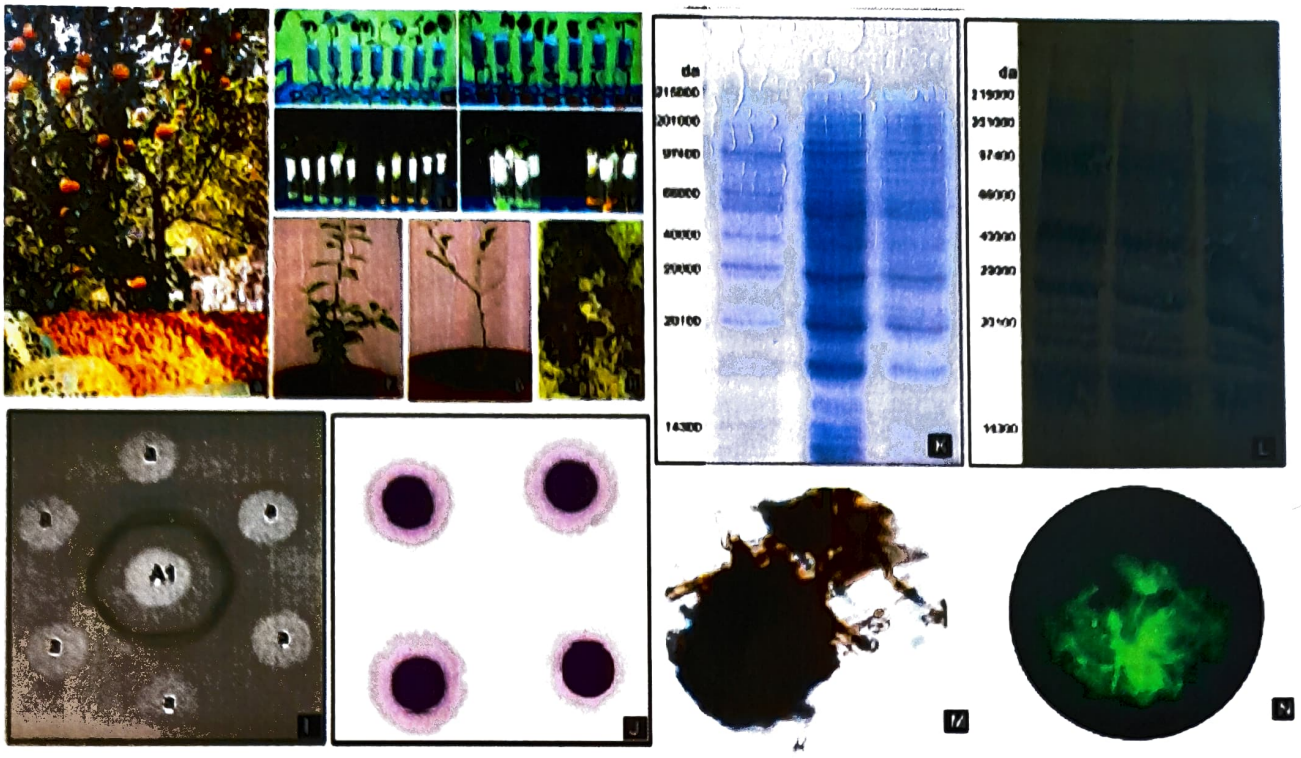

Figure 1: *C. reticulata* in Mirik orchard and harvested mandarin fruits in market place (A): Mandarin seedlings grown in distilled water (B, D & E - left) and culture filtrate of *M. phaseolina* (C, D & E - right); Healthy mandarin plants in pots (F), Infected mandarin plants showing symptoms 30 days following artificial inoculation with *M. phaseolina* (G); *M. phaseolina* (root rot pathogen) (H); Immunodiffusion (I, Dot immunobinding assay (J), SDS PAGE analysis (K) and Western blot analysis(L) of *M. phaseolina* using mycelial antigen (a) and PAb of M. phaseolina (A1); Microscopic observation of *M. phaseolina* under bright field (M). Mycelia of *M. phaseolina* treated with PAb of the pathogen and labeled with FITC (N)

Table 3: Effect of different pH on the growth of *M. phaseolina*

pH	Average dry weight of mycelia (mg)
4.0	310.50 ± 2.33
4.5	581.25 ± 3.40
5.0	616.00 ± 3.12
5.5	695.00 ± 2.61
6.0	437.55 ± 2.53
6.5	409.32± 3.86
7.0	325.12± 2.63
8.0	310.33± 1.95

Average of 3 replicates/treatment; Temperature 28°C; Incubation time – 12 days

Table 4: Effect of different temperature on the growth of *M. phaseolina*

Temperature (°C)	Average dry weight of mycelia (mg)
15	95.00 ± 2.43
20	181. 05 ± 2.40
25	246.00 ± 3.22
28	595.00 ± 2.82
30	737.55 ± 3.53
35	665.42± 2.85
40	125.25± 3.63

Average of 3 replicates/treatment; pH adjusted to 5.5 ; Incubation time – 12 days

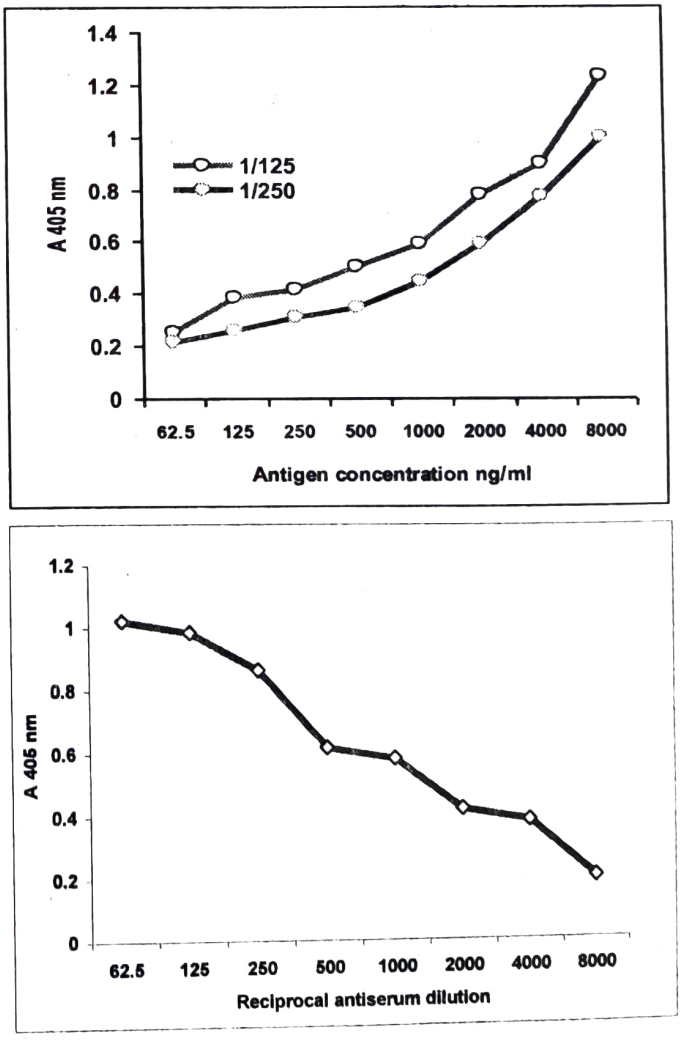

Figure 2: Optimization of ELISA by considering two variables, dilution of the antigen extract (A) and dilution of the antiserum (B)

Table 5: PTA-ELISA values showing reaction of PAbs of *M. phaseolina* with antigens of healthy and inoculated roots of *C. reticulata*

Citrus saplings	Antigen concentration (40 mg/L)	
Locality	Healthy	Inoculated *M.phaseolina*
Rangli Rangliot	0.812	1.264
Bijanbari	0.890	1.149
Sukhia Pokhari	1.115	1.774
Kurseong	0.972	1.880
Mirik	1.064	1.993
Kalimpong Block I	1.007	1.887
Kalimpong Block II	1.187	1.932
Gorubathan	0.938	1.872

PAb of *M. phaseolina* was used at 1:125 dilution; 7 days after inoculation; absorbance at 405 nm

be easily detected by ELISA, in both antisera dilutions. In time course experiment involving artificial inoculation of roots with the fungal pathogen (s).infection could be detected from 20h onwards in ELISA on the basis of significantly higher (p = 0.01) absorbance values of infected root extracts in comparison with healthy root extracts. Absorbance values in PTA-ELISA were also significantly higher for infected root extracts than for healthy controls up to a concentration of 2mg/L. Kitagawa *et al.* (1989) successfully used a competitive ELISA technique to develop an assay to identify *F. oxysporum* f. sp. *cucumerinum* among other *Fusaria*. Mycelial antigen of

Table 6: NCBI GenBank sequences of *M. phaseolina*

Acc. No.	Sequences	Country
JN241996	**310bp**	**India**
DQ314733	527 bp	India
DQ233666	495 bp	India
DQ233664	441 bp	India
DQ233663	519 bp	India
DQ233662	432 bp	India
EU250575	582 bp	China
HQ713771	511 bp	Switzerland
HQ380051	685 bp	Turkey
HM990163	534 bp	India
EF446288	562 bp	India
HQ660591	583 bp	China
HQ660590	584 bp	China
HQ660589	583 bp	China
JF710587	583 bp	China
FJ960442	582 bp	China
EF570501	642 bp	Canada

the pathogen was analysed on SDS-PAGE and then western blot analysis was done using homologous PAb.

Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiological techniques, as a number of constraints are removed. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. Differences in the nucleotide composition of the variable ITS region have been successfully employed to

Table 7: Similarity of rDNA sequences within groups of *M. phaseolina*

	2JN241990	DQ233663	HM990163	HQ660594	HQ660593	HQ660592	HQ660591	HQ660590	HQ660589	JF710587	DQ233662	EF446288	HQ713771	J960442	EU250575	EF570501	DQ314733	DQ233666	DQ233664	HQ380051
JN241996																				
DQ233663	0.35																			
HM990163	0.26	0.55																		
HQ660594	0.26	0.55	0.00																	
HQ660593	0.22	0.55	0.00	0.00																
HQ660592	0.25	0.60	0.00	0.00	0.00															
HQ660591	0.23	0.47	0.00	0.00	0.01	0.01														
HQ660590	0.34	0.27	0.10	0.10	0.11	0.13	0.07													
HQ660589	0.26	0.52	0.00	0.00	0.01	0.01	0.00	0.09												
JF710587	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00											
DQ233662	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00										
EF446288	0.25	0.42	0.01	0.01	0.02	0.02	0.00	0.04	0.01	0.01	0.01									
HQ713771	0.32	0.67	0.01	0.01	0.01	0.01	0.02	0.14	0.02	0.01	0.01	0.03								
J960442	0.33	0.43	0.03	0.03	0.04	0.05	0.02	0.02	0.03	0.03	0.03	0.01	0.05							
EU250575	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03						
EF570501	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00					
DQ314733	0.89	0.15	0.94	0.94	0.98	1.02	0.83	0.49	0.88	0.94	0.94	0.76	1.11	0.72	0.94	0.94				
DQ233666	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00	0.00	0.94			
DQ233664	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00	0.00	0.94	0.00		
HQ380051	0.26	0.55	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.01	0.01	0.03	0.00	0.00	0.94	0.00	0.00	0.00

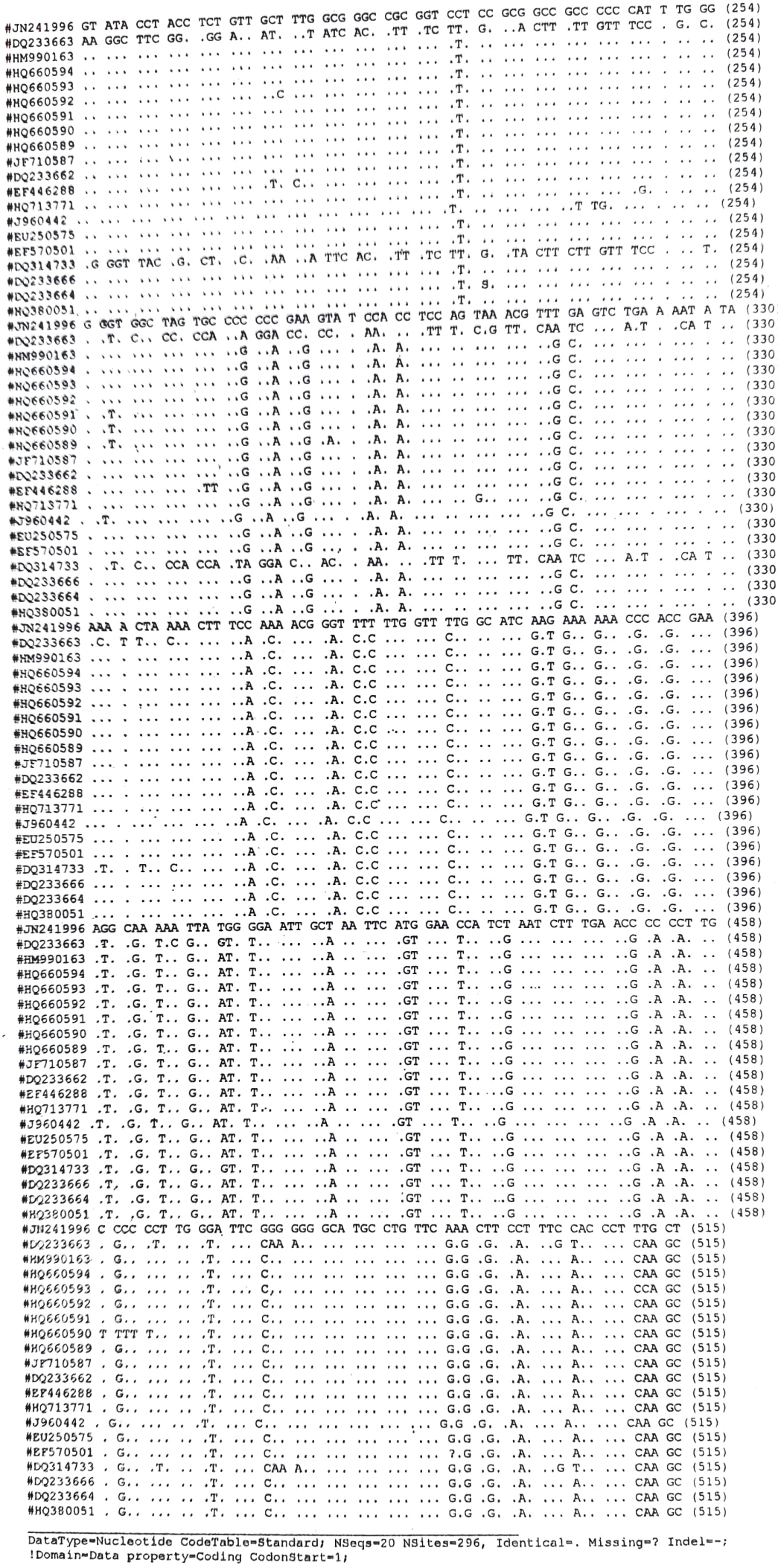

Figure 3: Nucleotide sequence alignments of the parts of the rDNA repeats encoding ITS region of different isolates of *M. phaseolina* used for analysis

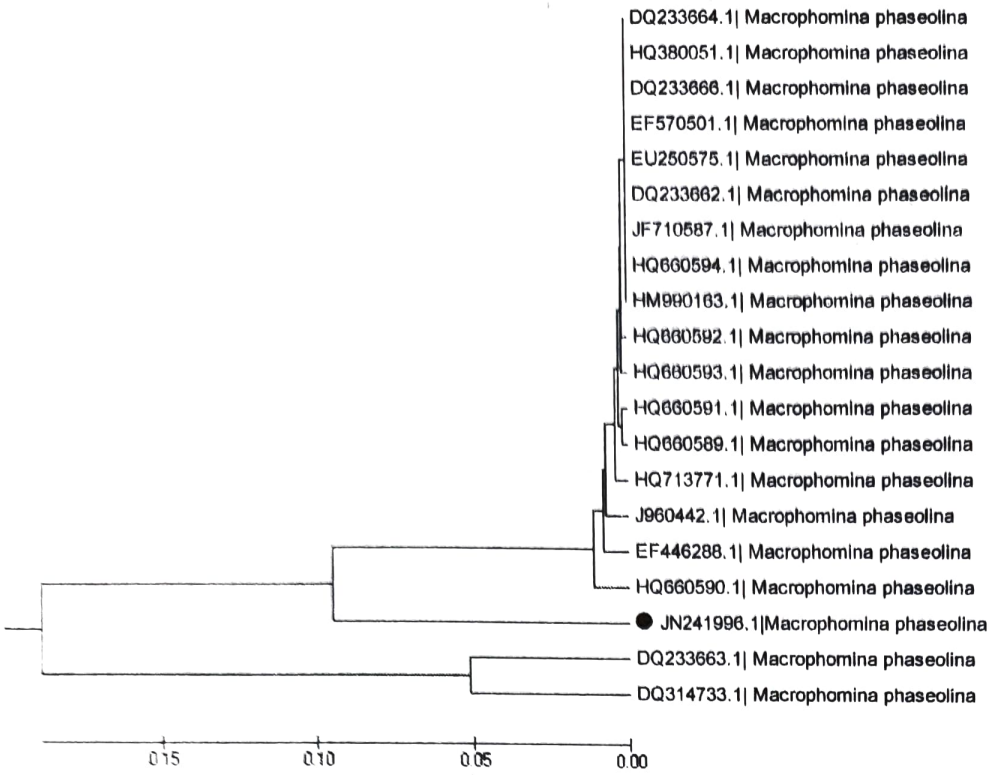

Figure 4: Phylogenetic placement of *Macrophomina phaseolina* (RHS/S565) with extype strains from NCBI genebank

design specific primer sets that amplify DNA selectively among and within species of plant pathogens (Nazar *et al.* 1991, Moukahmedov *et al.* 1994, Schilling *et al.* 1996, Moricca *et al.* 1998). In the broader context, taxon -selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al.* 1995). These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al.* 1991) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes.

In the present study, ITS regions of ribosomal genes for the construction of primers were used to identify *M. phaseolina.* ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers for *M. phaseolina.* Amplified products of size in the range of 550bp were produced by the primer pairs.

Amplification of DNA fragments of *M. phaseolina* with specific primers indicate the usefulness of molecular technique for their detection and identification. Using the specific primers ITS 1 and ITS 4, only a single band of 550 bp was generated in the amplification pattern of all the isolates. *M. phaseolina* as first described by (Pearson *et al.* 1986) suggested that isolates from one specific host are more suited to colonize it. Later, differences in pathogenicity among the isolates of soybean and sorghum have been observed (Cloud and Rupe 1991). This has been further confirmed with isolates from soybean, sorghum and cotton (Su *et al.* 2001). Isolates were clearly grouped according to the host origin. Additionally, no molecular variation could be observed among the isolates tested in PCR of the ITS region. The overall study was established the rDNA gene sequence of *M. phaseolina* a root rot pathogen of *C. reticulata* based on the bioinformatics tools with sequence analysis of ITS regions of the rDNA gene for rapid identification and development of rDNA markers for analysis of genetic variability within the outgrops. We have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, the present study shows that it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database. Cloud and Rupe (1991) working with isolates of soybean and sorghum, also observed differences in pathogenicity. This has been further confirmed with isolates from soybean, sorghum and cotton (Su *et al.* 2001). Isolates were clearly grouped according to the host origin. Additionally, no molecular variation could be observed among the isolates tested in PCR of the ITS region.

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Efficiency and effectiveness of physical and chemical mutagens in *Trigonella foenum graecum* L.

Jinu Devi Rajkumari
Department of Botany, Cotton College. Guwahati-1, Assam, India. E-mail: jinudrajkumari@yahoo.in

Abstract
Trigonella foenum graecum L was utilized to study the Chlorophyll mutations, mutagenic efficiency and effectiveness by physical mutagen gamma rays and chemical mutagen EMS. The frequency of chlorophyll mutation in M_1 & M_2 generation were more in gamma treated plants but the mutation spectra and mutagenic efficiency of EMS was higher than gamma. The most efficient mutagens were 0. 02% EMS, 0. 06% EMS and 3KR gamma radiation to induce mutation in *T. foenum graecum* L.

Keywords: Mutagens, Mutation, Mutation efficiency, mutagenic effectiveness

Mutation breeding has been widely used for crop improvement by developing new plant types with superior genotypes and better adaptive potentiality. Induced mutation have contributed a lot for creating additional variability and to the development of new cultivars (Broertjes and Harten, 1978). Different types of physical and chemical mutagens have been successfully employed to introduce mutation and more than eight hundred commercial cultivars have been released (Novak and Micke, 1987).

Trigonella foenum graecum L. is a small seeded, self pollinated annual herb which is having very high nutritional and medicinal values. The plant is also having insect and pest repellent property (Billaud and Adrian, 2001). Being a self pollinated crop the plant is having less genetic variability and it can be increased by adopting mutation breeding programme. Since the frequency of mutation depends on efficiency and effectiveness of mutagens, therefore an attempt has been made to estimate the efficiency & effectiveness of physical mutagen gamma rays and chemical mutagen EMS on *T. foenum graecum* L.

Materials and Methods

Seeds of *T. foenum graecum* L cv. PEB were subjected to gamma irradiation and EMS treatment. For gamma irradiation 1000seeds of uniform size and moisture content 11. 5% were arranged in monolayer and subjected to acute dose of 1KR, 2KR, 3KR, 4KR &5KR at. BBCI, Guwahati, Assam. The source of gamma is Cobalt ^{60} and average energy is 1. 25 Mev. For chemical mutagen the seeds were presoaked in distilled water for 12 hours then treated with freshly prepared aqueous solution of EMS (0. 02%, 0. 04 %, 0. 06%, 0. 08% &0. 1%) for 8hrs. Then the seeds were washed thoroughly with water to avoid residual effect of mutagen. Immediately after treatment seeds were sown in randomized block design (30 ×7. 5cm spacing) with three replications to raise M_1. M_1 plants were harvested individually and grown to raise M_2 as progeny rows. The biological effects of different treatments evaluated in 200 plants each of M_1 & M_2 generations. Seed germination, seedling survival percentage and percentage of chlorophyll mutation were scored on M_1 plant progeny and M_2 population basis. The mutagenic

Table 1: Frequency of chlorophyll mutants (on M_1 plant progeny & M_2 population basis), mutagenic efficiency and mutagenic effectiveness in treated and control population of *T. foenum graecum* L.

Treatments & dose	Total no. of M_1/M_2 plants scored	M_1 mutant frequency%	M_2 mutant frequency%	Mutagenic efficiency	Mutagenic effectiveness
Control	200/200	-	-	-	-
Gamma rays					
1KR	200/200	11.2	1.75	0.37	1.33
2KR	200/200	19.6	2.11	0.4	1.27
3KR	200/200	20.8	5.5	0.44	0.9
4KR	200/200	20.3	2.97	0.37	0.65
5KR	200/200	19.4	1.77	0.36	0.5
EMS					
0.02%	200/200	3.6	1.52	0.46	22.5
0.04%	200/200	8.4	1.99	0.58	26.3
0.06%	200/200	9.5	4.92	0.62	21.7
0.08%	200/200	11.6	1.98	0.55	18.1
0.10%	200/200	12.9	1.4	0.56	16.1

Table 2: Frequency & spectrum of chlorophyll mutations (M_1 plants progeny basis) in *T. foenum graecum* L.

Treatment & dose	Chlorophyll mutations			Total %
	Albino	Chlorina	Straita	
Gamma ray				
1KR	5.2	4.3	1.7	11.2
2KR	7.1	7.8	4.7	19.6
3KR	18.6	2.2	-	20.8
4KR	16.8	3.8	-	20.3
5KR	19.4	-	-	19.4
EMS				
0.02%	2.0	1.2	0.4	3.6
0.04%	3.1	5.3	-	8.4
0.06%	2.4	4.7	2.4	9.5
0.08%	6.2	3.5	1.9	11.6
0.1%	-	8.9	4.0	12.9
Control	-	-	-	-

efficiency and effectiveness were worked out following Konzak *et. al.* , 1965.

Results and Discussion

M_1 Generation

Seed germination and seedling survival percentage was progressively decreased with increase dose of gamma rays & EMS. Maximum reduction was observed at 5KR gamma and 0. 1% EMS. Sterility percentage was also positively correlated with mutagen doses and it may be due to degeneration of egg nucleus and abortion of embryo sac in mutagen treated plants. Frequencies of chlorophyll mutations were almost identical in different doses of gamma radiation except 1KR which showed lowest value 11. 2%. However, considerable increase of mutation frequencies were observed with increase dose of EMS and maximum was recorded in 0. 1 % (Table1). Dose dependent mutation were also reported earlier in several crops (Mitra and Bhowmik 1997; Venkateswarlu *et. al.*, 1988)

M_2 Generation

Gamma rays produced more viable mutants than EMS. Treatment with 3KR gamma showed highest mutation frequency and lowest was recorded in 0. 02% EMS. Relative frequency of chlorophyll mutation varied with dose of mutagen used. Although gamma rays created higher mutation frequency but gave narrow spectrum (Table 2) this result supported the findings of Kumar and Mani (1997). The present studies revealed that efficiency of EMS was markedly higher than the gamma rays in spite of gamma treatment recording high frequency of mutation than EMS. Higher mutagenic efficiency of EMS than gamma rays was reported earlier in rice (Kaul & Bhan, 1977). However, efficiency and effectiveness of a mutagen is also depends on genetic background and intracellular condition of the specimen treated. From the present investigation it can be concluded that most efficient mutagen doses are 0. 06% EMS, 0. 02%EMS and 3KR gamma ray for production of mutant in *T. foenum graecum* L.

Acknowledgement

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A homology model for 16S rRNA tertiary structure of *Frankia*

Sangita Saha[1], Saubashya Sur[1], Asim Bothra[2] and Arnab Sen[1]*

[1]NBU Bioinformatics Facility, Department of Botany, University of North Bengal, Siliguri-734013, West Bengal, India; [2]Cheminformatics Bioinformatics Laboratory, Department of Chemistry, Raiganj College (University College), Raiganj-733134, Uttar Dinajpur, West Bengal, India.

Abstract

Tertiary structure determination of biomolecule is important for knowing their function. Although different experimental prediction methods are present computational approach has an important role. *Frankia sp.*, is a nitrogen fixing bacterium promoting soil fertility.16S ribosomal nucleic acid molecule is not only important as a molecular signature for classification and identification of organisms but has a great impact on protein synthesis. Here we have developed 3D structure of 16S rRNA of three different strains of *Frankia* viz. *Frankia* sp. strain HFPCcI3, *Frankia alni* strain ACN14a and *Frankia* sp. strain EAN1pec through knowledge based coarsed to atomic approach. The occurrence of tertiary structural motifs like C-loop, E-loop, GNRA tetraloop, hook-turn, sarcin-ricin motif, K-turn, reverse K-turn were examined. The presence of such sort of motif stabilizes the complexed RNA structures through their Watson-Crick, Non-Watson Crick, tandem sheared stacking packages. Among them E-loop, K-turn, sarcin-ricin motif have significant functional importance for transporting the biomolecules, RNA-protein interaction, RNA-RNA interaction/RNA-drug interaction accordingly.

Keywords: Homology modeling, *Frankia*, 16S rRNA, GNRA, NMR,

The 16S rRNA acts as a novel model for not only taxonomic differentiation but also for understanding the clinical microbiology and infectious diseases and also for identifying the non-cultured bacteria (Clarridge III 2004). 16S rRNA genes can also be assigned as 16S rDNA genes. Both are in interchangeable form. It is most stable among all genetic material during evolution (Goudarzi *et al.* 2006) and thus is used for not only comparing among all bacterial world but also with the 16S rRNA genes of archeobacteria as well as 18S rRNA genes of eukaryotes (Clarridge III 2004). It also has functional association with protein synthesis and may directly or indirectly involve in the translational machineries to regulate the mRNA translation into proteins. But their function in protein synthesis is still unclear to us (Saraiya *et al.* 2009). There are limited numbers of tertiary structural information available in structural database relative to primary structure available in the sequence database. Since tertiary structure carry much more conserved structural-functional relationship in comparison to primary structure so there has a need to develop a 3D structure based analysis to explore its new look. Generally like protein the primary structure of RNA folds into its much more compact form and thus introduce the ribosomal subunit for interacting remotely. The building blocks of RNA structure are RNA-motifs. According to the Leontis *et al.* (2003) RNA motifs are the directed and the ordered arrays of non-WC base pairs forming distinctive folding of the phosphodiester backbones of the interacting RNA strands. The specific motif comprised with similar tertiary conformation with its similar sort of tertiary interaction i.e. they are much more conserved part of a structure. Here our main objective is to design 3D structure of 16S rRNA of three different *Frankia* strains from its 1D information. The three different *Frankia* strains are *Frankia alni* ACN14a, *Frankia sp.* CcI3 and *Frankia sp.* EAN1pec. *Frankia sp.* are facultative symbiont of actinorhizal plants. By late 19's century scientists have discovered that it has a capability to fix environmental nitrogen into the root nodules of non-leguminous plants (Benson and Silvester 1993). Thus it may arise its importance in ecological as well as in agricultural field in future. It can be used as a reducer of soil-droughts. Nitrogen fixative genes share a common evolutionary history with 16S rRNA. So the development of tertiary structure is much more prevalent over its primary one. Homology model of 16S rRNA structure was generated through *In-silico* approach. There are a number of solved 16S rRNA structure of different bacteria existed in structural database. But they were predicted through crystallography or NMR method. Both are time consuming and expensive one to use where as computational approach not only reduces the cost but also a faster one in this new emerging field.

Materials and Methods

The 16S rRNA gene sequences in FASTA format for the following organisms were taken from IMG database (http://img.jgi.doe.gov/). They are *Frankia alni* ACN14a, *Frankia sp.* CcI3 and *Frankia sp.* EAN1pec.

The secondary structures of all 16S rRNA were predicted through MFold web server of Mr. Zuker Version 2.3(http://mfold.rna.albany.edu//?q=mfold/RNA

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

Table 1: Number of loop, helix and single strand segment

Strain	Loop	Helix	SSS*
ACN14a	23	58	111
CcI3	18	51	100
EAN1pec	20	54	102

*SSS=single strand segment

-Folding-Form2.3). The folding temperature was changed into 30°C and other default parameters were used. The coarsed-grained structures of each all were generated by NAST (Nucleic Acid Simulation Tool). The full atomic structure of the above said generated coarse-grained structure were further evolved through the coarse to atomic package of NAST. Finally full 3D structure of 16S rRNA of the above three strains were obtained and here I have used 2QAL (PDB ID) as reference 16S rRNA model for structure prediction through homology modeling concept. 2QAL contains the 16S rRNA of *E. coli*. Energy Minimization of the generated 3D structure was performed with Gromacs package where it was modified with Amber Force Field 99 which is a nucleic acid supported force field. We have used the Gromacs 4.0.7 and fftw of 3.3.2 version in Ubuntu Linux Platform. The above three structures were energy minimized in Vacuum to get a much more stabilized and accepted form. The structures were then further validated through RCSB validation server (http://deposit.pdb.org/validate/). The structures were then annotated by RNAview software package which reveals their intra-atomic configuration in detail. Again the inbuilt tertiary structural features were studied from the generated 3D structures through RNAMotifScan to get into the structural-functional relationship.

Results and Discussion

We restricted our study to three different strains of *Frankia sp.* to understand their structural inbuilt of 16S rRNA for their functional importance at 3D level relative to 1D. According to the analysis of primary structure so far studied, it is revealed that among the *Frankia alni* ACN14a, *Frankia sp.* CcI3 and *Frankia sp.* EAN1pec the 16S rRNA primary structure is 97.8-98.9% conserved signifies their evolutionary sequence level conservation irrespective to the other factors (Mastronunzio *et al.* 2008) The generated 2D structure have their Gibb's free energy of -668.48kcal/mol, -662.44kcal/mol and -661.24kcal/mol for 16S rRNA of *Frankia alni* ACN14a, *Frankia sp.* CcI3 and *Frankia sp.* EAN1pec respectively at 30 °C. The negative Gibb's free energy of the generated secondary structures reveal about their stable conformation. The stable 2D conformation selection for tertiary structure prediction is much more important as it incorporate the primary building blocks like helix, loop.

These secondary structures were further used to develop the tertiary one. We have developed 3D structure of 16S rRNA of three different strains of *Frankia sp.* through knowledge based coarsed to atomic approach by NAST and c2a package (Jonikas *et al.* 2009). The energy minimized homology model have their potential energy of 1.505e+05 kJ/mol, 3.09e+05kJ/mol and 1.69e+kJ/mol for 16S rRNA of *Frankia alni* ACN14a, *Frankia sp.* CcI3 and *Frankia sp.* EAN1pec respectively. The validated report shows that the structures have 10-15% of outliers need to refine them in future as it is out of scope of present platform available so far. The annotated analysis of these partially stabilized structure shows their brief details of intra-atomic configuration which are given in a Table 1a,1b and 1c .

We analyzed the building blocks of RNA architecture i.e. the 3D motifs in these experimentally derived 16S rRNA tertiary structure. The result demonstrate that each of the three generated model consists of C-loop, E-loop, GNRA, K-turn, reverse K-turn, sarcin-ricin, hook-turn like most conserved 3D building blocks which are shown by Table 2. The presence of number of C-loops in all three structures show that the bases in the longer strand form non-WC base pairs with the bases in the shorter strand. There are also unpaired bases present as extruded. They have their unique functional importance in living creatures. Structural stability and conservation depends on the presence of structural motifs like C-loop, E-loop, GNRA, K-turn, reverse K-turn, hook-turn.

C-loop motif is an internal loop which is asymmetrical in nature. The presence of C-loop in the stem-loop structure reveals its importance in the tertiary interaction between the two hairpins. It also helps to twist the helical stem between two WC base pairs (Lescoute *et al.* 2005). Here the presence of WC base-pair acts as helix twister. The complex form of its tertiary nature stabilizes the 3D structure of the whole molecule. C-loop in 16S rRNA has affinity to interact with protein and it also acts as a complex in between the threonine synthetase and the synthetase itself. Thus it performs certain sort of cell regulation (Leontis and Westhof 2003).

GNRA is tetraloop in nature. In GNRA N stands for any nucleic acid and R stands for purine bases. It has a functional importance for tetraloop-tetraloop receptor interactions. It is thermodynamically stable in nature relative to the other tetraloops. There are high percentage of GNRA exists in RNA molecules e.g. 70% of tetraloop belongs to GNRA/ UNCG family in ribosomal RNA (Hendrix *et al.* 2005).

K-turns or kinks are recurrent internal loops which produce pointed kinks or twist in the helical region in

Table 2 Overview of base pair types

Strain	Base pair types												
	WW		HH		SS		WH		WS		HS		standard
	cis	trans	cis	trans	cis	trans	cis	trans	cis	trans	cis	trans	
ACN14a	108	6	1	1	1	0	5	27	8	3	5	13	95
CcI3	94	10	0	0	1	1	3	30	8	5	3	26	76
EAN1pec	90	6	0	0	2	3	2	30	5	5	8	27	83

Table 3 Glycosyl-conformation (syn base-position) in 16SrRNA of *Frankia*

Strain	Syn base-position
ACN14a	77, 93, 101, 114, 146, 147, 156, 169, 192, 207, 221, 248, 250, 254, 264, 276, 300, 301, 315, 331, 347, 366, 380, 381, 383, 388, 405, 407, 423, 446, 447, 489, 490, 503, 515, 516, 526, 554, 596, 597, 608, 610, 623, 628, 640, 642, 658, 667, 680, 707, 715, 734, 780, 819, 820, 822, 829, 834, 839, 882, 891, 929, 997, 1000, 1032, 1038, 1099, 1100, 1138, 1168, 1209, 1210, 1234, 1261, 1262, 1264, 1291, 1293, 1301, 1334, 1336, 1343, 1346, 1392, 1397, 1400, 1410, 1420, 1471, 1487, 1489
CcI3	20, 28, 29, 31, 38, 47, 62, 64, 112, 114, 122, 126, 127, 137, 141, 209, 218, 229, 247, 254, 286, 307, 308, 311, 323, 324, 325, 367, 378, 418, 425, 427, 507, 552, 562, 584, 586, 612, 630, 642, 689, 713, 714, 729, 733, 751, 753, 766, 767, 790, 794, 822, 823, 824, 825, 833, 850, 851, 852, 853, 857, 866, 870, 916, 931, 934, 945, 946, 971, 973, 1049, 1074, 1098, 1133, 1154, 1161, 1164, 1178, 1185, 1186, 1197, 1198, 1203, 1215, 1221, 1231, 1232, 1235, 1246, 1249, 1265, 1266, 1282, 1294, 1295, 1312, 1321, 1334, 1341, 1344, 1345, 1362, 1364, 1403, 1407, 1429, 1454, 1463
EAN1pec	16, 17, 26, 27, 59, 82, 100, 101104, 114, 116, 129, 145, 168, 171, 184, 185, 187, 191, 208, 211, 213, 223, 226, 253, 272, 304, 305, 314, 374, 414, 415, 419, 423, 477, 483, 488, 515, 539, 559, 596, 596, 596, 598, 653, 656, 658, 724, 773, 783, 850, 859, 866, 867, 894, 895, 897, 927, 950, 995, 1003, 1014, 1029, 1055, 1062, 1065, 1079, 1088, 1103, 1104, 119, 1124, 1127, 1146, 1156, 1158, 1186, 1213, 1215, 1221, 1222, 1225, 1290, 1297, 1324, 1340, 1344, 1347, 1363, 1374, 1375, 1388, 1393, 1411, 1436, 1440, 1442, 1446, 1463, 1464, 1465, 1466, 1479, 1481

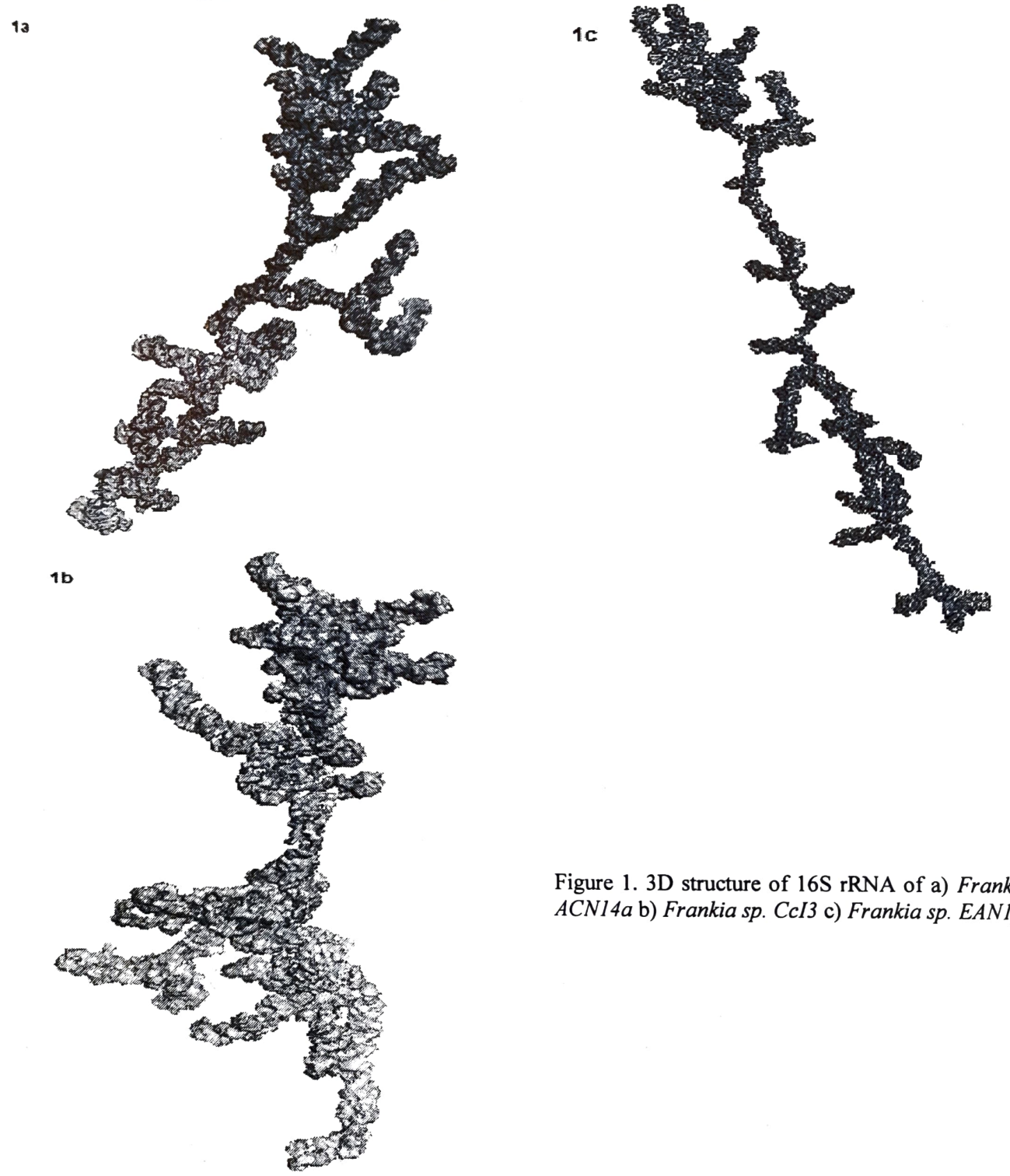

Figure 1. 3D structure of 16S rRNA of a) *Frankia alni ACN14a* b) *Frankia sp. CcI3* c) *Frankia sp. EAN1pec*

Table 4 Structural motifs for 16srRNA of *Frankia*

Tertiary Motif	ACN14a	CcI3	EAN1pec
C-Loop	X409-X423/X392-X405	Y639-Y646/Y676-Y686	Z554-Z572/Z582-Z598
	X1164-X1174/X1149-X1158	Y1163-Y1177/Y1145-Y1160	Z243-Z253/Z225-Z238
	X128-X143/X149-X168	Y1062-Y1067/Y1038-Y1054	Z944-Z948/Z1173-Z1177
	X1090-X1095/X34-X41	Y775-Y790/Y899-Y911	Z480-Z497/Z468-Z479
	X1448-X1456/X1394-X1403	Y1303-Y1321/Y1334-Y1350	Z160-Z168/Z175-Z183
	X661-X677/X645-X657	Y552-Y558/Y609-Y616	Z546-Z552/Z257-Z263
	X1476-X1482/X1489-X1495	Y105-Y121/Y93-Y115	Z361-Z364/Z353-Z356
	X785-X796/X762-X773	Y410-Y421/Y394-Y405	Z502-Z514/Z522-Z538
	X3-X7/X1370-X1374	Y699-Y710/Y625-Y635	Z694-Z705/Z613-Z624
	X183-X191/X170-X178	Y1256-Y1262/Y1207-Y1213	Z802-Z812/Z790-Z795
	X302-X306/X319-X323	Y1430-Y1447/Y1388-Y1405	Z634-Z649/Z652-Z670
	X589-X607/X563-X582	Y518-Y530/Y156-Y166	Z988-Z1001/Z952-Z970
	X225-X231/X214-X219	Y639-Y646/Y676-Y686	Z1277-Z1293/Z1258-Z1274
	X1278-X1290/X1307-X1325	Y1163-Y1177/Y1145-Y1160	Z554-Z572/Z582-Z598
	X884-X898/X844-X850	Y1062-Y1067/Y1038-Y1054	Z243-Z253/Z225-Z238
	X1108-X1118/X1125-X1139	Y775-Y790/Y899-Y911	Z944-Z948/Z1173-Z1177
	X409-X423/X392-X405	Y1303-Y1321/Y1334-Y1350	Z480-Z497/Z468-Z479
	X1164-X1174/X1149-X1158	Y552-Y558/Y609-Y616	Z160-Z168/Z175-Z183
	X128-X143/X149-X168	Y105-Y121/Y93-Y115	Z546-Z552/Z257-Z263
	X1090-X1095/X34-X41	Y410-Y421/Y394-Y405	Z361-Z364/Z353-Z356
	X1448-X1456/X1394-X1403	Y699-Y710/Y625-Y635	Z502-Z514/Z522-Z538
	X661-X677/X645-X657	Y1256-Y1262/Y1207-Y1213	Z694-Z705/Z613-Z624
	X1476-X1482/X1489-X1495	Y1430-Y1447/Y1388-Y1405	Z802-Z812/Z790-Z795
	X785-X796/X762-X773	Y518-Y530/Y156-Y166	Z634-Z649/Z652-Z670
	X3-X7/X1370-X1374		Z988-Z1001/Z952-Z970
	X183-X191/X170-X178		Z1277-Z1293/Z1258-Z1274
	X302-X306/X319-X323		
	X589-X607/X563-X582		
	X225-X231/X214-X219		
	X1278-X1290/X1307-X1325		
	X884-X898/X844-X850		
	X1108-X1118/X1125-X1139		
	X409-X423/X392-X405		
	X1164-X1174/X1149-X1158		
	X128-X143/X149-X168		
E-Loop	X251-X255/X241-X247	Y474-Y502/Y170-Y195	Z225-Z238/Z243-Z253
	X563-X571/X599-X607	Y495-Y502/Y170-Y175	Z1075-Z1080/Z1106-Z1111
	X1370-X1374/X3-X7	Y411-Y419/Y396-Y404	Z833-Z836/Z779-Z782
	X1476-X1481/X1490-X1495	Y236-Y243/Y255-Y257	Z1478-Z1487/Z1465-Z1473
	X225-X231/X214-X219	Y23-Y31/Y1181-Y1188	Z546-Z551/Z258-Z263
	X413-X422/X393-X402	Y302-Y308/Y317-Y323	Z808-Z811/Z791-Z795
	X302-X306/X319-X323	Y518-Y524/Y160-Y166	Z228-Z234/Z246-Z251
	X645-X651/X671-X677	Y552-Y557/Y610-Y616	Z694-Z704/Z615-Z624
	X56-X62/X962-X967	Y845-Y847/Y894-Y896	Z510-Z514/Z522-Z526
	X333-X335/X326-X328	Y676-Y686/Y640-Y646	Z1173-Z1177/Z944-Z948
	X183-X191/X170-X178	Y699-Y706/Y630-Y635	Z749-Z751/Z744-Z746
	X844-X848/X895-X898	Y590-Y593/Y579-Y582	Z468-Z476/Z486-Z497
	X409-X414/X401-X405	Y41-Y43/Y1092-Y1094	Z1277-Z1284/Z1266-Z1274
	X280-X283/X286-X289	Y111-Y115/Y117-Y121	Z160-Z167/Z176-Z183
	X589-X591/X580-X582	Y775-Y779/Y907-Y911	Z906-Z909/Z1188-Z1191
	X251-X255/X241-X247	Y186-Y193/Y478-Y484	Z353-Z356/Z361-Z364
	X563-X571/X599-X607	Y1317-Y1321/Y1334-Y1338	Z1052-Z1054/Z1043-Z1045
	X1370-X1374/X3-X7	Y1274-Y1283/Y1286-Y1295	Z1106-Z1111/Z1075-Z1080
	X1476-X1481/X1490-X1495	Y105-Y109/Y93-Y97	Z243-Z249/Z231-Z238
	X225-X231/X214-X219	Y1049-Y1054/Y1062-Y1066	Z510-Z514/Z522-Z526
	X413-X422/X393-X402	Y1388-Y1390/Y1445-Y1447	Z1188-Z1191/Z906-Z909
	X302-X306/X319-X323	Y643-Y646/Y676-Y679	Z1173-Z1177/Z944-Z948
	X645-X651/X671-X677	Y1334-Y1338/Y1317-Y1321	Z257-Z262/Z547-Z552

Continued to next page

Table 4 *continued*

	X56-X62/X962-X967	Y590-Y593/Y579-Y582	Z808-Z811/Z791-Z795
	X333-X335/X326-X328	Y170-Y175/Y495-Y502	Z694-Z704/Z615-Z624
	X183-X191/X170-X178	Y474-Y483/Y187-Y195	Z361-Z364/Z353-Z356
	X844-X848/X895-X898	Y552-Y557/Y610-Y616	Z468-Z476/Z486-Z497
	X409-X414/X401-X405	Y845-Y847/Y894-Y896	Z1277-Z1284/Z1266-Z1274
	X280-X283/X286-X289	Y117-Y121/Y111-Y115	Z160-Z167/Z176-Z183
	X589-X591/X580-X582	Y699-Y706/Y630-Y635	Z1052-Z1054/Z1043-Z1045
	X251-X255/X241-X247	Y775-Y779/Y907-Y911	Z589-Z598/Z554-Z561
	X563-X571/X599-X607	Y396-Y404/Y411-Y419	Z582-Z586/Z568-Z572
	X1370-X1374/X3-X7	Y22-Y25/Y1186-Y1189	Z634-Z640/Z664-Z670
	X1476-X1481/X1490-X1495	Y41-Y43/Y1092-Y1094	Z1075-Z1080/Z1106-Z1111
	X225-X231/X214-X219	Y254-Y257/Y235-Y243	Z944-Z948/Z1173-Z1177
	X413-X422/X393-X402	Y1445-Y1447/Y1388-Y1390	Z228-Z234/Z246-Z251
	X333-X335/X326-X328	Y1049-Y1054/Y1062-Y1066	Z522-Z526/Z510-Z514
	X1476-X1481/X1490-X1495	Y317-Y323/Y302-Y308	Z906-Z909/Z1188-Z1191
	X214-X219/X225-X231	Y93-Y97/Y105-Y109	Z257-Z262/Z547-Z552
	X302-X306/X319-X323	Y1286-Y1295/Y1274-Y1283	Z619-Z624/Z694-Z700
	X580-X582/X589-X591	Y522-Y530/Y156-Y162	Z792-Z795/Z802-Z810
	X393-X402/X413-X422	Y1163-Y1169/Y1154-Y1160	Z486-Z497/Z468-Z476
	X895-X898/X844-X848	Y676-Y686/Y640-Y646	Z1043-Z1045/Z1052-Z1054
	X599-X607/X563-X571	Y495-Y502/Y170-Y175	Z1266-Z1274/Z1277-Z1284
	X3-X7/X1370-X1374	Y236-Y243/Y255-Y257	Z175-Z182/Z161-Z168
	X645-X651/X671-X677	Y579-Y582/Y590-Y593	Z353-Z356/Z361-Z364
	X130-X138/X154-X162	Y1317-Y1321/Y1334-Y1338	Z554-Z561/Z589-Z598
	X170-X178/X183-X191	Y894-Y896/Y845-Y847	Z569-Z574/Z580-Z585
	X280-X283/X286-X289	Y609-Y616/Y552-Y558	Z664-Z670/Z634-Z640
	X454-X459/X425-X431	Y111-Y115/Y117-Y121	
	X34-X41/X1090-X1095	Y907-Y911/Y775-Y779	
	X69-X75/X943-X950	Y411-Y419/Y396-Y404	
	X1285-X1290/X1307-X1314	Y1092-Y1094/Y41-Y43	
		Y22-Y25/Y1186-Y1189	
		Y1274-Y1283/Y1286-Y1295	
		Y105-Y109/Y93-Y97	
		Y1049-Y1054/Y1062-Y1066	
		Y302-Y308/Y317-Y323	
		Y1388-Y1390/Y1445-Y1447	
		Y478-Y484/Y186-Y193	
		Y699-Y706/Y630-Y635	
GNRA	X281-X283/X286-X288	Y1157-Y1160/Y1163-Y1164	Z1272-Z1274/Z1277-Z1279
			Z744-Z746/Z749-Z751
K-turn	X409-X423/X392-X405	Y410-Y421/Y394-Y405	Z580-Z598/Z554-Z574
	X1489-X1495/X1476-X1482	Y631-Y645/Y677-Y704	Z522-Z538/Z502-Z514
	X225-X231/X214-X219		Z243-Z253/Z225-Z238
	X664-X689/X636-X655		Z1075-Z1080/Z1106-Z1111
			Z175-Z183/Z160-Z168
Hook-turn	X425-X431/X454-X459	Y474-Y483/Y187-Y195	Z1106-Z1111/Z1075-Z1080
	X943-X950/X69-X75	Y1317-Y1321/Y1334-Y1338	Z1181-Z1191/Z906-Z915
	X563-X571/X599-X607	Y105-Y109/Y93-Y97	Z547-Z552/Z257-Z262
	X214-X219/X225-X231	Y1445-Y1447/Y1388-Y1390	Z589-Z598/Z554-Z561
	X302-X306/X319-X323	Y552-Y558/Y609-Y616	Z230-Z235/Z245-Z251
	X409-X414/X400-X405	Y590-Y593/Y579-Y582	Z1435-Z1445/Z1388-Z1397
	X21-X31/X1178-X1189	Y1049-Y1054/Y1062-Y1066	Z944-Z948/Z1173-Z1177
	X789-X796/X762-X769	Y899-Y909/Y777-Y790	Z582-Z586/Z568-Z572
	X1476-X1481/X1490-X1495	Y317-Y323/Y302-Y308	Z522-Z526/Z510-Z514
	X183-X191/X170-X178	Y1286-Y1295/Y1274-Y1283	Z1277-Z1284/Z1266-Z1274
	X326-X328/X333-X335	Y640-Y646/Y676-Y686	Z1258-Z1268/Z1282-Z1293
	X130-X141/X150-X162	Y1163-Y1169/Y1154-Y1160	Z1043-Z1045/Z1052-Z1054
	X3-X7/X1370-X1374	Y894-Y896/Y845-Y847	Z791-Z795/Z802-Z811
	X671-X677/X645-X651	Y699-Y710/Y625-Y635	Z353-Z356/Z361-Z364
	X1307-X1320/X1282-X1290	Y394-Y402/Y413-Y421	Z614-Z624/Z694-Z705
	X1164-X1174/X1150-X1158	Y170-Y175/Y495-Y502	Z664-Z670/Z634-Z640
	X286-X289/X280-X283	Y1178-Y1188/Y23-Y31	Z277-Z280/Z271-Z274
	X580-X582/X589-X591	Y518-Y524/Y160-Y166	Z468-Z476/Z486-Z497
	X844-X848/X895-X898	Y1109-Y1112/Y1133-Y1135	Z160-Z167/Z176-Z183
		Y1430-Y1435/Y1398-Y1405	Z1029-Z1032/Z1037-Z1040
		Y117-Y121/Y111-Y115	Z833-Z836/Z779-Z782
		Y1256-Y1262/Y1207-Y1213	
		Y41-Y43/Y1092-Y1094	

the RNA structure. K-turn is generally helix-loop-helix element which also revealed in our designed structure. It presents in the minor groove side of 16S rRNA and thus helps to fold the RNA structure in more complexed form. The number of listed K-turns comprised with two stems of which one bears connonical base-pairs and the other with non-cannonical base-pair. It helps to come closer two minor groove. It has functional importance to produce local as well as long range tertiary interaction to stabilize the complexed RNA structure (Leontis and Westhof 2003) and may participate in protein-RNA interaction (Szep *et al.* 2003).

Reverse kink-turn produce bend at the major groove of complexed structure. Thus it recalled as independent RNA-motifs (Leontis and Westhof 2003).

Sarcin-ricin motif is generally prevalent in 23S rRNA but in some 16S rRNA also include such kind of motif for its functional importance. 16S rRNA of *Frankia* CCI3 *and* EAN1pec. has very low percentage of existence of this motif where as *Frankia* ACN14a haven't for a single one. It is also reported that *Frankia* sp. is antibiotic resistance [] but the molecular basis of the symbiosis is largely unknown because genetic manipulation of *Frankia* has not been possible. But why *Frankia* ACN14a haven't such sort of structural motif is still unclear to us. They have the major functional significance for RNA-RNA, RNA-protein, RNA-drug interactions (Leontis *et al.* 2002). Thus it has a major target for pharmaceutical field.

E-loop is generally important for its transport activity. It has co-relation with transporters mainly ABC-transporters (Okuda *et al.* 2010). Thus the high percentage of existence of such kind of motif reveals that it may involve in signal peptide transport in some way or may involve for its symbiotic association through transportation machineries.

Hook-turn presents at the end of helix having hook like conformation i.e. it bears a sharp turn like a hook mainly at the end of WC- basepair of helix which ends with G on short strand-site or else A on large-strand side (Szep *et al.* 2003). They stabilize the structures through RNA-tertiary interactions but not used as cap for helical segments.

Conclusion

The tertiary structure prediction reveals that atomic constitution of 16S rRNA is too large to handle in a single platform relative to present RNA webservices. There is need to develop a service for such sort of large molecules to achieve a much more stabilized structure through *In-silico* approach. A short survey of tertiary structural motif shows that the sarcin-ricin motif, K-motif, E-loop should be examined in more detail in future. Our main objective for future case studies are exploring functional importance in much more detailed fashion. Here we give some pictorial representation of 16s rRNA of *Frankia sp.* with a structural-functional association.

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