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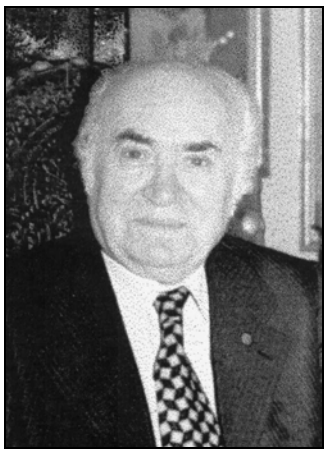
In memoriam



GHEORGHE ZARNEA

(1920–2012)

The Academician *Gheorghe Zarnea*, a founder of General Microbiology education in Romanian universities, was one of the most prominent personalities of the University of Bucharest in the second half of the last century. During his entire life, he was a true inspiration for students, disciples and collaborators, as well as for all those around him. Endowed with an outstanding intelligence, work capacity and imagination, with unique tenacity and leadership skills, he surpassed multiple limitations and obstacles. During his entire scientific, teaching and organizational activity, he proved to be a demanding person, yet capable of empathy, a good counsellor, a wise man in the true meaning of the word. He believed in people, even when they have not responded in the same manner to the gesture of the great Professor.

Born in Bucharest on September 22nd, 1920, *Gheorghe Zarnea* graduated from "Aurel Vlaicu" High school and the University of Medicine (1945), becoming Doctor of Medical Science (1966) and Senior Lecturer of Medical Science at the Faculty of Biology, Bucharest (1970).

He went through the entire scientific hierarchy (from Assistant to Head of Department) and all didactic stages (from Assistant to Associate Professor) at the Faculty of Medicine; in 1948, he got transferred to the Faculty of Biology within the University of Bucharest, where he became Professor in 1967.

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The **teaching activity** of Professor *Gheorghe Zarnea* is reflected in courses taught over four decades and Microbiology textbooks for bachelor, master and doctoral students from all academic centres in the country, especially in Bucharest. We had the privilege to attend his lectures full of information, well summarized. We were impressed by his pedagogical innate talent, his way of teaching, the precision, high clarity and beauty of the language he used; we were also impressed by the way he addressed the auditory, by the perfect attire always used when he came in the amphitheatre "Dimitrie Brândză" of the Botanical Institute within the University of Bucharest. We were impressed by the laboratory he established and led for a long period of time, by his interaction with students and collaborators. Due to his way of speaking and to all the things he used to do, the Professor of Microbiology managed to captivate us; we were always willing to listen to him speaking, to "steal" something of the secrets he used while teaching. His proverbial punctuality, the elegance he proved in conversation, the manner of examination and the accuracy with which he assessed the students' knowledge remained in the memory of those who knew him, in class and in laboratory, as a Professor, Head of Department and Dean. Originally, he illustrated the General Microbiology course, subsequently introducing in the curriculum also other disciplines in the field (Virology, Immunobiology, Medical Bacteriology, Ecology of Microorganisms, and Industrial Microbiology) for students from biology, biochemistry and ecology departments.

The **scientific activity**, also impressive and widely appreciated, carried out during nearly 70 years, has its beginning at the "Dr. I. Cantacuzino" Institute, where he benefited from the direct supervision of Professors C. Ionescu-Mihăieşti, D. Combiescu, M. Ciucă and Al. Ciucă. Very useful proved to be for him the specializations he attended in France ("Pasteur" Institute) and in Denmark (Staatensserum Institute), where he acquired various methods and work techniques in Medical Microbiology. The research directions he embraced have been mainly represented by biology, immunobiology, rickettsial pathogenicity and epidemiology; in the 50s of the last century, he participated in a field campaign in endemic areas with typhus in northern Moldavia, contributing to the eradication of this disease in Romania.

At the request of Academician Traian Săvulescu, in 1948, *Gheorghe Zarnea* got transferred as a Lecturer at the Faculty of Biology within the University of Bucharest, in order to organize the General Microbiology education, as well as the scientific research in this field. He initiates new areas of fundamental and applied research on: the biology of useful microorganisms such as nitrogen fixing bacteria, microorganisms producing enzymes with biotechnological importance or producing combustible gases (methane, hydrogen); bacterial genetic engineering, targeting the genetic reprogramming of economically important bacteria, by bacterial protoplast fusion or gene transfer to plant protoplasts; the biology of

extremophilic organisms (halophytes); the energetic metabolism in prokaryotes; the microbial bio fuel cells; the bacterial and fungal bioconversion of cellulose.

The laboratories created by Professor *Gheorghe Zarnea* at the Faculty of Biology within the University of Bucharest and at the Institute of Biology belonging to the Romanian Academy have played and are still playing a key role in the formation of numerous specialists who have worked and are currently working in different laboratories of Microbiology from areas such as: health, pharmacy, agriculture, food industry, traditional and modern biotechnology, wastewater treatment, etc. Thus, Professor *Gheorghe Zarnea* is the creator of the Microbiology school, being known and appreciated both nationally and internationally. His students from the Faculty of Biology and the Institute of Biology in Bucharest have cherished and admired Professor *Gheorghe Zarnea* both for his scientific authority and his pedagogical talent and erudition; for all bachelor and doctoral students whom he formed, Professor *Gheorghe Zarnea* represented a true model of teacher and scientist of high academic standard, an example of self-criticism, refinement and eloquence; he also represented a model in terms of lifestyle, as he showed moderation, discretion and wisdom. This is what all former colleagues and collaborators from the Faculty of Biology in Bucharest emphasised on the occasion of the teacher's 90th anniversary (Natura, **52**, *2*, 2010: 11-13).

His entire scientific activity is materialized in more than 200 original articles, manuals, handbooks and dictionaries published as sole author or in collaboration, among which we mention some of reference: Medical Bacteriology (1961), Microbiology (1963), General Microbiology (1970), Bioengineering of Microbial Enzyme Preparations (1980), Treaty of General Microbiology in 5 volumes (1983, 1984, 1986, 1990, 1994), Biological Revolution (1985 ed.), Principles and Techniques of General Microbiology (1992), Principles and Techniques of Immunobiology (1993), Immunobiology (1995), General Virology (1997), Encyclopaedic Dictionary of Microbiology and Cell Biology (2011).

Together with another great Romanian microbiologist – Professor Napoleon Topală, from the Faculty of Biology within the "Al.I. Cuza" University in Iaşi, Professor *Gheorghe Zarnea* initiated, since 1976, the series of national symposia of industrial microbiology and biotechnology, held in Iaşi, Bucharest and Galati until 1994; these national symposia were attended by experts from all academic centres in the country and not only by them, the results being published in communications presented in 10 volumes.

For some works, Professor *Gheorghe Zarnea* was awarded the Ministry of Education's Prize (1963) and the "Emanoil Teodorescu" Prize of the Romanian Academy (1980, 1983).

In 1973, the Central Institute of Biology was founded, the first general director of which was Professor *Gheorghe Zarnea* until 1985; the Central Institute consisted of the Institute of Biology in Bucharest, the Centre for Biological Research in Cluj-Napoca and the Biological Research Centre in Iaşi. Professor

Gheorghe Zarnea coordinated also the scientific research of the three faculties of biology in the country. During this time, we got to know him better, admiring his leadership skills over an impressive research team, his organizational capacity, and the way he chose and trained young researchers. As a teacher, *Gheorghe Zarnea* stood out due to his impressive work capacity, the exigency applied both to collaborators and to himself. We were about to know these qualities of the Professor during the visits made by the general director at the research centres and resorts in Cluj-Napoca, Iaşi, Sinaia, Pângărați, Sulina, etc. We accompanied Professor *Gheorghe Zarnea* acting as specialized referee for doctoral committees chaired by Dean *Gheorghe Zarnea* or in which he acted as scientific advisor; in the same manner, Professor *Gheorghe Zarnea* participated as official referee in doctoral committees chaired by us at Iaşi, Cluj-Napoca and Bucharest.

As a general director of the Central Institute of Biology, he initiated and organized genetic engineering and biotechnology laboratories; within the Institute of Biology in Bucharest, the studies of high theoretical and practical value made during this period represented pioneering research works in our country. Among these, a special significance is represented by the analysis of the processes of somatic hybridization by fusion of protoplasts to bacteria, yeast and plants, as well as those aimed at the study of the Ti plasmid from *Agrobacterium tumefaciens* in order to be used as a vector for plant cell cloning, studies conducted under Professor's thorough supervision.

For his valuable scientific work, for his outstanding Treaty of Microbiology, as well as for his contribution to the management of biological research in our country, in 1991 Professor *Gheorghe Zarnea* was elected as corresponding member of the Romanian Academy, being tenured in 1994. In this high quality, as a member of the Romanian Academy, we got to know him better as a person, colleague and leader: Chairman of the Department of Biological Sciences (1995-2005), Honorary Director of the Institute of Biology of the Romanian Academy (1988) and Honorary Chairman of the Department of Biological Sciences belonging to the Romanian Academy (2005).

As Chairman of the Department of Biological Sciences, Academician *Gheorghe Zarnea* acted as a true and close colleague, taking always into consideration our suggestions. He showed us respect, we showed him respect, he has respected and supported by means of his scientific authority the management of the Romanian Academy when in crisis; it is a pity that, in his turn, he did not receive the same support in solving the problems of the department he managed in an unparalleled manner for many years.

For outstanding achievements in scientific, editorial and organizational work, Professor and Academician Gheorghe Zanea was honoured with various awards and titles, including The Order of Scientific Merit (1971, 1978), the Order of the "Star of Romania" with the rank of Officer (2000), Doctor Honoris Causa of

"Vasile Goldis" Western University in Arad (whose Associate Professor he was for many years).

When he reached the venerable age of 90 years, the Nature Journal (biology) dedicated to Academician *Gheorghe Zanea the 2nd* no. of volume 52 (2010), wherein the following ones wrote: the teams of the Microbiology Laboratory from the Faculty of Biology, within the Institute of Biology Bucharest and Professors Marin Andrei (Bucharest), Aurel Ardelean (Arad), Marin Falcă (Piteşti) and Constantin Toma (Iaşi).

For all of us, Professor and Academician *Gheorghe Zanea* was and remains a role model both as a teacher and a researcher. He stopped working only on June 16th, 2012, when he said goodbye forever, being buried in Belu cemetery in Bucharest.

Now that you left, dear sir, our secondary education loses a prestigious Professor, Biology loses a remarkable scientist, your students and collaborators lose a sincere supervisor, a model of devotion on the altar of science. The Romanian Academy loses an eminent leader who created so many lasting works. May the coming generations preserve and honour the memory of the one and only *Gheorghe Zanea,* professor and scientist, with the same purity and dedication that come from the works inherited from him.

Constantin TOMA
Aurelia BREZEANU

EFFECT OF EMS ON GENETIC PARAMETERS AND SELECTION SCOPE FOR YIELD ATTRIBUTES IN M_2 MUNGBEAN (*VIGNA RADIATA* L.) GENOTYPES

RAJIB ROYCHOWDHURY[1,2], SUDIPTA DATTA[2], MOMEZUL HAQUE[2], TAMOGHNO KANTI MUKHERJEE[2], DIPIKA MAJI[2], PARENEETA GUPTA[2], DIPIKA DAS[2] and JAGATPATI TAH[2,*]

In the present study, seeds of mungbean (*Vigna radiata* L. Wilczek), a self-fertilized pulse crop, were treated with four doses (0.2%, 0.4%, 0.6% and 0.8% as w/v) of EMS to analyze the genetic variability and heritability for the studied attributes like plant height, days to flowering, pods/plant, seeds/pods and seed yield/plant in second mutant (M_2) generation. Significant increase in mean values of the studied traits was noticed in mutants. The analysis of variance (ANOVA) revealed highly significant ($P < 0.01$) differences among the characters, indicating the presence of substantial genetic variability, which was higher in treated population than in the control. In general, the phenotypic coefficient of variation (PCV) was higher than its genotypic counterpart (GCV) for all the studied characters. The highest GCV (0.537) for seeds/pods and highest PCV (0.635) for plant height were recorded in 0.4% EMS treatment. The lowest GCV (0.179) and PCV (0.214) were recorded in control for pods/plant. Highest broad sense heritability estimate (92.33%) was observed with 0.4% EMS for plant height. The expected genetic advance was high (42.39%) with the same treatment for days to flowering. The lowest heritability (38.43) and genetic advance (5.37) were noticed in control for seed yield/plant. EMS at 0.4% and 0.6% concentration gave the maximum values of all the genetic parameters. The increased genetic variability in treated population for these traits has a high scope for selection and can be exploited for the further improvement of mungbean.

Key words: Genetic variability, heritability, *Vigna radiata*, selection response, yield component.

INTRODUCTION

Mungbean (*Vigna radiata* L. Wilczek) belongs to the angiospermic dicot family: Papilionaceae, is an important self-fertile pulse crop of South-Eastern Asia and occupies a pivotal position in meeting the protein needs of people in developing countries like India (Wani and Khan, 2006). This pulse crop is widely

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grown in India, Pakistan, Bangladesh, Sri Lanka and Thailand. It is also known as the crop of sub-continent and up to three crops per year can be grown (Malik, 1994). Diversifying the limited genetic variability for agronomic traits of interest, specially yield and its associate attributes and developing new mungbean cultivars are demand of this modern era. Due to lack of sufficient natural variability for yield and its component traits in *V. radiata*, conventional methods of breeding have limited scope. Induced mutations have been used to generate genetic variability and have been successfully utilized to improve yield and yield components of various crops like *Oryza sativa* (Singh *et al.*, 1998), *Dianthus caryophyllus* (Roychowdhury and Tah, 2011a), *Vicia faba* (Ismail *et al.*, 1977), *Vigna unguiculata* (Mensah and Akomeah, 1992), *Cajanus cajan* (Srivastava and Singh, 1996), *Vigna mungo* (Singh and Singh, 2001) and *Lens culinaris* (Khan *et al.*, 2006). These reports show that mutagenesis is a potential tool to be employed for crop improvement.

The knowledge of the extent to which the desirable attributes are heritable is a prerequisite for any crop improvement programme, especially for mutation breeding. For this purpose, inducible mutation is a suitable source of producing variation through mutation breeding procedure (Domingo *et al*., 2007) which can produce several improved mutant varieties with high demanding economic values (Din *et al*., 2004). Various metrical traits with agro-economical value like seed weight, number of branches, leaves, flowers, leaf area, etc. are very much complex in nature because they confirm polygenic inheritance and are greatly influenced by minute fluctuation of environmental components. Genetic improvement of any crop is largely depending on the magnitude of several genetic parameters like analysis of variance (ANOVA) of each mean value, phenotypic and genotypic variance, phenotypic and genotypic coefficient of variation (PCV and GCV), broad sense heritability (H^2) and genetic advance (GA) on which the breeding methods are formulated for its further improvement. Development of high-yielding varieties requires a thorough knowledge of the existing genetic variation and heritability of agronomic traits and their interrelationship which helps in understanding yield components and yield potential in mungbean. The observed variability is a combined estimate of genetic and environmental causes, of which only the former one is heritable. Assessment of genetic variation is the most appropriate statistical tool to find out the magnitude of heritability, genetic coefficient of variation and response to selection using appropriate selection intensity for traits of interest. Analysis on genetic variability reveals about the presence of variation in their genetic constitution, and it is of outmost important as it provides the basis for the scope of effective selection. Wide spectrum of genetic variability has been induced in *Vigna radiata* using both chemical and physical mutagens in order to utilize it in pulse-crop improvement and inheritance studies (Patil, 1966; Ashri, 1970; Gowda *et al*., 1996). The extent of variability is measured by genotypic coefficient of variance (GCV) and phenotypic coefficient of variance (PCV) which provides

information about relative amount of variation in different characters. Hence, to have a thorough comprehensive idea, it is necessary to have an analytical assessment of metrical traits. Since heritability is also influenced by environmental factors, the information on heritability alone may not help in pin pointing characters enforcing selection. Makeen *et al*. (2007) evaluated mungbean genotypes to estimate genetic variability, heritability and genetic advance for agronomic characters and reported highly significant differences for all traits with greater magnitude of heritability for plant height and seed weight. Similarly, Siddique *et al*. (2006) reported highly significant genetic variation for days to flowering, maturity, pods per plant and seed yield among their studied mungbean genotypes. Rohman *et al*. (2003) reported that plant height and days to flowering were mostly governed by additive genes effects. Sriphadet *et al*. (2005) reported 89.9, 98.9, 93.7 and 93.2% heritability for leaves number per plant, seed hardness, pod-length and pod-width, respectively. Seed yield is reported to be positively correlated with traits like days to flowering, plant height, branches per plant, pods per plant and pod-length. Malik (1994) has reported a positive correlation of a number of pods and branches per plant with seed yield. Similarly, Khan *et al*. (2001) reported a strong association among branches per plant and pods per plant leading to increased yield per unit area. A positive and statistically significant relationship between seed yield per plant and days to 50% flowering, pods per plant, seeds per plant, harvest index and 1000 seed weight is reported by Celal (2004). However, estimates of heritability alone do not provide an idea about the expected gain in the next generation, but have to be considered in conjunction with estimates of genetic advance, the change in mean value between generations (Johnson *et al*., 1955). Simply, heritability gives the information on the magnitude of inheritance of metrical attributes, *i.e.* polygenic inheritance, while genetic advance will be helpful in formulating suitable selection procedures.

In the present study, therefore, an attempt has been made to determine the genetic variation, its components, especially phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV), broad-sense heritability (H^2) and genetic advance (GA) for yield and its associate components in M_2 generations of mungbean. These estimates could be useful in developing reliable selection indices for important agronomic traits of *Vigna radiata*.

MATERIALS AND METHODS

The seeds of experimental plant material (*Vigna radiata* L. Wilczek) were obtained from Globe Nursery, Kolkata. The field experimentation was conducted during June-September of 2009 at the Crop Research Farm, The University of Burdwan, Burdwan, India. Information regarding the meteorological characteristics like temperature, seasonal rainfall, relative humidity, geographical position, etc.

of the test location is given in Table 1. As per the protocol of Roychowdhury and Tah (2011b), seeds of mungbean were treated with four different concentrations (0.2%, 0.4%, 0.6% and 0.8% as w/v) of potent chemical mutagen: ethyl methane sulphonate (EMS) to induce mutations. The untreated seeds were presoaked in distilled water and were sown as control. One hundred seeds for every treatment and control were sown in the field in a complete randomized-block design (CRBD) with three replicates to raise the first mutant (M_1) generation. Plot size for a mungbean treatment in each replication was 3.5 m^2. Each plot had 3 m long three rows with row to row and plant to plant distance of 50 cm and 30 cm, respectively. Such a field design is most frequently used in plant breeding programmes. Soil test of field for available nutrients was carried out prior sowing the experimental treatments through Soil Testing Lab, Gov. of West Bengal, Cooch Behar, India (Table 2). Based on the soil analysis, 20% Zinc sulfate as 30 kg/ha and NPK as 50:100:50 kg/ha were applied as a basal dose during final land preparation in experimental field. Urea (nitrogen source), di-ammonium phosphate (P_2O_5 source) as phosphate fertilizer and potash sulfate (K_2O source) as potassium fertilizer were also applied. Required amount of chemical fertilizers was applied at the time of soil preparation. Pesticides were sprayed to protect the crop from pests especially white fly, a vector for Mungbean Yellow Mosaic Virus.

The phenotypic variance of the test genotype might be influenced partially by soil heterogeneity and partially by environment, and the complete randomized-block design provides an efficient mechanism for Analysis of Variance (ANOVA) to account for these sources of variation. For raising M_2 generation, ten M_1 progenies were selected which showed significant deviations in mean values in the positive direction from the mean values of the control, particularly for the yield and its associate components. Seeds from each selected M_1 progeny were bulked and thoroughly mixing them. A random sample of this bulk was sown to obtain M_2 progeny. Three replications of each M_2 mungbean treatment were maintained in the experimental field. Normal recommended cultural practices and plant protection measures were followed. Data collected for plant height (cm) at maturity, days to flowering, pods per plant, seeds per pods and seed yield (g) per plant in the M_2 generation were subjected to statistical analysis in order to assess the extent of induced variation due to mutagenic action. Significant differences were identified using the Least Significance Difference estimated from the error mean square and tabulated 't'- values at the 1% level of significance.

For statistical analysis of genetic parameters, we considered the analysis of variance of each mean value (Table 3), coefficient of variation (CV), critical difference (CD), phenotypic and genotypic variance, phenotypic and genotypic coefficient of variation (PCV and GCV), broad sense heritability (H^2) and genetic advance (GA). Mean values were subjected to analysis of variance (ANOVA) to test the significance for each traits as per Panse and Sukhatme (1967). Phenotypic and genotypic variances were estimated according to Lush (1940). The genotypic

and phenotypic coefficients of variation (GCV and PCV) were computed according to the method advocated by Singh and Chaudhary (1985). Heritability in broad sense was determined according to the methodology given by Allard (1960). The estimate of the expected genetic advance (GA) expressed as a percentage of the mean value with an assumed 5% (2.06 after Kang *et al*., 1983) intensity of selection pressure was computed by the formula given by Singh and Chaudhary (1985) as:

$$GA = k.\ H^2.\ \sqrt{\sigma_g},$$

where $k = 2.06$, constant for 5% selection intensity (*i.e.* the highest-performing 5% are selected), H^2 = broad-sense heritability, σ_g = genotypic variance of the treated population.

Table 1

Meteorological characteristics like temperature, seasonal rainfall, relative humidity, wind speed, geographical position, altitude and soil type about the test location used for evaluation of mungbean treatments/genotypes in 2009

PARAMETER	**JUNE**		**JULY**		**AUGUST**		**SEPTEMBER**	
Average temperature (^oC)	Max	Min	Max	Min	Max	Min	Max	Min
	39.2	28.1	42.1	29.5	38.8	27.6	34.6	26.3
Average rainfall (mm)	65		69		51		42	
Relative humidity	Max	Min	Max	Min	Max	Min	Max	Min
	79	51	82	59	68	46	64	41
Wind speed (km/h) & direction	8.1, S & SE		7.2, SE & NE		6.4, NE & S		6.4, SE & N.	
Latitude	23.53^O N, 22.56^O S							
Longitude	83.25^O E, 86^O W							
Elevation (msl)	45 m							
Soil type & pH	Neutral (pH 6.6 – 7.3)							

Table 2

Soil characteristics and available macro and micro elements/nutrients based on soil test of experimental location

Parameters →	Organic Carbon (%)	Available P_2O_5 (kg/ha)	Macro elements			Micro elements					Exchangeable Na (mmolc/100g)	Dissoluble salt EC (dS/m)
			N	**K**	**P**	**Ca**	**Mg**	**Bo**	**Fe**	**Zn**		
Actual amount	1.37	76	789	296	18.7	24.83	6.1	0.46-0.51	6.3-7.7	1.3-1.5	16.5	0.21

Status	High	High	High	Adequate	Adequate	Adequate	Adequate	Low	Adequate	Adequate	Adequate	Adequate
Threshold level	0.75	22.5-56	217-544 kg/ha	125-283 kg/ha	3-20 ppm	10-40 meq/100g	5-15 meq/100g	≤ 0.7ppm	≤4.5 ppm	≤1.0 ppm	15	0-15

Table 3

Analysis of Variance (ANOVA)

Source	**d.f.**	**Sum of Square (SS)**	**Mean Sum of Square (MSS)**	**Expectations**
Treatment (t)	t-1	SS_t	SS_t /t-1	MSS_t/MSS_e
Replication (r)	r-1	SS_r	SS_r /r-1	MSS_r/MSS_e
Error (e)	(t-1) (r-1)	SS_e	SS_e /(t-1) (r-1)	

where d.f. = degree of freedom, SS = Total sum of square, MSS = Mean sum of square, g = number of genotypes or treatments, r = no. of replications.

Table 4

Mean, standard error (S.E.), maximum and minimum range, mean sum of square (MSS), F-value, Coefficient of Variance (CV %) and Critical Difference (CD) values for metrical attributes in mungbean (*Vigna radiata* L.) treatments

Characters	**Mean ± S.E.**	**Range (Min - Max)**	**MSS**	**F value**	**CV%**	**CD**
Plant height (cm) at maturity	63.5±2.36	58.6 – 69.4	110.76**	61.928	7.62	2.54
Days to flowering	56.34±0.93	48.4 – 64.9	14.92**	29.72	5.66	3.78
Pods per plant	32.98±0.72	25.48 – 37.38	86.40**	19.782	9.31	1.92
Seeds per pods	8.62±1.04	7.31 – 9.66	1.96**	12.478	8.46	3.61
Seed yield (g) per plant	15.19±0.19	12.40 – 16.93	21.37**	17.66	9.14	0.76

** indicates significant at 1% level of significance.

Table 5

CHARACTERS	**PARAMETERS**	**TREATMENTS**				
		CONTROL	**0.2% EMS**	**0.4% EMS**	**0.6% EMS**	**0.8% EMS**
Plant height (cm)	Mean (X) ± S.E.	63.3±2.62	61.7±3.31	69.4±3.34	64.5±3.66	58.6±3.89
	Shift in X					
	GCV%	0.392	0.266	0.506	0.416	0.197
	PCV%	0.441	0.339	0.635	0.498	0.283
	H^2 %	76.32	81.65	92.33	88.43	83.92
	GA as % of X	18.24	21.43	27.48	25.77	23.81

Days to flowering	Mean (X) ± S.E.	48.4±0.88	52.6±0.63	56.5±0.79	59.3±0.37	64.9±0.94
	Shift in X					
	GCV%	0.239	0.283	0.317	0.375	0.408
	PCV%	0.248	0.312	0.366	0.433	0.480
	H^2 %	66.35	73.73	90.12	82.66	87.38
	GA as % of X	36.36	38.28	42.39	40.71	41.55
Pods per plant	Mean (X) ± S.E.	25.48±0.55	33.23±0.83	37.14±0.85	37.38±0.62	31.65±0.62
	Shift in X					
	GCV%	0.179	0.224	0.297	0.299	0.198
	PCV%	0.214	0.283	0.386	0.391	0.248
	H^2 %	43.81	51.59	59.32	59.78	46.55
	GA as % of X	8.77	10.78	12.06	12.63	9.96
Seeds per pod	Mean (X) ± S.E.	8.94±1.07	7.31±1.12	9.66±1.02	9.10±1.23	8.08±1.17
	Shift in X					
	GCV%	0.439	0.413	0.537	0.498	0.424
	PCV%	0.505	0.468	0.612	0.586	0.482
	H^2 %	58.86	56.32	76.88	66.55	62.74
	GA as % of X	26.92	24.57	34.73	32.21	30.18
Seed yield (g) per plant	Mean (X) ± S.E.	12.40±0.12	15.20±0.20	16.74±0.24	16.93±0.19	14.66±0.18
	Shift in X					
	GCV%	0.266	0.321	0.346	0.354	0.288
	PCV%	0.332	0.398	0.412	0.424	0.366
	H^2 %	38.43	41.86	44.13	42.68	40.12
	GA as % of X	5.37	7.24	8.86	7.97	6.98

RESULTS AND DISCUSSION

The estimate of mean, maximum and minimum range, mean sum of square (MSS), F-value, coefficient of variation (CV), critical difference (CD) for five studied metrical attributes in mungbean treatments are given in Table 4. Analysis of variance (ANOVA) showed that the genotypes differed significantly ($P < 0.01$) for all the studied characters (Table 4) which indicate that the treatments were genetically divergent after mutagenic treatment. So, there is a huge scope for selection of promising mutant lines with different metrical attributes from the present gene pool. Similar result was reported by Sirohi and Kumar (2006) in mungbean. The highest mean value (63.5) was noticed for plant height at maturity followed by days to flowering (56.34) and the lowest mean value (8.62) was for seeds per plant. Mean sum of square (MSS) for all the characters studied was significant at the probability of 1% level. The range of F-value was 12.478 – 61.928 indicated that this range follows the magnitude of mean values for all attributes. The coefficient of variation (CV as percentage) indicated that there were significant differences among the treatments for the characters under study. It also reveals that the value of CV ranges from 5.66 for days to flowering to 9.31 for pods per plant. All the CV values for five metrical attributes were below the value of 10. Rahim *et al.* (2008) also found medium to high CV for days to maturity and days to 50% flowering. The significant critical difference (CD) values indicate that *Vigna radiata* L. Wilczek cultivar was suitable for the location where prevailing

environmental effects were favourable. The higher CD value indicates the higher stability in that environment (Roychowdhury, 2011). Here, days to flowering showed higher CD value (3.78), plant height at maturity showed moderate (2.54) and seed yield (g) per plant represented lower CD value, *i.e.* 0.76. Significant genetic variation for morphological traits like days to flowering, maturity and plant height is also reported by Rohman *et al*. (2003), Siddique *et al.* (2006) and Rozina *et al*. (2008) in mungbean.

The estimation of mean, genotypic (GCV) and phenotypic (PCV) coefficient of variation, broad sense heritability (H^2) and genetic advance (GA) of mungbean treatments are given in Table 5. A wide range of variation in studied five traits was observed with regard to control and four different doses of EMS. There was a significant increase in the mean values and genetic variability of studied characters in all the mutagenic treatments over the control in M_2 generation. In the present study, increase in the mean values of various traits may be due to the selection of normal-looking plants in M_2 which led to the elimination of aberrant plants and also due to changes induced at genetic level. Gaul (1964) suggested that the selection process should be delayed until the M_3 or later generations following mutagenic treatment. However, here the selection of progenies was on the basis of desirable mean and greater variance in the early generation was found to be highly useful, leading to the desirable improvement of yield and its components. Here, the increase in the number of pods was due to an increase in the number of flowers. Flower shedding was not noticed in the mutants. Similar increases in the number of pods of some other varieties of mungbean have been reported by Tickoo & Chandra (1999) using EMS, nitroso-methyl urea (NMU), hydroxyl-amine (HA) and gamma rays as mutagens. In most of all cases, we see that with the increase in EMS concentration, the mean value rises over control for corresponding traits. The magnitude of mean values for all traits are: 0.4% EMS > 0.6% EMS > CONTROL > 0.2% EMS > 0.8% EMS for plant height, 0.8% EMS > 0.6% EMS > 0.4 % EMS > 0.2% EMS > CONTROL for days to flowering, 0.6% EMS > 0.4% EMS > 0.2% EMS > 0.8% EMS > CONTROL for pods per plant, 0.4% EMS > 0.6% EMS > CONTROL > 0.8% EMS > 0.2% EMS for seeds per pods, 0.6% EMS > 0.4% EMS > 0.2% EMS > 0.8% EMS > CONTROL for seed yield per plant. Only days to flowering showed complete sequentially increase in mean value from control to lower EMS dose (0.2%) to its higher doses accordingly. For most of characters, 0.6% and 0.4% EMS doses were responsible for giving the highest value.

In general, the phenotypic coefficient of variation (PCV) was higher than its genotypic counterpart (GCV) for all the studied characters. This resemblance between PCV and GCV in almost all the characters suggests that the environment had little effect on those characters' expression and it was consistent with Jalgaonkar *et al.* (1990). The GCV provides a measure for comparing genetic variability in various metrical characters. The highest GCV (0.537) for seeds per

pods and the highest PCV (0.635) for plant height were recorded in 0.4% EMS treatment. The lowest GCV (0.179) and PCV (0.214) were recorded in control for pods per plant. For plant height, GCV/PCV was the highest (0.506/0.635) in 0.4% EMS and the lowest (0.197/0.283) in 0.8% EMS, whereas for days to flowering, the highest value (0.408/0.480) in 0.8% EMS and the lowest value (0.239/0.248) in control. Seeds per pods showed maximum GCV/PCV (0.537/0.612) in 0.4% EMS and minimum (0.413/0.468) in 0.2% EMS. In case of pods and seed yield per plant, the highest GCV/PCV (0.299/0.391 and 0.354/0.424, respectively) was recorded in 0.6% EMS and lower value (0.179/0.214 and 0.266/0.332) in control. We see that most of the lowest GCV and PCV were encountered in control, whereas 0.4% and 0.6% EMS were responsible for its highest value. High values of GCV suggested better improvement for selection of traits. However, the estimation of heritable variation with the help of genetic coefficient of variation alone may be misleading. Burton (1951, 1952) suggested that the genetic coefficient of variation together with heritability estimates gave a better picture of the extent of heritable variation. Heritability (H^2) and genetic advance (GA) estimates were interpreted as low, medium and high as per the classification of Johnson *et al.* (1955).

The values of heritability increased and differed from trait to trait. The highest broad sense heritability estimate (92.33%) was observed with 0.4% EMS for plant height. The expected genetic advance was high (42.39%) with the same treatment (0.4% EMS) for days to flowering. The lowest heritability (38.43) and genetic advance (5.37) were noticed in control for seed yield per plant. Kaul & Kumar (1983) obtained low heritability values for grain yield in rice. The high estimates of heritability in the metrical traits have been found to be useful from the point of view of plant breeding, as this enables selection to be based on phenotypic performance. The ranges of heritability in each character according to lower magnitude were: 76.32 (Control) – 92.33 (0.4% EMS) for plant height, 66.35 (Control) – 90.12 (0.4% EMS) for days to flowering, 56.32 (0.2% EMS) – 76.88 (0.4% EMS) for seeds per pods, 43.81 (Control) – 59.78 (0.6% EMS) for pods per plant and 38.43 (Control) – 44.13 (0.4% EMS) seed yield per plant. Most of the control plants show lower value of heritability and 0.4% EMS was responsible for giving its higher value. High heritability was recorded for plant height and days to flowering, moderate heritability for seeds per pods and low for seed yield and pods per plant. Roychowdhury and Tah (2011a) also reported high heritability for plant height and moderate for seeds per inflorescence in case of *Dianthus caryophyllus.* High heritability combined with high genetic advance as percent of mean was observed in 0.4% EMS for days to flowering. This indicates the lesser influence of environment in the expression of this character and prevalence of additive gene action in its inheritance, hence amenable for simple selection. High heritability with moderate genetic advance as per cent of mean was recorded in 0.6% and 0.8% EMS for days to flowering indicating that this character was governed by additive gene interaction. High heritability coupled with low genetic advance as per cent of

mean was recorded in all EMS treatment, specially 0.4% EMS for plant height indicating non-additive gene action for this trait. Miah and Bhadra (1989) reported high values for expected genetic advance for seeds per pods. Makeen *et al*. (2007) and Sriphadet *et al*. (2005) have also reported moderate to high heritability for various morphological traits in mungbean. Due to high heritability estimates, the traits are expected to remain stable under varied environmental conditions and could easily be improved through selection (Khattak *et al*., 1997; Siddique *et al*., 2006).

CONCLUSIONS

With regards to genetic parameters, variability was higher in EMS treatments than in the control set. The values of the coefficients of variation (phenotypic and genotypic), heritability and expected genetic advance increased in the treated population as compared to control. EMS at 0.4% and 0.6% concentration gave the maximum values of the genetic parameters. High heritability was observed for plant height and days to flowering indicated that in the present material, the scope of improvement for these traits by simple selection would be effective. High heritability coupled with high genetic advance expected in the next generation for days to flowering suggesting this character was governed by additive genetic effect to a great extent and improvement of it would be effective through selection. But, plant height expressed non-additive gene interaction, hence it needs to be a complex selection process and heterosis breeding would be recommended.

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ESTIMATION OF GENETIC VARIABILITY, HERITABILITY AND GENETIC ADVANCE IN AROMATIC FINE GRAIN RICE

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A study on genotypes was conducted in Randomized Block Design in three replications under rainy condition during 2010. The data were recorded for 15 qualitative characters viz., days to 50% flowering, number of tillers per hill, number of panicles per m^2, number of spikelets per panicles, panicles length, flag leaf length, flag leaf width, plant height, days to maturity, grain yield per plant, grain yield per plot, biological yield, harvest index and test weight to study genetics variability, heritability and genetic advance. Analysis of variance among 41 genotypes showed highly significant differences for yield contributing traits viz., number of panicles per m^2, plant height, days to 50% flowering, days to maturity, biological yield harvest index, number of spikelets per panicles. Grain yield per plant, panicles length, flag leaf length, number of panicles per hill, number of tillers per hill, test weight grain yield per plot and flag leaf width indicated the presence as substantial amount of genetic variability in study material and there is scope for selection. On the basis of mean performance yield traits it was conducted that "NDR-9542" was best performer for yield. The results showed higher GCV and PCV in parameters like grain yield per plot, flag leaf width, biological yield, days to 50% flowering and plant height. Maximum heritability was encountered for characters viz. plant height, days to 50% flowering, days to maturity, panicles length and number of panicles per hill. High genetic advance was observed in number of panicles per m^2, plant height and days to 50% flowering. Thus these characters could be improved by selection in breeding programme for crop improvement.

Key words: Genetic variability, heritability, genetic advance, coefficient of variation, aromatic rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is principal food crop of South-Eastern countries and supports nearly one half of the world population. It holds the key for the food security and prosperity. India is a natural repository for long and short-grained aromatic rices, which are conserved by the formers over centuries. It is the primary

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staple food crop through out Asia and other part of the world. Today the demand for increase in productivity and quality of rice in available marginal land is very high. It is leading food source in terms of colonies being consumed for mankind and feed about 60% of the world's population (FAO, 2007). India share in the world 21.6% in rice production, China 1st and India 2nd position in rice production in the world. India has the area under rice 43.77 m.ha. and production 96.43 m.ha tonnes and in the world rice production 63.5 m. tonnes. (Agricultural statistics at a Glance 2008). Thus, there is a challenging need to improve rice yield to meet the growing demand. During the past two decades, significant progress has been made in increasing the yield and other qualities.

A well planned plant-breeding programme for developing high yielding genotypes requires complete knowledge on the genetic and genetic variation available in the population. In all these stages, estimation of genetic variability, heritability and genetic advance is necessary. Yield is a complex character being governed by a large number of cumulative and dominant genes and highly influenced by environment. This necessitates thorough knowledge variability during to genetic factors, actual genetic variation heritable in the progeny and the genetic advance that can be achieved through selection. Moreover, heritability estimate along with genetic advance was more useful than heritability estimate alone in predicting resultant effect for the selection of the best individual form of segregating population.

MATERIALS AND METHODS

Seeds of Rice were obtained from Directorate of Rice Research, Hyderabad, A.P., India. Aromatic fine grain observational nursery consisting of 41 entries aromatic rice was grown in randomized block design with three replications each with plot size of 7.5 m^2 at Field Experimentation Center of the Department of Genetics and Plant Breeding, Sam Higginbottom Institute of Agriculture, Technology and Sciences, Deemed to-be University, Allahabad during Kharif (June–November 2009). The recommended standard culture practices were followed to raise the crop. Data on days to 50% flowering, number of tillers per hill, number of panicles per hill, number of panicles per m^2, number of spikelets per panicles, panicles length, flag leaf length, flag leaf width, plant height, days to maturity, grain yield per plant, grain yield per plot, biological yield, harvest index, and test weight were recorded following standard evaluation system or method. The mean value subjected to analysis of variance to test the significance for each character as per methodology advocated by Panse and Sukhatem (1967). The estimates of phenotypic and genotypic coefficient of variation were classified as low, medium and high (Sivasubramanian and Madhavamenon, 1973) (less than 10% = low, 10–20% = moderate, greater than 20% = high). Heritability (broad

sense) estimates for yield component of rice were worked by Burton and De Vane 1952. The estimation method of genetic advance was given by Johnson *et al*. (1955).

RESULTS AND DISCUSSION

The analysis of variance revealed highly significant differences among the genotypes for the entire test characters, indicating the existence of high variability among the varieties. Thus this ample scope for selection of different quantitative characters for rice improvement. The estimates of phenotypic and genotypic variance were obtained for different characters and they are presented in Table 1. Estimates of phenotypic and genotypic variance revealed that number of panicles per m^2 exhibited the highest phenotypic and genotypic variance followed by plant height, days to 50% flowering, days to maturity, biological yield and harvest index. Phenotypic variance was higher than genotypic variance for all characters of yield and yield contributing characters indicated the influence of environmental factors of these traits on the basis of mean performance of yield and yield contributing traits, NDR 9542 was the highest yielder followed by IR77512-2-1-2-2, WAS 197-B-4-1-25 and they are presented in Table 2.

The phenotypic coefficient of variation (PCV) and the genotypic coefficient of variation (GCV) were of the same magnitudes for all the characters, indicating the least effects of environmental factors.

High genotypic coefficient of variation and the phenotypic coefficient of variation were observed for grain yield / plot, days to 50% flowering and plant height. These are in confirmation with the earlier finding of Chaubay and Singh (1994). Genetic coefficient of variability along with heritability gave an idea from selection (Burton, 1952). The character, which exhibited high heritability and genetic advance, indicated the broad sense of additive gene effects in its inheritance and such characters could be improved by selection (Panse and Sukhatme, 1967). In the present study, almost all the characters though had high heritability values but plant height, days to 50% flowering, days to maturity, panicles length and number of panicles per hill, suggesting preponderance of non-additive gene action in the inheritance of these traits. Similar results were also reported by Mehetre *et al.* (1996).

High heritability coupled with high genetic advance number of panicles/m^2, plant height, days to 50% flowering, days to maturity and biological yield. The high heritability with high genetic advance observed for these characters might be due to additive gene effects. Hence, selection in the segregating generation would be very effective for them. Similar results were also reported by (Kavitha and Reddy, 2002) and Kumar *et al.* (2007).

Table 1

ANOVA for different characters in 41 aromatic rice short grain genotypes

S. No.	Characters	Mean sum of squares		
		Replication (d. f. = 2)	**Genotypes (d. f. = 40)**	**Error (d. f. = 80)**
1.	Days to 50% flowering	36.32	394.00*	8.03
2.	No. of tillers / hill	1.03	2.34*	0.31
3.	No. of panicles / hill	0.67	3.57*	0.19
4.	No. of panicles / m2	12.13	1076.76*	75.36
5.	No. of spikelet / panicles	18.06	42.05*	11.60
6.	Panicles length (cm)	0.48	8.39*	0.40
7.	Flag leaf length (cm)	0.24	7.56*	1.66
8.	Flag leaf width (cm)	0.16	0.06*	0.01
9.	Plant height (cm)	4.39	462.71*	2.29
10.	Days to maturity	34.76	266.97*	5.96
11.	Grain yield / plant (g)	65.34	10.08*	3.700
12.	Grain yield / plot (kg)	0.02	0.46*	0.05
13.	Biological yield (g)	117.95	195.3*	31.15
14.	Harvest index (%)	16.73	51.17*	16.74
15.	Test weight (g)	1.86	1.18*	0.75

* As significant at 5 % level of significance.
Abbreviations: No. – Number, g – gram, kg – kilogram, cm – centimeter, % – percentage, d.f. – degree of freedom.

Therefore, selection based on the characters number of panicles per m^2, plant height, days to 50% flowering, days to maturity, panicles length, biological yield, number of panicles per hill, grain yield / plot, number of tillers per hill and number of spikelets per panicles having high heritability coupled with high genetic advance may bring about the desired improvement in yield.

Table 2

Estimates of variability, heritability and genetic advance in aromatic rice

S. no.	Characters	VG	VP	GCV	PCV	h^2 (%)	GA
1.	Days to 50% flowering	128.66	136.68	11.68	12.04	94.1	22.67
2.	No. of tillers/hill	0.68	0.98	4.29	5.16	69.0	1.41
3.	No. of panicles/hill	1.13	1.32	6.49	7.02	85.3	2.02
4.	No. of panicles/m^2	333.80	409.16	6.68	7.40	81.6	33.91
5.	No. of spikelets/panicles	10.15	21.75	2.19	3.21	46.7	4.48
6.	Panicles length (cm)	2.66	3.07	6.13	6.58	86.8	3.13
7.	Flag leaf length (cm)	1.97	3.63	4.54	6.16	54.3	2.13
8.	Flag leaf width (cm)	0.02	0.02	10.62	12.89	67.9	0.21
9.	Plant height (cm)	153.44	155.83	11.18	11.27	98.5	25.32
10.	Days to maturity	87.00	92.96	7.89	8.15	93.6	18.59
11.	Grain yield/plant (g)	2.13	5.83	2.57	4.26	36.4	1.81
12.	Grain yield/plot (kg)	0.14	0.19	14.65	17.33	71.5	0.64
13.	Biological yield (g)	54.73	85.87	9.86	12.35	63.7	12.16
14.	Harvest index (%)	0.17	1.55	1.33	4.04	10.8	0.28
15.	Test weight (g)	0.14	0.89	1.87	4.66	16.1	0.31

Abbreviations: g – gram, kg – kilogram, cm – centimeter, VG – Genotypic Variance, VP – Phenotypic Variance, PCV – Phenotypic Coefficient of Variation, GCV – Genotypic Coefficient of Variation, GA – Genetic Advance, h^2 – heritability.

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USING SSR MARKER TO IDENTIFY THE MYMV RESISTANCE GENE IN MUNGBEAN [*VIGNA RADIATA* (L.) WILCZEK]

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Yellow mosaic disease (YMD) is caused by Mungbean yellow mosaic virus (MYMV) major disease of mungbean in India as well as in other mungbean growing countries in Asia. In the present investigation Simple Sequence Repeats (SSR) and Bulk segregant analysis (BSA) techniques were used to analyse the F_2 individuals of susceptible VBN (Gg) 2 × resistant KMG 189 to screen and identify the Mungbean yellow mosaic virus (MYMV) resistant gene in mungbean. Two DNA bulks, namely resistant bulks and susceptible bulks, were setup by pooling equal amount of DNA from five randomly selected plants of each disease response. Parental survey study was carried out by using forty five Azukikibean SSR primers. This study revealed that six SSR markers showed polymorphism between the parents. These six SSR primers were used in the bulk segregant analysis. Out of these six SSR primers, none showed polymorphism between the parents and bulks. These SSR primers can produce polymorphism between parents and are not able to distinguish the bulks. This attributes the fact that these SSR primers are heterologous probes developed in azuki bean which can produce polymorphism between parents and are not able to distinguish the bulks.

Key words: Marker Assisted Breeding, mungbean, Mungbean Yellow Mosaic Virus, Simple Sequence Repeats.

INTRODUCTION

Mungbean [*Vigna radiata* (L.) Wilczek] (2n = 22) is one of the most important pulse crops which is native to India. The yield of mungbean has been stagnant over years. Improvement in yield of mungbean is becoming difficult mainly due to the occurrence of pest and diseases (Karthikeyan *et al*., 2011). Among the various diseases Yellow mosaic disease is caused by Mungbean yellow mosaic virus (MYMV), which is a Begomovirus transmitted through white fly, *Bemesia tabaci*, causing significant yield losses in mungbean, leading to a yield

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penalty of cent percent under; and the disease occurs throughout the Asian countries. It remains unsuccessful in controlling the disease and developing a resistant variety for MYMV through conventional breeding methods due to rapid explosion of new isolates of MYMV and also to the complexity of mechanism in controlling MYMV resistance (Selvi *et al*., 2006: Karthikeyan *et al*., 2012). At this situation Plant geneticists consider molecular marker assisted selection a useful additional tool in plant breeding programs to make selection more efficient to control this disesease (Maiti *et al.* 2011). One of the advances in molecular marker technology is Microsatellites or simple sequence of repeats (SSRs) are simple sequence of tandemly repeats which can presently be a short motif of di-nucleotides or tetranucleotides repeated and contained in 1-6 base pairs (bp) in length. Recently SSRs have become a popular type of co-dominant molecular marker in genetic studies (Lixia *et al.* 2009). Plant breeding application has also been useful in integrating genetic, physical, and sequence based maps of different crops, and provided breeders and geneticists with efficient tool to link phenotypic and genotypic variations. Therefore, they represent new sources of informative genetic markers (Khai and Lang 2005). Silva *et al.* (2008) reported SSR markers provided a rapid and technically simple way for identifying markers linked to disease resistant genes. With this background knowledge, the present study was carried out with the objective of identifying the association of resistance with SSR markers.

MATERIALS AND METHOD

Plant materials. VBN (Gg) 2 is an agronomically superior high yielding variety but highly susceptible to MYMV developed at Vamban. KMG 189 is a field resistant genotype to MYMV. The present investigation was carried out with 203 F2 individuals. These were derived from crossing between MYMV Susceptible parent VBN (Gg) 2 and MYMV resistant parent KMG 189 during Kharif 2009 at National Pulse Research Centre, Tamil Nadu Agricultural University, Vamban.

Phenotyping of F2 individuals. In the field condition MYMV infection can be evaluated by infector row method. The test material (203 F2 individuals) was scored after 80% of plants showed MYMV incidence. In F_2 generation, each individual was scored for MYMV infection using 1-9 rating scale suggested by (Singh *et al.,* 1988) and adopted. The mean disease score was calculated as disease rating and frequency.

DNA extraction and SSR analysis. DNA was extracted from leaves using the method described by (Karuppanapandian *et al*., 2006). Equal quantities of DNA were bulked from five resistant individuals and five susceptible individuals to give two DNA bulks, namely resistant bulks (RB) and susceptible bulks (SB),

respectively. A total of 45 azukibean SSR primers were used for DNA amplification in this experiment. The chromosome wise Azukibean SSR primers are listed in Table 1. PCR reaction mixtures were prepared with the volumes of 15 µL containing 3.00 µL of the extracted DNA, (25 ng/µL), 1.50 µL (10X) assay buffer, 1.00 µL (10µm) Primer, 0.50 µL (2.5 mM) dNTPs (Bangalore Genei Ltd., India), 0.20 µL (3 units/µL) Taq polymerase (Bangalore Genei Ltd., India) and 8.30 µL Sterile distilled H_2O. The PCR reaction was carried out in a DNA thermal cycler (BIO OVEN III) programmed to run the following temperature profile: 94°C for 5 minutes Initial denaturation then 35 cycles consisting each of a denaturation step for 1 min at 94°C, an annealing step for 1 min at 55°C: an extension step for 2 min at 72°C and the final extension for 5 min at 72°C. Agarose gel (3%) electrophoresis was performed to separate the amplified products. Seven micro litre of PCR amplified product was loaded with 3µl of loading dye. The voltage was maintained at 120 volts for 3 hours. The staining is done with Ethidium bromide solution separately after agarose gel electrophoresis and the bands were visualized and documented in a gel documentation system (Alpha Imager^{TM}1200, Alpha Innotech Corp., CA, and USA).

Table 1

List of Azuki bean SSR primers surveyed on VBN (Gg) 2 X KMG 189

S. No	SSR locus name	Forward Primer (5'-3')	Reverse Primer(5'-3')	Chromosome
01	CEDG254	CGATGTCTCTTGCTTCAAGG	GTGAAGGACTAGCCAAGTTTG	1
02	CEDG133	GCATACATAATGTGGTGAGATG	GTCTCGTGCCTTTCACAC	1
03	CEDG275	CACACTTCAAGGAACCTCAAG	GTAGGCAACCTCCATTGAAC	2
04	CEDG026	TCAGCAATCACTCATGTGGG	TGGGACAAACCTCATGGTTG	2
05	CEDG065	GGAATTTTGAGAACGGATTTGC	CCACCGACCACGGCCTTC	2
06	CEDG284	GGTGCTAACGTTGGAAACTGAG	CACTCCATTCTGAGGATCAATCC	2
07	CEDG043	AGGATTGTGGTTGGTGCATG	ACTATTTCCAACCTGCTGGG	3
08	CEDG117	GTACACTTCCACTAATCCAAAATT	TGGTACCTTCCTTATCTGAAATTA	3
09	CEDG186	GGATGGGAGAGTAAGAAG	GCATGGCATGATGACTTG	3
10	CEDG088	TCTTGTCATTTAGCACTTAGCACG	TTGTTGTTTACTAAGAGCCCGTGT	4
11	CEDG008	AGGCGAGGTTTCGTTTCAAG	GCCCATATTTTTACGCCCAC	4
12	CEDG165	GCTCTGTCAGTTCCCACTAC	GGTCCTGAACCCAGATGAAC	4
13	CEDG055	CAAACACTTTTGTAACTCCC	GCTTCTAACCTTGATCCTTC	4
14	CEDG127	GGTTAGCATCTGAGCTTCTTCGTC	CTCCTCACTTGGTCTGAAACTC	4
15	CEDG115	GGCTCATTGTACCACTGGATAT	ATGCCTCCTTTCAGGTGATTGT	5
16	CEDG264	GATTCCCTTCCTAGCTATGG	CTGCTGGACATGAAGATTCAG	5
17	CEDG015	CCCGATGAACGCTAATGCTG	CGCCAAAGGAAACGCAGAAC	6
18	CEDG191	CAATAAGCAATCTGTGGAGAG	CTGCAGGAAACTTGGAATTGC	6
19	CEDG143	GATGAACTCGTCTCGCTCATCG	CTGGACGCGTCTACTCAGAC	7
20	CEDG201	CGGGTAGACAAAGAGATACACG	CTAGCAGAAACAGGAGATCCTC	7
21	CEDG247	GTAGACACTGATCATCACC	GACCATCATCGATACGATTC	8

S. No	SSR locus name	Forward Primer (5'-3')	Reverse Primer(5'-3')	Chromosome
22	CEDG125	TGGAATATACTGTTTAATAGAG	AGATTAATTTGATCACTCATTC	8
23	CEDG251	ATATCTCAAAACCCTTCCTG	CCTCAATAACAATGATACGAC	8
24	CEDG257	GACTACTCTCAAGACCAAAG	GATGGTTGTAGATAACACTCC	8
25	CEDG265	GTAAAACAAACACACAGGAC	GCTCTCAACGAGAATGAAC	8
26	CEDG269	CTGTTACGGCACCTGGAAAG	GCAGAGACACACCTTAACCTTG	8
27	CEDG270	GTGCGTCACTAGTCCATTGC	GCAGAAGATTGAATCCTGGACC	8
28	CEDG286	CGAGCAGAACACTGATCATG	CCTCTTAGAGGTCATTGCTC	8
29	CEDG030	TGAGGGAATGGGAGAGAGGC	TCCGCAGATAGAGGCTCACG	8
30	CEDG304	ACCACTTCATAATCCCTGAG	GTTGCATGCTATATTTTGGTTCAC	9
31	CEDG011	GTCCGACTTTATGTGTGGAG	TTTCTAGTTCCAGCCCCGAC	9
32	CEDG080	CACGTTGGAGGAAGTGACGC	CATCGCCACCACAGAACCA	9
33	CEDG166	GGTACAACATTCTTCTATTTG	GGCTTATGAGTTTATCTTATC	9
34	CEDG150	GAAGGGAATGAAAATGAAACCC	GTTCAATCCATTCAGTCTCC	10
35	CEDG116	TTGTATCGAAACGACGACGCAGAT	AACATCAACTCCAGTCTCACCAAA	10
36	CEDG134	CTCCGTGTTGAAACAATGACG	GGTCTTTCTGATCTACGAACTTG	10
37	CEDG198	CAAGGAAGATGGAGAGAATC	CCTTCTAAGAACAGTGACATG	10
38	CEDG243	GACAACTCATCCATTCTTGAG	CCTATGGATAGTGATACAGC	10
39	CEDG273	GTTTAGCTTCTTCTGCTG	CCAAACTGTCAATATCTGC	11
40	CEDG014	GCTTGCATCACCCATGATTC	AAGTGATACGGTCTGGTTCC	11
41	CEDG042	CACAGTGGTTTGGGCAACAG	TCAGAGGTTCCCATTTCCCG	11
42	CEDG168	CTGCTTGGTGTTGAAGCTTC	CATTCTACATTCCAGACCTGC	**11**
43	CEDG100	CCCATCAAGTAACTACATAACA	ATGTGGGACTGGACAAATAAAA	**11**
44	CEDG295	CAAAGGTTAGATCCAACATCG	GGTTAGTCATCAACAACTCC	**11**
45	CEDG013	CGTTCGAGTTTCTTCGATCG	ACCATCCATCCATTCGCATC	Un mapped

RESULTS AND DISCUSSION

Genome level studies of mungbean have been very limited. Genome maps are valuable in providing insights into genome organization, inheritance and linkage of traits. Genome maps are therefore helpful in determining breeding strategies for traits of interest. Identification of resistant genotypes needs careful, repeated and thorough screening under ideal epiphytotic conditions, which is time consuming and laborious. Molecular markers associated with MYMV resistance would hasten the process of identification of resistant genotypes (Selvi *et al*., 2006). At the same time, one of the most time-consuming requirements in marker development is the need to screen the entire mapping population with every primer has been reduced by bulked segregant analysis (Malik *et al.*, 2007). The screening of contrasting bulks made from individuals of a same phenotype of a segregating

population suggests that the testing of the entire population is required only when polymorphisms between the bulks are detected (Michelmore *et al.*, 1991). The bulk segregant analysis is considered for saving time particularly when used with PCR based techniques. Recently Simple sequence repeat (SSR) is a popular tool in genetic studies. SSR markers prepared by polymerase chain reaction are widely used for mapping genes. The SSR marker had also been used effectively for tagging disease resistant genes such as Powdery mildew resistance in mungbean (Zhang *et al.*, 2008), Cercospora leaf spot in mungbean (Chankaew *et al.* 2010), *Phytophthora* resistance in soybean (Sandhu *et al*., 2005) and Asian rust in soybean (Silva *et al.*, 2008).

Microsatellites have been identified in *Vigna* species based on database searches (Yu *et al.*, 1999) and microsatellite libraries have been specifically developed from cowpea (Li *et al.*, 2001), mungbean (Kumar *et al.*, 2002) and azuki bean (Wang *et al.*, 2004). Though the number of these SSRs is very limited in mungbean (Swag *et al.*, *2006:* Lixia *et al.*, 2009). SSRs from azuki bean [*V. angularis* (Willd.) Ohwi & Ohashi] (Wang *et al.*, 2004), common bean (Blair *et al.*, *1997*) and cowpea (Li *et al.*, 2001) can be used in both mungbean and blackgram. As high as 72.70 per cent and 78.20 per cent of the azuki bean SSRs amplify the genomic DNA of mungbean and blackgram respectively (Chaitieng *et al.*, 2006).The high proportion of azuki bean SSR primer pairs that amplify DNA fragments in other Asian *Vigna* indicates their close relationship whereas in species of section *Ceratotrophis* 67-78% primer pairs succeeded in amplification. In addition, azuki bean SSR markers are useful in helping to improve the linkage maps of other Asian *Vigna* (Chaitieng *et al*., 2006: Lixia *et al.*, 2009). This enabled a saturated genome map for azuki bean developed (Han *et al.*, 2005) and azuki bean markers have enabled the second Asian *Vigna* linkage map to be developed that has resolved all linkage groups (Lixia *et al.* 2009). This is the usefulness of these azuki bean markers that they have used on this present study.

In the field condition, MYMV infection can be evaluated by MYMV disease rating scale suggested by Singh *et al*., 1988. Based on the rating scale MYMV score recorded for VBN (Gg) 2 was 5.1-7.0 and the score for KMG 189 was 1.0-2.0.The mean for this trait among the F_2 individuals was 5.68. This ranged from 1–9 among the F_2 individuals with the CV of 0.373. Besides 203 F2 individuals were categorized as 30 resistant individuals, 41 moderately MYMV resistant individuals, 57 MYMV moderately susceptible individuals, 56 susceptible individuals and 19 highly susceptible individuals. The frequency distribution of MYMV score among the F_2 individuals is shown in Figure 1. The MYMV susceptible VBN (Gg) 2 and MYMV resistant KMG 189 parents were initially screened fourty five SSR primers. Among the fourty five SSR primers DNA amplification was obtained only with 35 primers. Out of the 35 primers only 6 primers CEDG 243, CEDG 257, CEDG 115, CEDG 008, CEDG 269 and CEDG 201 (17.14%) showed polymorphism between the parents (Figs. 2, 3). These six SSR primers were taken

for bulk segregation analysis, none showed polymorphism between the parents and bulks (Fig. 4). Similar to the present finding recently Sudha (2009) screened 106 azukibean and 11 mungbean SSR primers in an interspecific population (F2 and F3) crossing between a ricebean TNAU RED and a mungbean VRM (Gg)1. Anushya (2009) screened 35 azukibean and four mungbean SSR primers a in the same population F_9 generation. The findings of both Sudha (2009) and Anushya (2009) are similar to the above results. This attributes the fact that these SSR primers are heterologous probes developed in azuki bean which can produce polymorphism between parents and are not able to distinguish the bulks.

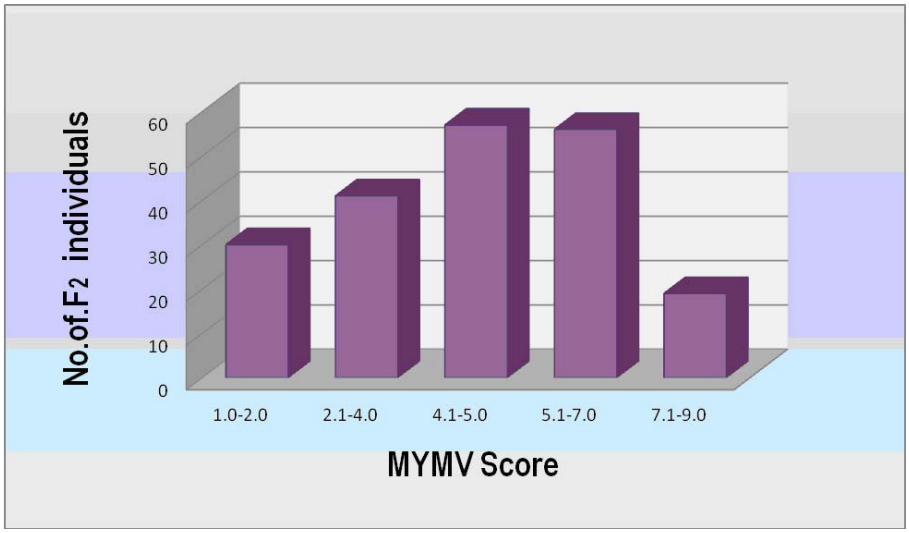

Fig. 1. Frequency distribution of MYMV score surveyed on F_2 individuals. (1.0-2.0: resistant, 2.1-4.0: moderately resistant,4.1-5.0: moderately susceptible,5.1-6.0: susceptible,7.1-9.0: highly susceptible).

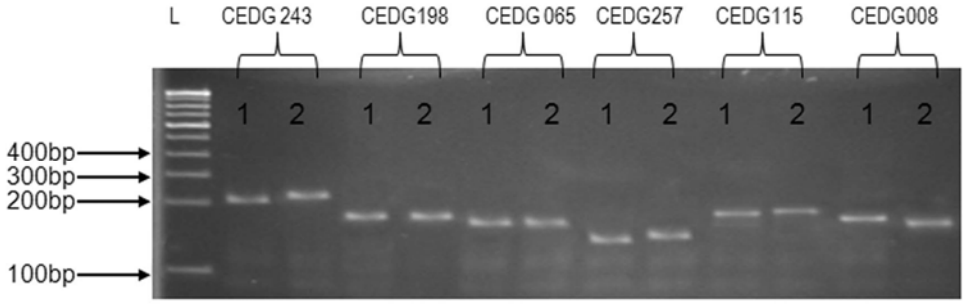

Fig. 2. Parental survey of mungbean lines VBN (Gg) 2 (1) X KMG 189(2) with SSR primers CEDG 243, CEDG 198, CEDG 065, CEDG 257, CEDG 115 and CEDG 008.

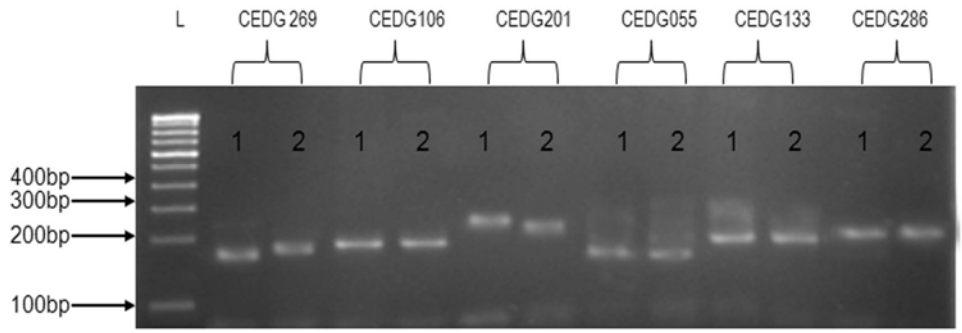

Fig. 3. Parental survey of mungbean lines VBN (Gg) 2(1) X KMG 189(2) with SSR primers CEDG 269, CEDG 106, CEDG 201, CEDG055 and CEDG 113,CEDG 286.

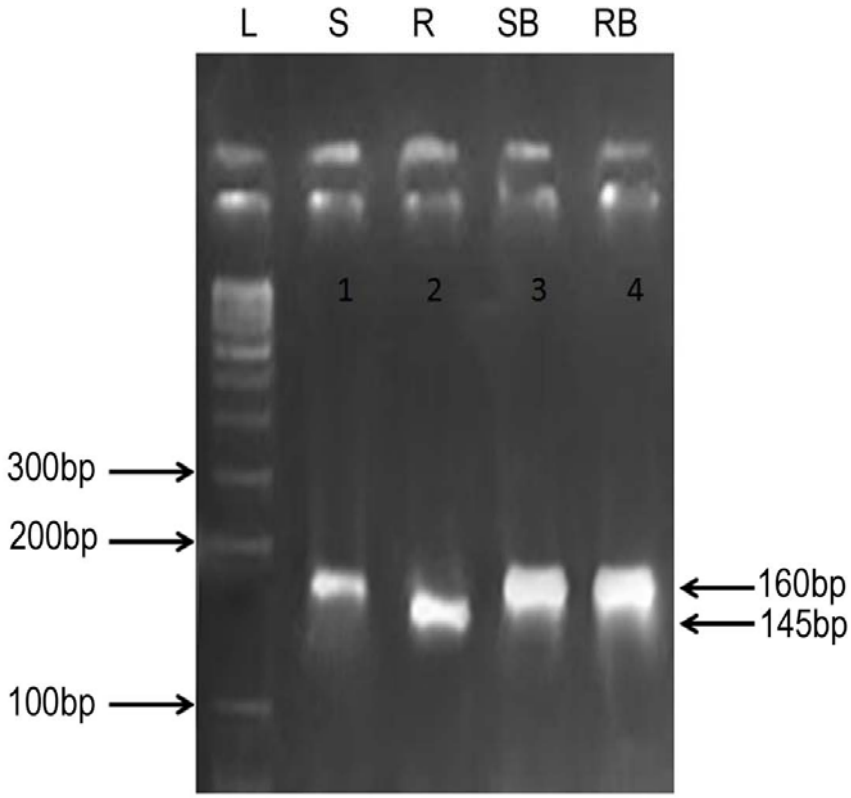

Fig. 4. Bulk segregant analysis with SSR primer CEDG 008 showing polymorphism between susceptible mungbean parent (SP) VBN (Gg) 2(1), resistant mungbean parent (RP) KMG 189 (2) and it does not show the polymorphism between susceptible bulk (SB)(3) and resistant bulk (RB)(4).

CONCLUSION

In the present investigation Azukibean SSR markers are used to identify the important trait (Yellow Mosaic Disease) in mungbean, but it could not give consistent results as at the same time the above findings revealed. To improve the mungbean crop wide range of the genome level studies are required. Presently available markers are not sufficient to identify and mapping the MYMY resistance gene in mungbean. To develop the large number of mungbean markers can help control this economically important disease.

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INDUCTION OF DROUGHT TOLERANCE WITH SEED PRIMING IN WHEAT CULTIVARS (*TRITICUM AESTIVUM* L.)

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Delay in planting and low precipitation (< 300mm annual) in wheat (*Triticum aestivum* L.) farming is the major problem in the irrigated and rainfall lands of Iran. A factorial experiment for evaluating the effects of seed priming on wheat cultivars was carried out under laboratory, greenhouse and at two field conditions during seasons of 2008-2010. Arrangement of treatments were Zarrin, Shariar, Sardary and Azar cultivars as A factor, and priming treatments including DW, osmotic solutions (10% PEG, 2.5% KCl, 4% MN, 10% Urea, 5% NaCl W/V) and plant growth inducers (20ppm IAA, 1000ppm CCC) with non-primed seed as a control established B factor. At the second year of field conditions two separate experiments were done under drought stress and well watered conditions. Irrigation of drought stress was withheld at booting stage. Maximum amount of absorbed water was related to Shariar, which was 15.5 g at DW. At all cultivars the most increased seed weight was seen for primed seed with CCC and IAA. Irrespective of cultivar seedling related traits revealed that CCC increased plumule and their radical dry weights (11.5 and 8.0mg) and lengths (17.2 and 17.8cm). In opposite, Urea pretreatment had negative effects for seedling growth. All priming treatments at four cultivars increased grain yield and its components, chlorophyll content and nitrogen absorbed under field and green house conditions as compared to control. Primed seed with potassium chloride at drought stress had the low variation percentage for traits of relative water content (–9.3%), total dry matter (–10.7%) and grain yield (–4.0%) than well watered conditions. Potassium chloride improved drought tolerance at all wheat cultivars. There were significant correlations for traits of spike per square meter (0.91**), grain per spike (0.92**) and total dry matter (0.79*) with grain yield. Therefore, it seems that these traits could be used as indirect criteria for selection of high grain yield of cultivars for primed seed.

Key words: drought stress, hydro- and osmo- priming, plant growth inducers, wheat.

Abbreviations: PEG, Polyethylene glycol; CCC, 2-Chloroethyl-trimethyl-amonium chloride; Ethephon; 2-Chloroethyl phosphonic acid; KCl, Potassium chloride; NaCl, Sodium chloride; IAA, Indole-3 acetic acid; K_2SO_4, Potassium sulfate; DW, Distilled water; MN, Micronutrient.

INTRODUCTION

In irrigated lands, winter wheat and sugar beet fallow is the dominant rotation in 130000 hectares of West Azerbaijan province of Iran. Planting of winter wheat

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was delayed after harvesting of sugar beet. In addition, low precipitation and inadequate moisture of seed zone under rainfall conditions is the major limiting factor that reduces grain yield potential. Therefore, seed priming is a technology that enhances rapid (7-10d) emergence and early establishment in both regions. Rapid and uniform field emergence is an essential prerequisite at two irrigated and rainfall conditions to reach the yield potential, quality, and ultimately profit in annual crops. Seed priming has been common pretreatment that reduces the time between seed sowing until emergence and synchronized seedling emergence (Parera and Cantliffe 1994). Seed priming can be accomplished through different methods such as hydro-priming (soaking in DW), osmo-priming (soaking in osmotic solutions such as PEG, potassium salts, eg., KCl, K_2SO_4) and plant growth inducers (CCC, Ethephon, IAA) (Capron *et al.,* 2000; Chiu *et al.*, 2002; Harris *et al.*, 1999; Chivasa *et al.*, 1998).

Several documents confirmed that seed priming have many benefits including early and rapid emergence, stand establishment, higher water use efficiency, deeper roots, increasing in root growth, uniformity in emergence, germination in wide range of temperature, break of seed dormancy, initiation of reproductive organs, better competition with weed, early flowering and maturity, resistance to environmental stresses (such as drought and salinity) and diseases (*Sclerotium rolfsii*), higher grain yield in wheat (*Triticum aestivum* L.) (Ghana and Schillinger 2003), corn (*Zea mays* L.) (Subedi and Ma 2005), canola (*Brassica napus* L.) (Farhoudi and Sharifzadeh 2006) pearl millet (*Pennisetum glaucum* L.) and chickpea (*Cicer arietinum* L.) and rice (*Oriza sativa* L.) (Harris *et al.*, 1999 and 2005), lettuce (*Lactuca sativa* L.) (Cantliffe *et al.*, 1984) under field and laboratory conditions. Inversely, longevity of primed seed was decreased (Bruggink *et al.*, 1999).

Singh and Agrawal (1977) before sowing treated wheat seed with DW during 12h, found that 11kg/ha nitrogen uptake was increased. Misra and Dwivedi (1980) found that seed soaking in 2.5% KCl for 12h before sowing 15% increased wheat grain yield. Paul and Choudhury (1991) observed that seed soaking with 0.5 to 1% solutions with KCl or K_2SO_4 significantly increased plant height, grain yield and its components in wheat genotypes. Kulkarni and Eshanna (1988) stated that pre-sowing seed treatment with IAA at 10ppm improved root length, rate of germination, and seedling vigor. The objective of this study was to evaluate several priming solutions on early growth, grain yield and its components under laboratory and field conditions. Specific objectives were to determine the effect of seed priming on improving winter wheat cultivars to drought stress under field conditions.

MATERIALS AND METHODS

To evaluate the effects of seed priming on seedling and whole plant growth, responses of four wheat cultivars in hydro, some-priming and plant growth

inducers were studied. Seed newly harvested was used. Wheat cultivars including Sardary and Azar (for rainfall conditions) Zarrin and Shariar (for irrigated conditions) treated with eight priming media: 1 – Hydropriming (DW), 2 – Osmopriming (2.5% KCl, 10% Urea, 5% NaCl, 4% MN and 10% PEG 8000 W/V), 3 – Plant growth inducers (20ppm IAA, and 1000ppm CCC). A non-treated as a check for four cultivars was also included. All priming media were prepared in distilled water and seeds soaked at 25˚C. The duration of soaking for hydro, osmopriming and plant growth inducers were 16h and 30min, respectively. 500g seed of each cultivar was placed in 36 individual one liter capacity bashers and immersed in liquid priming media. After soaking weight of seeds was recorded and rinsed three folds with tap water. All seed lots were surface sterilized with 10% Sodium hypochlorite solution for 10 minutes, then rinsed with sterilized water and air dried at room temperature (25˚C) for 20 days. After air-drying, the weights of seeds were recorded again, and amount of moisture absorbed during soaking was derived (Subedi and Ma 2005; Ghana and Schillinger 2003).

Laboratory experiment. Germination test of dried seed was measured at laboratory with using a factorial experiment based on Completely Randomized Design for 36 combination treatments with five replications. Factor A and B included four wheat cultivars and nine priming media+control, respectively. For each treatment 100 seeds were placed on five 90mm diameter of Petri dish. Two filter paper of Whatman No. 2 was moistened with 10mL of distilled water. Seed was kept at germinator in 20˚C for 10 days under 16/8h day/night light. After this period plumule and radical lengths, and dry weights of them were measured.

Greenhouse experiment. Plants were grown in 0.5L plastic pots (5cm diameter) filled with a mixture of soil, Peat moss, Vermiculite and Perlite (3:1:5:1 v/v). Selection of greenhouse treatments was based on germination performance in the laboratory experiment. As germination was negative effect by Urea and NaCl, these treatments were removed. The arrangement of factors was the same of laboratory experiment. Three uniform seeds of each treatment were planted on 25 February 2008. At seedling emergence (10 days after planting), one gram of NH4NO3 fertilizer were applied for per pot at each irrigation. Pots were regularly watered. The temperature inside the greenhouse was maintained at 25/15˚C (day/night regime ± 3˚C) with 10h photoperiod. At 60 days after planting, when plants were at five leaves stage, plants were removed and oven dried at 80˚C for 24h and then nitrogen uptake was measured (Bremner and Mulvaney 1982). Leaf chlorophyll content was measured using SPAD-502.

Field experiments. They were carried out in West Azerbaijan agricultural research center in 2009-2010. The experimental field station was located at latitude 45º 22'N, 75º 32' 36° 58′, longitude 46° 6′ and altitude 1371m, by a typical silty loam texture.

At the first year, seed lots used in the laboratory experiment were planted with factorial experiment based on Randomized Complete Blocks design with five replications. Chemical fertilizers were applied pre-planting according to soil analysis, therefore 100kg per hectare NH4NO3 was applied before planting. At the booting stage, 1.5L.ha-1 of 2-4-D was used for weed control.

At the second year the same treatments were planted at two separate factorial experiments based on Randomized Complete Blocks Designs under drought and well-watered conditions. In drought experiment water was withheld at the booting stage and irrigation was done after 150 ± 5mm evaporation from the Class A Pan. Well watered plots were irrigated after 75 ± 5mm evaporation from the Class A Pan (Table 1). To determine above ground biomass, four central rows were harvested upon maturity. Total dry matter, grain yield, 1000-kernel weight, spike/m^2, grain per spike, relative water content (Gonzalez 1999) and plant height were measured.

Analyses of variance for all data of laboratory, greenhouse and field experiments were conducted by Mstat-c software. Treatments were considered significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

Seed Soaking. A determined weight of seed lots were put inside the prepared solutions. After soaking and re-drying increased weight was measured (Table 2). The greatest amount of absorbed water within cultivars was observed for Shariar with DW and the lowest amount corresponded to Zarrin and Shariar with CCC pretreatment. Priming with CCC and IAA pretreatments had the shortest time and the lowest absorbed water to the other types, but the most increased seed weight. In general, increased weight of primed seed lots was due to activation of cell respiration (Bewley and Black 1994), repairs of macromolecules (Osborn 1993), movements of acquired materials (Gallardo *et al.*, 2001), activation of cell cycling (Vasquez-Ramos and Sanchez 2004) and weakening of seed coat structure for radical emergence (Cantliffe *et al.*, 1984). Water absorption is the first stage of germination, at the second stage or retardation stage, seeds start the replication of DNA (Bray *et al*., 1989), increasing of protein and RNA synthesis (Gallardo *et al.,* 2001), availability to more ATP (Mazor *et al.*, 1984), rapid embryo growth (Dahal *et al.,* 1990) than control seeds.

Seedling Vigor and Plant Stand. Radical lengths of Shariar and Zarrin at pretreatments with IAA and CCC were 22.3, 22.0, 22.0 and 22.5cm, respectively (Fig. 1-A). Cell divisions intensified at radical tip with priming CCC and IAA (Farooq *et al*., 2006; Fu *et al*., 1988). The trend of variation between cultivars and pretreatments for plumule length was similar with radical length, however, at urea pretreatment decreased (Fig. 1-B). Irrespective of cultivar, pretreatments of CCC,

IAA and DW with 8.1, 8.0, 8.1g had more radical dry weights (Fig. 1-C). Within pretreatments, potassium chloride had the most plumule dry weight 12.6g and Urea 7.1g was the lowest value (Fig. 1-D). Increased plumule dry weight due to osmopriming was reported by Harris *et al*., (2004). It caused rapid plant stand during germination and ultimately production of more dry matter. Rapid germination in primed seeds caused increasing disintegration including of Alfa amylase enzyme, more levels of ATP, synthesis of RNA and DNA, increasing number and efficiency of Mitochondria (Bittencourt *et al*., 2005).

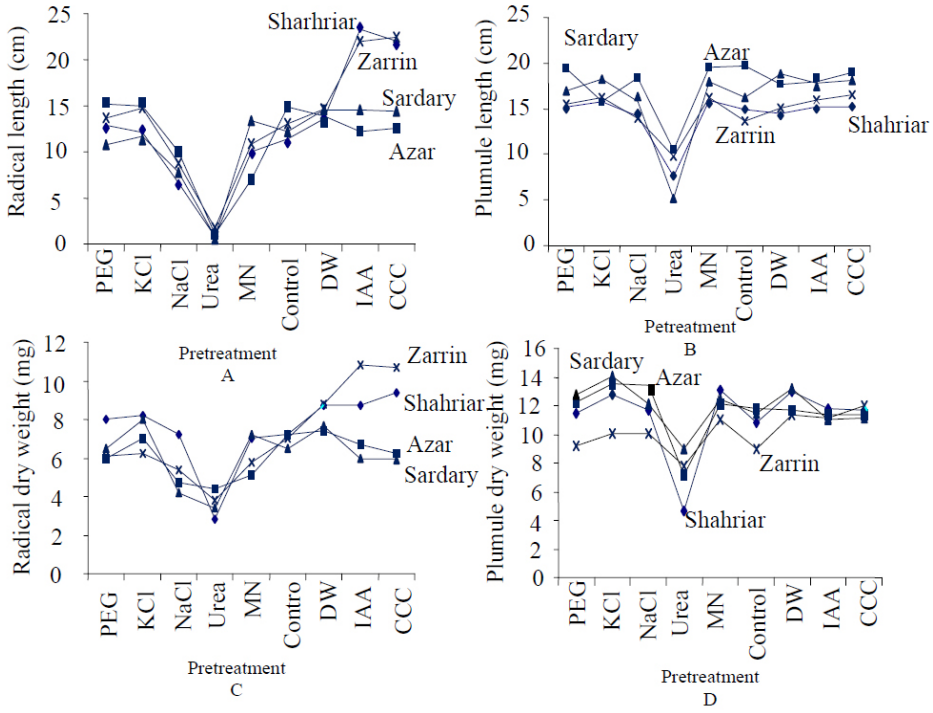

Fig. 1. The effects of different seed pretreatments in wheat cultivars on seedling related traits.

Response to Nitrogen. Azar and Sardary at IAA pretreatment had the most chlorophyll percentage and Zarrin and Sharriar were the highest values in DW (Fig. 2-B). All pretreatments at four cultivars had more nitrogen absorption than control. The highest nitrogen absorption with 57.3% was measured for Shariar and the lowest amount for Sardary with 47.6% (Fig. 2-A). Priming with CCC and IAA in comparison to the other pretreatments at four cultivars had more nitrogen absorption. The reason behind that is probably the increase of the radical length, which was seen in the laboratory evaluation. The increase of nitrogen absorption at priming with plant growth inducers may finally cause improvement of grain yield.

Absorbed nitrogen directly depends on leaf chlorophyll content, and increased chlorophyll content, in turn, improves metabolism and photosynthesis (Kulkarni and Eshanna 1988).

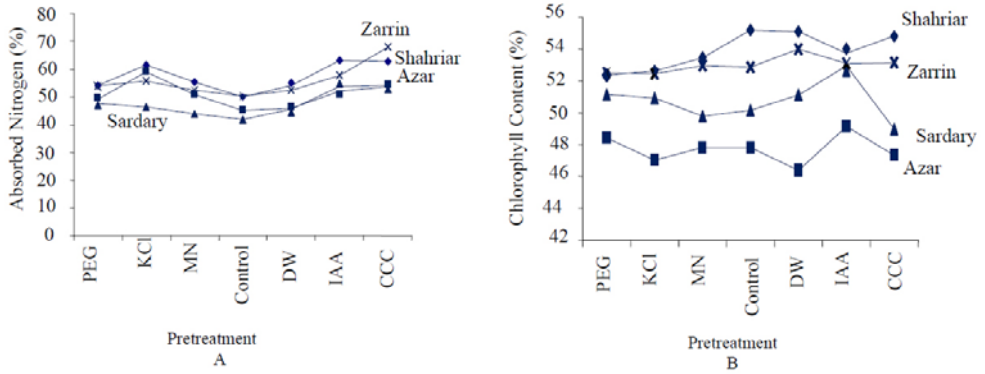

Fig. 2. The effects of different seed pretreatments in wheat cultivars on traits of chlorophyll content and absorbed nitrogen under green house conditions.

Morpho-physiological traits. Under field conditions, all of pretreatments at four cultivars had more grain yield than control and among them CCC had the highest value with 591g/m^2. Responses of cultivars varied with the type of pretreatments. Therefore, the grain yield of Shariar was 635, 625 and 613g/m^2 for hydropriming, CCC and MN, respectively. Zarrin with IAA and CCC had 628 and 620g/m^2 grain yield, respectively. Pretreatment of CCC for grain yield of Sardary and Azar were 590 and 520g/m^2, respectively (Fig. 3-A). The increase of grain yield with pretreatments was due to the expansion of leaves, which results in more photosynthesis, assimilation and ultimately more total dry matter. Accumulated materials in plants were effective during seed set and grain filling (Haris *et al*., 1999 and 2004). Many researchers reported the increase of grain yield in wheat cultivars due to pretreatments, as 37% in (Misra and Dwibedi, 1980), and 15% in (Haris *et al*., 1999 and 2004). Success in seed priming depends on type of cultivar, osmotic potential solution, time of priming, environment of temperature, seed vigor, the rate of seed re-drying and the conditions of primed seed storage (Parera and Cantliffe 1994).

The highest grain per spike with 66 grain was counted for Shariar and Zarrin, and the lowest values corresponded to Azar and Sardary with 36 and 30 grain, respectively (Fig. 3-B). Irrespective of cultivar, pretreatments of IAA, CCC, DW and MN had 54, 53, 52 and 51 grain per spike, respectively. Pretreatments of CCC for 1000-Kernel weight with 45g and CCC, DW and MN with 372, 371 and 366 spike per square meter were the highest values (Fig. 3-C and D). The range of variations for spike per square meter was between 277 and 392, related to Sardary and Zarrin, respectively.

The most total dry matter was measured for pretreatments DW, IAA and CCC with 1198, 1185.3 and 1196.6g/m^2, respectively (Fig. 3-E). The increased number of spike per square meter at all pretreatments is a reason of raising total dry matter. The lowest plant height was obtained for IAA and CCC pretreatments (Fig. 3-F). The effect of CCC on inter-node distance resulted in short plant height, which is a genetic trait (Musa *et al*., 2001).

Inducing of drought tolerance stress. The most variation percentage of grain yield under drought stress compared to well watered were measured for Shahriar 39% primed with IAA, Azar 23% primed with CCC, Sardary 32% primed with PEG and Zarrin 45% primed with CCC. In contrast, pretreatment of potassium chloride at four cultivars had the lowest variations percentage (Table 3). Potassium ion induced tolerance under drought stress (Khajeh-Hosseini *et al*., 2003). Depending on the cultivar responses of grain yield components to pretreatments were different. The variations percentage of grain yield components within primed seeds with potassium chloride at four cultivars showed that under drought stress 1000-kernel weight had the lowest value. In contrast, except to Sardary maximum variations percentage was seen for spike per square meter (Fig. 4). Saha *et al*., (1990) reported that performance of grain yield at primed seeds of soybean had differed and depended on cultivar type. The increase of grain yield at primed seeds in wheat, barley, rice, sorghum, chickpea, millet were stated by different researchers (Harris *et al.*, 2001; 2004; Misra and Dwibedi 1980; Paul and Choudhury 1991).

Table 1

General characteristics, summary of water inputs (rainfall and irrigation), class A pan evaporation and maximum and minimum temperatures in 2009-2010 under field conditions

Number of cultivars	4	Month	Tmax (°C)	Tmin (°C)	Rain (mm)	Irr. (mm)	Evap. (mm)
Number of pretreatments	7						
Number of combination treatments	28	October	26.1	9.2	5.5		124.1
Total plots	280	November	15.6	9.3	27.7		45.2
Density of plants	400	December	8.9	1.5	32		
Intervals between blocks	1.3m	January	7.7	-0.7	38.4		
Intervals between rows	0.2m	February	5.9	-3.8	16.6		
Rows per plot	6	March	14.4	-0.4	44.6		
Plot size	1.2×2m^2	April	15.9	5.3	63.6	25	57.7
Harvest area per plot	1m^2	May	20.2	6.9	34.1	33	130.9
Replications per experiment	5	June	28.6	10.7	3.8	110	232.3
Ec of water irrigation	0.024ds/m	July	33	15.7	4.4	130	314.6

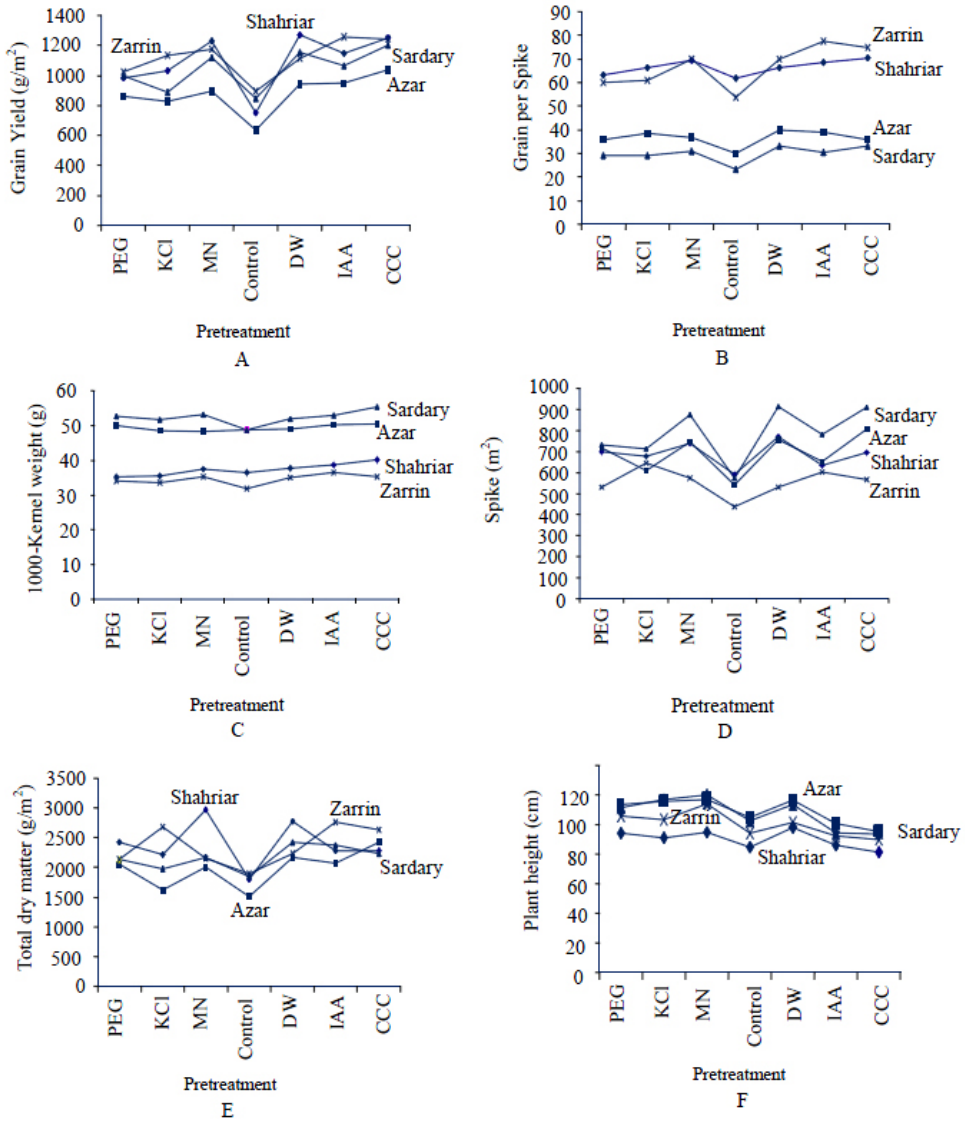

Fig. 3. The effects of different seed pretreatments in wheat cultivars on morpho-physiological traits under field conditions.

All pretreatments had lower total dry matter variations under drought stress compared to well-watered (Table 3). Among them Potassium chloride had the lowest value, which induced more drought tolerance stress. Pretreatments resulted in high total dry matter through effect on growth period. Primed seeds after sowing had faster germination, rapid establishment, and uniformity in growth. Such a plant

expands root system at short time compared to the control and with uptaking more water and nutrient materials produce photosynthetic organs that rapidly reach the autotrophic stage (Duman 2006). Flag leaf of relative water content at primed with potassium chloride and control were the maximum and minimum variations (Table 3), respectively.

Table 2

Changes in weight and moisture content of wheat seed cultivars after soaking and air-drying for 20 days in 25˚C

Cultivar	Seed weight (g)	Pretreatment							
		DW	NaCl 5%	Urea 10%	KCl 2.5%	PEG 10%	MN 4%	IAA 20ppm	CCC 1000ppm
Sardary	water absorbed	9.68	2.43	3.42	10.44	11.48	12.04	3.60	2.28
	Increased seed weight	0.42	0.33	0.32	0.96	1.12	1.02	1.20	1.12
Azar	water absorbed	13.32	2.55	2.96	9.52	7.76	12.40	4.4	3.68
	Increased seed weight	0.16	0.17	0.05	0.20	0.40	0.28	0.76	0.84
Zarrin	water absorbed	10.56	2.78	3.1	8.88	6.32	11.20	2.68	1.92
	Increased seed weight	0.52	0.22	0.25	0.80	1.16	0.52	0.96	1.20
Shariar	water absorbed	15.12	3.35	3.77	13.32	9.88	13.32	4.88	1.96
	Increased seed weight	0.4	0.12	0.15	0.60	0.88	0.44	0.96	1.24

Table 3

Variations percentage for traits of grain yield, total dry matter and relative water content in primed seeds of wheat cultivars under drought stress in comparison with well watered

Grain yield (g/m^2)							
Cultivar	PEG 10%	KCl 2.5%	MN 4%	Control	DW	IAA 20ppm	CCC 1000ppm
Shariar	-17.6	-7.1	-25.1	-36.1	-33.0	-39.0	-32.0
Azar	-22.5	-1.9	-7.4	-15.6	-9.1	-16.6	-23.9
Sardary	-32.4	-2.3	-8.8	-27.7	-16.5	-16.7	-17.1
Zarrin	-27.3	-4.3	-37.7	-33.4	-32.9	-40.8	-45.4
Total dry matter (g/m^2)							
Shariar	-14.2	-12.3	-11.0	-18.1	-14.3	-15.5	-13.3
Azar	-12.7	-10.1	-13.8	-17.4	-17.2	-12.4	-14.0
Sardary	-13.9	-9.8	-10.3	-15.8	-10.7	-11.7	-12.2
Zarrin	-15.7	-10.4	-18.1	-20.2	-11.0	-13.6	-12.3
Relative water content (%)							
Shariar	-28.0	-9.1	-24.9	-37.1	-19.6	-22.7	-11.7

Azar	-25.4	-10.5	-17.3	-25.6	-14.6	-18.9	-12.3
Sardary	-19.5	-9.6	-16.2	-27.1	-11.0	-17.9	-13.6
Zarrin	-37.0	-8.0	-34.0	-35.0	-21.8	-34.5	-9.7

Variation (%) = [(Mean cultivar under stress-Mean cultivar under well watered)/Mean cultivar under well watered]×100.

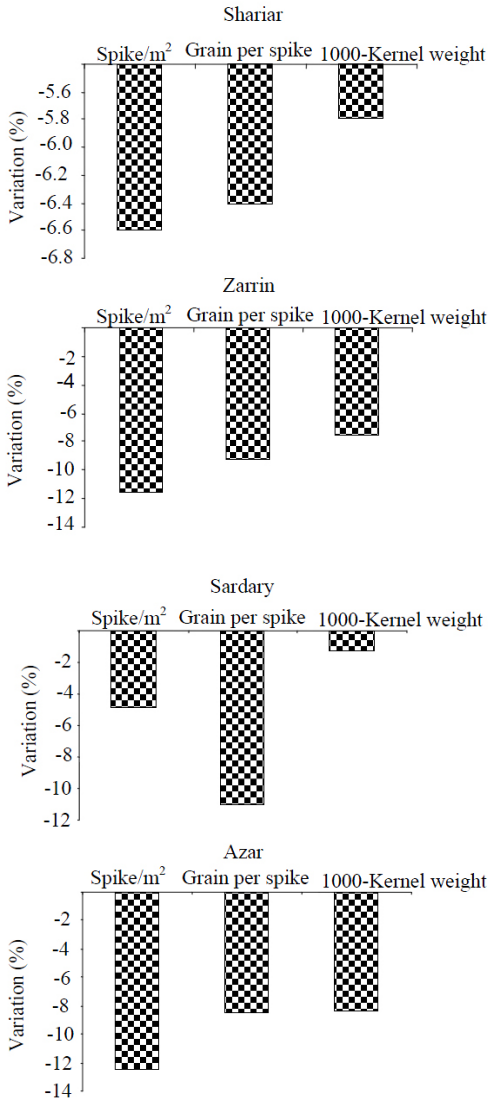

Fig. 4. Variations percentage of grain yield components of primed seed with using of Potassium chloride in wheat cultivars under drought stress in comparison with well watered.

Correlation coefficients of traits. Traits of spike per square meter, grain per spike and total dry matter with grain yield had positive and significant differences (Table 4). With increasing of these traits, grain yield increases as well. Spike per square meter and grain per spike at potassium chloride pretreatment had the highest variation percentage. Therefore, these traits could be used as an indirect criterion for the selection of high grain yield. Chimenti and Hall (1994) observed a positive correlation between leaf area and grain yield in sunflower under drought stress, and used it as an indirect selection in screening tolerant genotypes under drought stress.

Table 4

Correlation coefficients of wheat cultivars traits primed seed with using of different pretreatments

Trait	Grain yield (g/m^2)	Spike/m^2	Grain per spike	1000-Kernel weight (g)	Total dry matter (g/m^2)
Spike/m^2	0.91**				
Grain per spike	0.92**	0.83*			
1000-Kernel weight (g)	0.54	0.74	0.59		
Total dry matter (g/m^2)	0.79*	0.79*	0.76*	0.29	
Relative water content (%)	0.50	0.66	0.29	0.33	0.55

* and **: significant differences at $p \leq 0.05$ and 0.01 probability levels, respectively.

CONCLUSION

Responses of wheat cultivars were different to pretreatments. Seed priming with IAA and CCC at 30 minutes had positive effects increasing the related seedling, nitrogen absorption and grain yield traits. It also increases the components of grain yield more than other pretreatments at 18 hours. In opposite, Urea pretreatment had negative effect on seedling related traits compared to control. Therefore, we excluded Urea pretreatment in green house and field experiments. The most variation percentages for grain yield under drought stress compared to well-watered case were for Shariar, Azar, Sardary and Zarrin with IAA 39%, CCC 23%, PEG 32% and CCC 45%, respectively. In contrast, Potassium chloride pretreatment at four cultivars showed the minimum variations for grain yield, total dry matter and relative water content traits. Potassium ion induced resistance to drought stress. This pretreatment, except for Sardary, had the strongest effect on spike per square meter. The trend of variations for pulmule

length at laboratory experiment was similar with plant height at field conditions. In the case of CCC pretreatment, the pretreatment decreased inter-node distance and subsequently plant height, but in other pretreatments it was increased. Seed priming improved grain yield up to 40 percent. Increase of 25% in absorbed nitrogen causes better vegetative growth and total dry matter compared to control. Factors that influence seed priming are type of variety, osmotic potential of media, time of priming, seed vigor and the rate of dried seed during priming, storage conditions, climate and the type of planting primed seed. Pretreatments with increasing seed vigor and rapid growth at seedling stage under field conditions has direct effect on grain yield. In addition, improving the germination percentage and uniformity emergence, pretreatment results in suitable density with increased tiller number and grain per spike. Under drought stress conditions, it is recommended that seeds be primed with potassium chloride. It is suggested that proteomic techniques be used to identify molecular mechanisms under drought stress for primed seeds.

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MITOTIC INDEX AND DNA CONTENT AS BIOLOGICAL TOOLS ON DETECTION OF MUTATION IN TREE SPECIES (*JATROPHA CURCAS* L.)

D. DHAKSHANAMOORTHY, R. SELVARAJ[1]

The aim of the present study was to investigate the effect of different doses of gamma rays (5Kr, 10Kr, 15Kr, 20Kr and 25Kr) and ethyl methane sulphonate (EMS) [1%, 2%, 3% and 4%] on mitotic index and DNA content in *Jatropha curcas* because these two traits are important in tree species to access the altered genotype in early days itself. In the present study, the mitotic index and DNA content ranged 3.66-0.93 and 1.95-1.35, respectively for gamma rays and EMS treatments. All treatments of gamma rays and EMS showed reduced mitotic index and DNA content when compared to that of control which recorded 29.28 and 2.27 respectively. From the present investigation, we conclude that gamma rays and EMS may change genetic material at molecular level and that result in the altered genotype in *J. curcas*. To study the growth (flowering and fruiting) and yield parameters in the mutant populations, several mutation generations (M_1, M_2 …) to be investigated and that is not feasible in *J. curcas* since it is a perennial tree crop. In this respect, the results of the present study will be useful to access the mutagenic effects in tree species.

INTRODUCTION

Jatropha curcas (Euphorbiacea), or simply *Jatropha* (Kattamanakku in Tamil), is a tree thought to be native from Central America (Fairless, 2007) and possibly from Brazil. It is now almost pantropical and has been listed as a weed in Australia, South Africa, India, Brazil, Fiji, Honduras, Panama, EI Salvador, Jamaica, Puerto, Rico, and other parts of Caribbean. *J. curcas* has been found to be a highly promising species which can yield oil seed (non-edible) as a source of energy in the form of biodiesel owing to its short gestation period, easy adaptation to different kinds of marginal and semimarginal lands, drought endurance and avoidance by animals. This Euphorbia is a "drought resistant" plant which grows on wasteland and could easily be cultivated by low income farmers. It is grown as a shrub and it could benefit energy provision to remote areas. In this respect, *J. curcas* is considered a strategic crop for developing countries such as India and

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Brazil. Even in good irrigation, fertilization and soil tillage conditions, *J. curcas* may reach the flowering stage only after 1 year of planting and can produce an abundant crop. Consequently, the farmers have not shown interest in cultivation of *J. curcas* because it lacks an improved germplasm in terms of agronomic characteristics such as flowering, maturity and high seed yield and oil content. In addition, several pests and diseases have already been observed at the industrial production level.

Mutation breeding in tree species is not conserved attractive because of lacunae in conventional breeding like time-consuming, unpredictable results, long juvenile phase, high heterozygosity and fear in loss of the unique genotype. However, studies on induced mutation in *J. curcas* have been performed by Pandey and Datta (1995); Sakaguchi and Somabhi (1987), Dwimahyani and Ishak (2004) and Dhakshanamoorthy *et al*., (2011) and in other plants such as *Pinus* (Thapa, 2004), perennial rye (Akgun and Tosum, 2004), cashew (Klarizze, 2005) and grapevine (Charbaji and Nabulsi, 1999). While *J. curcas* germplasm is being harvested all over the world with the purpose of crop improvement, little is known about its genome. Hence, in the present investigation, we studied the effects of gamma rays and EMS on mitotic index and genomic DNA in *J. curcas.*

MATERIAL AND METHODS

The seeds of *J. curcas* were treated with different doses of physical and chemical mutagens viz., control, 5Kr, 10Kr, 15Kr, 20Kr and 25Kr of gamma rays and 1%, 2%, 3% and 4% of EMS respectively. The treated seeds were sown at 1cm depth in plastic trays (23×27cm, 6cm in height) filled with river sand, red soil and farm yard manure in the ratio of 3:2:1. After germination, the root tips from control and treated plants were fixed in acetic acid and alcohol in the ratio of 1:3 and stored at room temperature for 24hrs. Then, the root tips were washed with distilled water for 10 minutes to remove the fixatives. Haematoxylin stain was used for squashing by following the technique described by Marimuthu and Subramanian (1960). The number of chromosomes and mitotic index were observed under the light microscope (Carl Zeiss) and the important stages photographed using digital camera (Cannon). For genomic DNA investigation, the fresh leaf material was harvested from five month-old plants treated with gamma rays and EMS along with control. Genomic DNA extracted by adopting the CTAB method outlined by Doyle and Doyle (1990) with minor modifications suggested by Dhakshanamoorthy *et al.* (2009). The genomic DNA isolated was quantified spectrometrically by measuring absorbance at 260 nm.

RESULTS AND DISCUSSION

In *J. curcas,* the diploid chromosome number was 2n = 22 and the size of the chromosome was smaller (Plate 1) than of the species belonging to the family "Euphorbiaceae". Mitotic index (MI) was reduced by gamma rays and EMS treatments. The average MI of control, gamma rays (5, 10, 15, 20 and 25Kr) and EMS (1, 2, 3 and 4 per cent) were found 29.28, 3.66, 4.50, 1.86, 1.43, 1.60, 1.50, 1.36, 1.03 and 0.93 respectively. The highest MI frequency was found to be 29.28 in the control while lowest frequency was 1.43 and 0.93 at 20Kr of gamma rays and 4 per cent of EMS, respectively. In terms of MI, the differences between control and treatments of gamma rays and EMS were not significant at 5 per cent level ($p < 0.05$). In terms of total number of dividing cells, it was significantly ($P > 0.05$ per cent level) reduced by all the treatments of gamma rays and EMS than control (Table 1). Among the mutagenic treatments, 25Kr of gamma rays (7.06) and 4 per cent of EMS (2.13) were shown a higher reduction for the total number of dividing cells than control (15.83). The critical difference for total number of dividing cells was 3.635.

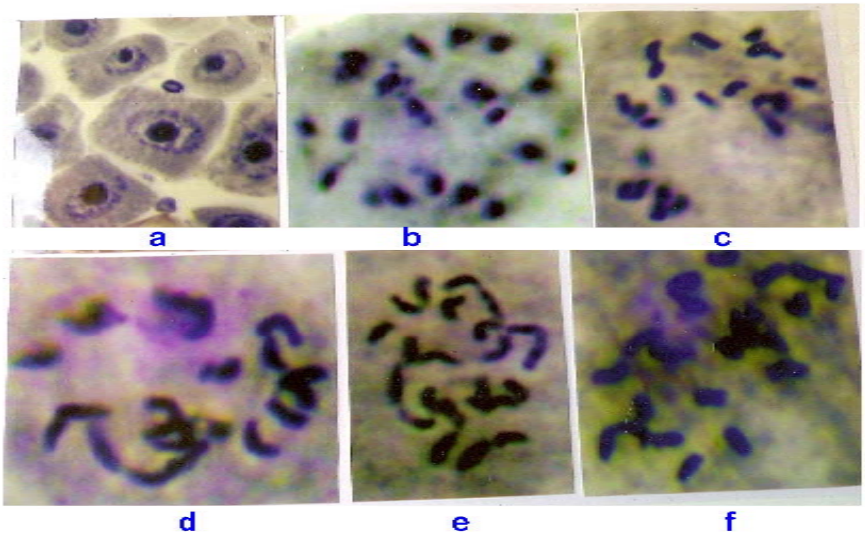

Photo –a: Showing DNA content of somatic cells (2-DNA)

Photos –b to f: Showing metaphase polar views of different metaphase plates with variations in chromosome size and morphology. All the metaphase showing 2n=22 chromosome

Plate 1 – Photomicrographs of *Jatropha curcas* L.

The A_{260}/A_{280} absorbance ratio of gamma rays and EMS treated plants was ranged from 1.83 to 1.97 indicating high purity of genomic DNA (Table 2). Further, it was confirmed by agarose gel electrophoresis which showed intact genomic DNA bands without RNA and other contaminations (Fig. 2a and b). The A_{260}/A_{280} ratio was not significant at 0.05 per cent level. It was observed that as the mutagens dose / concentration increased, DNA concentration decreased. Due to the effect of mutagens, all the treatments reduced the DNA concentration as compared to control. The DNA concentration ranged from 1.353 to 2.273µL/mL. It was decreased from 1.953 at 5Kr of gamma rays to 1.780 at 25Kr of gamma rays and from 1.924 at 1 per cent of EMS to 1.353 at 4 per cent of EMS as compared to control (2.273) (Fig. 1b). The level of significant was 0.05 per cent and their critical difference was 0.228 (Table 2). Similar trend (DNA concentration) was also observed for DNA yield. The DNA yield was not significant at 0.05 per cent level. All the treatments of gamma rays and EMS reduced the DNA yield as compared to control. The maximum reduction was 1780 and 1353 at 25Kr of gamma rays and 4 per cent of EMS when compared to that of control (2273).

Table 1

Effect of mutagens on mitotic index of *J. curcas*

S. No.	Treatments	Number of cells counted	Total number of dividing cells	Mitotic index (%)
1.	Control	50.66	15.83 ± 2.92	29.28 ± 3.00
	Gamma rays			
2.	5Kr	86.00	14.66 ± 1.00	3.66 ± 1.53
3.	10Kr	39.33	9.50 ± 1.50	4.50 ± 1.80
4.	15Kr	49.33	10.86 ± 1.00	1.86 ± 0.40
5.	20Kr	52.00	8.73 ± 0.90	1.43 ± 0.49
6.	25Kr	54.33	7.06 ± 0.92	1.60 ± 0.53
	EMS			
7.	1%	50.00	13.80 ± 1.96	1.50 ± 0.50
8.	2%	44.66	8.26 ± 0.80	1.36 ± 0.15
9.	3%	45.00	4.50 ± 0.77	1.03 ± 0.25
10.	4%	49.00	2.13 ± 0.60	0.93 ± 0.05
F – Value			4.883	1.129
C.D (p = 0.05)			3.635	NS
Level of significant (p)			0.05	0.05

± – Standard Deviation (S.D); three replicates

Table 2

Effect of mutagens on DNA content of *J. curcas*

S. No.	Con.	A_{260}/A_{280} ratio	DNA con. (µg/µl tissue)	DNA yield (µg/ml tissue)
1.	Control	1.90 ± 0.016	2.273 ± 0.04	2273 ± 40
	Gamma rays			
2.	5Kr	1.94 ± 0.027	1.953 ± 0.05	1953 ± 50
3.	10Kr	1.87 ± 0.015	1.930 ± 0.02	1930 ± 20
4.	15Kr	1.89 ± 0.010	1.943 ± 0.03	1943 ± 30
5.	20Kr	1.83 ± 0.005	1.830 ± 0.01	1830 ± 10
6.	25Kr	1.91 ± 0.023	1.780 ± 0.02	1780 ± 20
	EMS			
7.	1%	1.89 ± 0.012	1.924 ± 0.04	1924 ± 40
8.	2%	1.97 ± 0.015	1.886 ± 0.02	1886 ± 20
9.	3%	1.89 ± 0.013	1.550 ± 0.01	1556 ± 10
10.	4%	1.85 ± 0.016	1.353 ± 0.02	1353 ± 20
F – Value		1.547	7.188	7.191
C.D (p = 0.05)		NS	0.228	1.360
Level of significant (p)		0.05	0.05	0.05

± – Standard Deviation;
Three replicates/DNA diluted two hundred times to measure/Con. = Concentrations.

In *J. curcas*, the basic chromosome number is 11 and with diploid (2n = 22). Carvalho *et al.* (2008) Soontornchainaksaeng and Jenjittikul (2003) and Jha *et al.* (2007) reported the same chromosome number in *J. curcas.* Carvalho *et al.* (2008) characterized the morphology of chromosome in *J. curcas,* according to the arm size. They observed the occurrence of very small homomorphic chromosomes that has been often considered to be obstacle for accurate cytogenetical characterization of most plant species Ohmido *et al.* (1998). Due to this limitation in *J. curcas*, we could not go ahead further to investigate the stages related to mutagenic treatments. However, the mitotic index test is a cytogenetic test which was performed in the present study to examine the genotoxic effects and extensive size for value mutagenic agents in a short time in different environments. The mitotic index assays are used to characterize proliferating cells and to identify materials that inhibit or induce mitotic progression.

The highest mitotic index (MI) frequency was found to be 28.28 per cent in control than all the treatments of gamma rays and EMS. It is known that various rays and chemical matters have positive or negative effects on living organisms. This effect can occur both spontaneously on nature and artificially by mutagens. In this study, the inhibition of mitotic activity of different concentration of gamma rays and EMS on embryonic roots of J. curcas were tested and observed. All the treatments of gamma rays and EMS reduced the mitotic index as compared to

control. This is due to the fact that the gamma rays cause ionization by affecting the molecules and ions in cells of the living creatures. EMS was found to react with the genetic material by alkylating DNA bases and phosphate groups (Thengane, 1984), resulting in the reduction of mitotic index. This might also be due to the mitodepressive action of the chemicals indicating thereby the EMS used interferes in the normal cell cycle resulting in a decrease in the number of dividing cells. By the way, the gamma rays and EMS disturb the normal cell cycle process by preventing biosynthesis of DNA and /or microtubule formation. This effect could be formed by decreased ATP level or suppression of the engine of energy production (Jain and Andsorbhoy, 1988). The inhibition of certain cell cycle specific proteins remain as a possible mutagenic target site which inhibit DNA polymerase as well as other enzymes resulting in antimitotic effect. Rank and Nielsen (1997) have reported that the chromosome bridges and fragments lead to structural changes in chromosomes of crop plants. Hence, the higher concentrations of EMS may become chromotoxic and clastogenic in crop plants; therefore, its higher concentration is not suggestive. This is caused to change the chromosome size and its number resulting in induction of mutation. The percentage of MI decreased with an increase in the doses / concentrations of gamma rays and EMS. Increasing dosages of gamma rays caused same effects in *Secale cereale* (Sarakan and Toker, 1991), *Hordeum vulgare* (Okamoto and Tatara, 1995; Eroglu *et al.* 2007), *Vicia faba*, *Zea mays* Clowes and Hof, 1996) and *Solanum surattense* (Kumar and Roy, 1990). Their results coincide with the present study with regard to effects of gamma rays.

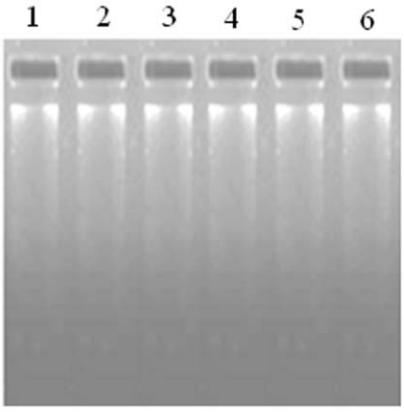

Fig. 2a – Gel electrophoresis (2%) showing intact genomic DNA isolated
from control and treatment of gamma rays,
(Lane-1: Control, Lane-2:5Kr, Lane-3:10Kr, Lane-4:15 Kr, Lane-5:20Kr and Lane-6:25Kr).

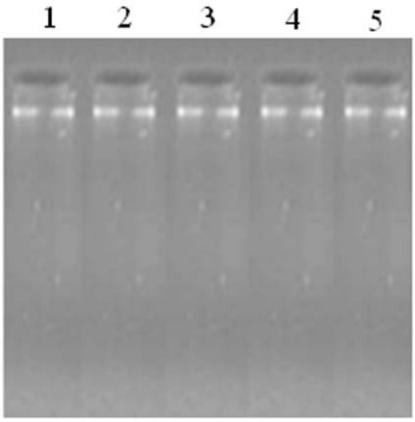

Fig. 2b – Gel electophoresis (2%) showing intact genomic DNA isolated from control and treatments of EMS, (Lane-1: Control, Lane-2% EMS, Lane-3:2% EMS, Lane-4:3% EMS and Lane-5: 4%EMS).

In this modified CTAB method, the A_{260} /A_{280} ratio (1.83-1.97) and intact genomic DNA in agarose electrophoresis obtained is free from RNA, protein and other contaminations that coincides with a previous study in *Jatropha* species by Dhakshanamoorthy and Selvaraj, 2009. A ratio of absorbance (A260/A280) in the range 1.8–2.0 indicates a high level of purity (Pasakinskiene and Pasakinskiene, 1999). DNA content in the gamma rays and EMS treated populations reduced significantly as compared to control. The higher dose / concentration of gamma rays and EMS showed a more reduced DNA content than control. This is due to genomic DNA damage (*e.g.* single and double strand breaks, modified bases, a basic sites, oxidized bases, bulky adducts, DNA–protein cross-links) by gamma rays and EMS treatments. Mutants induced by gamma irradiation are often generated by deletion of large DNA fragments, up to 6 Mb (Naito *et al.* 2005). The mutagenic agents (EMS) can induce different extensions of genomic lesions, ranging from base mutations to larger fragment insertions or deletions (Kim *et al.* 2006). The total DNA content decreased with increasing gamma rays dosage. Similar results were observed by a previous study in barley seeds by Jyoti *et al.* (2009) and Sarduie-Nasab *et al*. (2010).

CONCLUSION

In conclusion, DNA content in control and treated populations showed a decreased trend as similar to that of mitotic index. This may be due to the effects of

gamma rays and EMS on genetic material at molecular level resulting in altering the genotype towards the achievements of our goal to improve the agronomic traits (flowering, fruiting and yield) through this type of conventional breeding approach. The present study coincides with previous report in *J. curcas* (Dhakshanamoorthy *et al.* 2011) and they observed disappearance of normal bands and appearance of new bands in gamma rays treated plants which were detected using PCR-RAPD marker. The present study has revealed that there was a correlation between the mitotic index and DNA content in respect of mutagenic treatments and a conclusion was also drawn that gamma rays and EMS can be used as mutagenic agents in *J. curcas* for improving the agronomic traits either in positive or negative direction. To the best of our knowledge, this is the first report in *J. curcas* using gamma rays and EMS as mutagenic agents and further investigation is on progress to study the growth and yield parameters in M_2-generation.

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PRE-DISPERSAL REPRODUCTIVE ECOLOGY OF *LAGOTIS CASHMERIANA* (ROYLE) RUPR. (SCROPHULARIACEAE) – AN ENDANGERED ALPINE ENDEMIC ANGIOSPERM OF KASHMIR HIMALAYA, INDIA

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Lagotis cashmeriana is a self-incompatible, dichogamous herb in alpine habitats of the Kashmir Himalaya with sparsely distributed population. Population density ranged from 10.64 ind. m^2 in Harmukh range to 15.6 ind. m^2 in Apharwat populations. Most of the individuals remained vegetative and only a small percentage (0.67% to 9.72%) progressed into reproductive phase. Herbivores damaged about 11 to 18% of individuals in different populations. The species exhibited obligate out-breeding nature and its flowers were foraged principally by a *Bombus* species. While the number of flowers produced per plant was 39.5, but only 10.3 seeds were produced per plant. About 60% of fruits possessed two seeds and 40% enclosed only one seed. Both seeds were filled in only 45% of the two-seeded fruits while in 55% of fruits only one seed was filled and viable. About 55.4% of the pollen mother cells showed abnormal meiosis and mean pollen viability was only 38.1%. Thus, reduced seed set because of abnormal pollen mother cell meiosis, and non-viable pollen together with scarce number of reproductive individuals, low pollinator visitation frequency, hence poor reproductive success contribute to threatened status of this species.

Key words: endemic; endangered; Kashmir Himalaya; pollen mother cell meiosis; pollen viability; seed set.

INTRODUCTION

It has long been recognized that the study of reproductive ecology is not only crucial to understanding of the causes that lead to restricted occurrence of endemic species, but it also provides vital information that is indispensable for conservation of threatened species (Young *et al.* 2007). Nonetheless, insufficient understanding of reproductive biology is repeatedly cited as a shortcoming of endangered species' recovery plans (Schemske *et al.* 1994; Clark *et al.* 2002).

However, only a few studies of rare plants describe the limits to reproduction at all stages from flower production to the dispersal of germinable seed (Massey & Whitson 1980; Crowder 1978; Pavlik *et al.* 1993). Consequently, study of the

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factors that critically affect reproductive success needs to be considered in the design of conservation and management strategies (Godt & Hamrick 1995; Hamrick *et al.* 1991; Bosch *et al*. 1998; Navarro & Guitian 2002; Neel 2002).

In fact, many such studies have shown that narrow endemics are susceptible to extinction for a variety of reasons, including habitat destruction, biotic interactions, and genetic collapse (Schemske *et al.* 1994), and many of these factors increase the vulnerability of the species through their deleterious effect on the reproductive success of such species. Many factors, including decreased pollen quality (Byers 1995), poor stigma receptivity and the absence of pollinators (Kwak & Jennersten 1991; Burd 1994; Evans *et al.* 2004) are known to decrease sexual reproduction and seed production. Such decreases can result in endangerment or extinction of plant species, particularly where populations are scattered, small and isolated (Wagner & Mitterhofer 1998). Consequently, the study of the factors that critically affect reproductive success of a species needs to be considered in the design of conservation strategies (Godt & Hamrick 1995). These studies have the potential to offer vital information urgently required for successful conservation (Wiens et al. 1989; Navarro & Guitian 2002).

In view of the significance of detailed reproductive ecological studies in understanding and addressing the intrinsic and extrinsic causes that threaten endemic species, the present study examined the current status of the existing populations of *L. cashmeriana* in respect of their population density, fate of individuals constituting the populations, extent of herbivory across different populations, breeding behaviour, pollen mother cell meiosis, pollen viability, insect visitation frequency, and seed set. The main objective of the study was to identify key bottlenecks that contribute to restricted occurrence of the species in the Kashmir Himalaya and also limit its population density so that the same is used in formulating effective recovery and restoration strategies for this narrow endemic species.

MATERIALS AND METHODS

Study area. The Valley of Kashmir is an oval plain valley that lies between 32° 20′ to 34° 50′ North latitude and 73° 55′ to 75° 35′ East longitude, covering an area of about 16,000 sq. km. It is formed by a girdling chain of the Himalayan mountains, namely the Pir Panjal range in the south and the Great Himalayan range all along the southeast through northeast to the west. The climate of the Valley is predominantly temperate, changing to sub-alpine and alpine higher up in the mountains. In spite of its being geologically younger and constituting only 0.48%

of the total land mass of India, the Valley is one of the 28 endemic centres of India with about 152 (*ca*. 8%) endemic taxa representing more than 3% of the total Indian angiosperm endemics (Dar & Aman 2003).

Study species. *Lagotis cashmeriana* (Royle) Rupr. commonly known as *Kashmir Hare's Ear*, occurs from an altitude of 3,000 to 4,000 m in glacier-fed open or shady wet places, rock cervices, steep grassy slopes, and in habitats characterized by loose soil with relatively less pebbles. It is a fleshy perennial herb; 10.5 ± 1.25 cm tall (mean and SD based on n = 20. Flowers arranged on terminal (3.68 ± 1.36; n = 20) cm long portion of cylindrical flowering spike. Each spike bears 39.5 ± 6.77 (n = 20) number of flowers. Flower bracteate, (1 ± 0.24) × × (0.52 ± 0.17; n = 20) cm, zygomorphic, hermaphrodite, heterostylous (protogynae), dark-blue to violet coloured; anther 2, exerted, epipetalous (Fig. 1); ovary superior, bicarpellary syncarpous with axile placentation (Fig. 2); ovule anatropous; stigma globose without any trichomes. Fruit ovate-obovate, acute, (0.65 ± 0.14) × (0.40 ± 0.09) cm, solid and hard. Each fruit on an average bears 1.6 ± 0.16 (n = 10) seeds. About 60% fruits possess two seeds while 40% enclose only one. In fruits with two seeds about in 45% cases both seeds are filled while in 55% cases only one seed is filled/viable while the other was found to be abortive or not at all present as its space or cavity was found empty. Such abortive seeds were shrunk, linear, irregularly curved, and soft, with hair like, long tip. Seeds ovate, obtuse and yellowish.

Fig. 1 – *Lagotis cashmeriana* flowers with projecting receptive stigma.

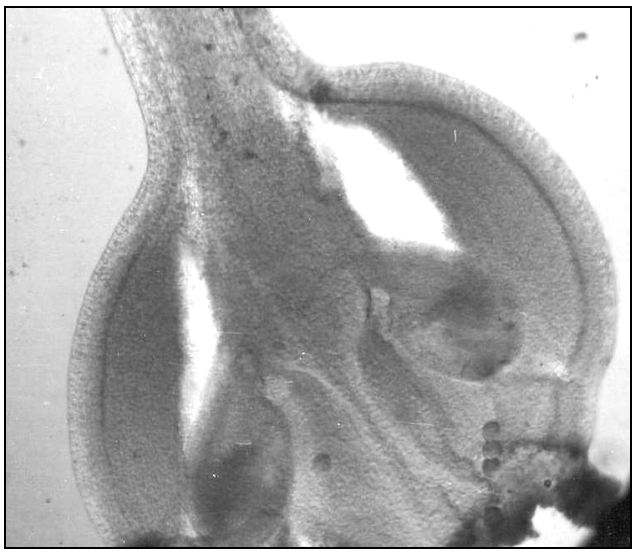

Fig. 2 – *Lagotis cashmeriana* ovary
with axile placentation and anatropous ovules.

Experimental sites. Various characteristics (altitude, habitat, nature of soil, exposure, stability, etc.) of different experimental sites were recorded (Table 1).

Variability in vegetative and reproductive traits. In order to record the extent of variation in different traits across the studied populations, 15 randomly selected healthy individuals were tagged and inspected weekly throughout the growing season. The onset and duration of various vegetative phenophases (sprouting, seed germination), sexual phases (bud formation, anthesis, pattern of anthesis), flowering period and type of dichogamy were noted. Characteristics of floral parts (pedicels, sepals, petals, stamens, carpels) were determined using magnifying lenses (10x, 20x) and dissection microscope. Also attributes, such as plant height, rhizome length, number of leaves, length and breadth of largest and smallest leaves, flower number, percentage fruit set and number of seeds per plant were recorded.

Population density and fate of individuals. To comprehend why populations of this species squeeze and each individual is able to survive and contribute to population growth, demographic data was collected during 2004-2006 from a total of 60 quadrats at 6 selected sites in Gulmarg, Naranag and Sonamarg areas for *Lagotis cashmeriana* in high altitude localities in the Kashmir Himalaya. These quadrats were established across six populations immediately after melting of snow in the month of June (sprouting/seed germination) to study population density (numbers/m^2) and fate (survival and reproduction) of individuals**.** The seedlings after attaining distinctive appearance were marked using colour paint following

Khushwaha *et al.* (1981). The marked seedlings were again counted on the subsequent census to record mortality and survival of the individuals. The seedlings without colour marking on each sampling date constituted the new recruitments, which after counting were marked with the colour paint in the permanent quadrats to distinguish between already recruited and new recruits on the successive sampling dates. Seedlings were distinguished from sprouted individuals on the basis of leaf texture, colour and presence of bristles. Marked plants in quadrats were monitored from sprouting to senescence throughout the growing season and survey was conducted after every 15 days. The life stage of individuals (reproductive and vegetative), mortality by herbivory, part herbivored and other related details were also noted. We believe that this would be useful to understand about the increase or decrease in the population size and how many individuals make it contribute to the population growth in future.

Breeding behaviour. To ascertain self- or cross-compatibility nature of *Lagotis cashmeriana*, thirty plants each with 16-41 floral buds were randomly selected from the Apharwat and Khillanmarg populations for pollination experiments. Five randomly selected plants in each of these populations were then subjected to one of the following controlled hand-pollination treatments (Kearns & Inouye 1993) in their natural environment: (i) unemasculated flowers tagged for open pollination (16-38 buds) (ii) emasculated and bagged to prevent pollination (21-36 buds), (iii) emasculated and hand-pollinated with self pollen (23-36 buds) (iv) emasculated and cross pollinated with pollen from different individual (21-33) (v) unemasculated and bagged before anthesis to enforce selfing (19-41) (vi) emasculated flowers left open to pollinate (21-30). These treatments were used to test for open pollination, apomixis, geitonogamy, cross-compatibility, autogamy or self-compatibility and other breeding characteristics of the species. Emasculations, done with the help of blunt ended forceps, were performed at appropriate time (just prior to floral opening or immediately on flower opening for appropriate treatments). Pollination experiments were performed between 9:00 am to 1:00 pm when flowers open and mature anthers are about to disperse their pollen. Fully mature pollen laden anthers were gently tapped to shower pollen on the receptive stigma. Butter paper bags with fine pores were used for bagging purposes. The bags were removed after the stigmas became completely non-receptive (approx. 5 days). The resulting fruits from these treatments were collected and number of fruits and number of seeds (wherever formed) per fruit was determined at the end of growing season.

Insect visitors to flowers. To record frequency of pollination, floral visitors to *Lagotis cashmeriana* populations were monitored in three populations located at Apharwat, Hangduph and Thajwas. In 10 m × 10 m area, 36 observation sessions during different times of a day (from 8 am to 6 pm), each of 30 minutes were conducted for 6 days by one or more observers, which totalled to about 18 hours of observation. Number of visits an insect made, time it spent on one flower, number

of flowers it visited in a single visit, and the number of plants it visited were recorded. To standardize these observations, we calculated the number of floral visitors per flower on hourly basis as the total number of visitors observed divided by the number of flowers. An insect landing anywhere on the inner petals, pistil or stamen was treated as one insect visit. Visitation rate was calculated as the total number of visits made to each population divided by the number of flowers to yield number of insects per flower per hour. Representative floral visitors were trapped, anaesthetized with NaCN and scrutinized for pollen load on their body parts. The pollinator was identified at the Department of Zoology, University of Kashmir, Srinagar, Jammu & Kashmir, India.

Pollen mother cell meiosis. To find out pollen viability through meiotic behaviour, floral buds collected from randomly selected plants from Apharwat, Khillanmarg and Thajwas populations were fixed in Carnoy's fluid (glacial acetic acid: absolute ethanol in the ratio of 1: 3) between 7 am to 11 am. The fixative after becoming turbid due to extraction of the bud contents was replaced by fresh fixative after 60-90 minutes. The buds were kept in it for 22 hours and thereafter transferred and preserved in 70% ethanol at 4 ºC. The anthers were squashed in 2% propionocarmine and slides scanned using simple microscope (Olympus) and selected PMCs were photographed. The meiotic behaviour of the species was analysed by scrutinising the dividing pollen mother cells at different developmental stages. The number and percentage of the abnormal PMCs was worked out from the total number of PMCs scanned and found at different meiotic stages.

Stigma receptivity. To record the initiation and longevity of receptivity, stigmas at different developmental stages were fixed in FAA (formaldehyde: acetic acid: alcohol) (Tangmitcharoen & Owens 1997) for 3-4 hours and later transferred to 70% alcohol for storage. The stigmas were stained with aniline blue in lactophenol (Hauser & Morison 1964) and scanned under light microscope (×400) for pollen deposition. Number of non-germinated, number of germinated, total number of pollen grains on stigma surface were determined to obtain percentage of germinated pollen grains on sigma surface. The stigmas with germinated pollen grains were considered as receptive. The morphological features associated with the receptive and non-receptive stigma vis-à-vis anther developmental stages were noted down.

Pollen viability and abundance. Fresh pollen collected from Apharwat, Khillanmarg and Thajwas populations were checked for their viability and longevity of viability. By using aniline blue in lactophenol (Hauser & Morison 1964) the percentage of stained grains pollen in random but non-overlapping microscopic fields (three replicates for each staining treatment) was determined (Bernardello *et al*. 2004) using an Olympus microscope at ×100 magnification. The mean pollen abundance was worked out for this species by selecting five reproductive plants and calculating pollen number per anther, flower and plant.

Table 1

Habitat characteristics of the experimental sites supporting six different populations of *Lagotis cashmeriana* in the Kashmir Himalaya

Characters	Study sites					
	Apharwat (Gulmarg)	**Booj Ki Lakad (Sonamarg)**	**Hangduph (Sonamarg)**	**Harmukh range (Naranag)**	**Khillanmarg (Gulmarg)**	**Thajwas (Sonamarg)**
Altitude	3270 m	3280 m	3040 m	3580 m	3200 m	3200 m
Habitat	Very steep slope, composed of thick soil layer mixed with small as well as big rocks throughout	Extremely sharp rocky slope just below the bare rocky peak of the rage, composed of thick layer of soil	Steep slope alongside a deep gorge, composed of thin patches of soil on and among big rocks	Very steep rocky slope, composed of very few patches of soil, otherwise composed of big, hard, sharp sedimentary rocks throughout	Very steep slope alongside a gorge, composed of thick layer of soil upon a rock cover which at many places emerge and form part of surface of this site	Extremely steep and much smaller slope alongside a deep and narrow gorge, composed of thick soil layer on rocky slope, some of which emerge on the surface
Soil	Deep, relatively hard and moderately moist, devoid of any sand, light-brown	Less deep, much sandy, loose, moderately-moist, light-brown to dark coloured	Moderately deep, pebbled, hard, moist, mostly mixed with good amount of sand, dark-coloured	Thin layer, sandy, less-hard, moderately moist, light-brown to dark coloured	Less deep, relatively loose, moist, mixed with little sand, light-brown to dark coloured	Much deep, pebbled, less hard, less moist, light-brown to dark coloured
Exposure	Well-exposed	Well shaded, less-exposed	Well-exposed to partially shaded	Well-exposed	Partially-shaded	Well-exposed to partially shaded
Stability	Least stable, threatened further by construction activities of GDA (Gulmarg Development Authority)	Relatively stable due to presence of sparse *Betula* trees	Relatively stable, however constantly narrowed by alongside gorge	Least stable due to dominance of unheld big rocks	Less stable, constant narrowing by the erosion via along side gorge	Least stable, already very small patch further narrowed constantly by along-side deep gorge with gushing waters
Grazing	Very frequent	Very frequent	Very frequent	Less frequent	Very frequent	Very frequent

Reproductive output. 10 randomly selected and tagged reproductive individuals from all the five populations were periodically inspected to record data on plant height, number of flowers, percentage fruit set and number of seeds per plant for the calculation of reproductive output.

Statistical analysis. The data for demography and extent of herbivory aspects of *Lagotis cashmeriana* was recorded from 10 replicate quadrats for each population. It was converted to numbers/m^2 and then subjected to statistical treatment so as to work out mean and standard error for each parameter in each population. Also mean and standard error for different vegetative and reproductive traits were worked out from 15 plants in each population. The breading experiment details have been worked out for 12 plants in total. The mean and standard error for stigma receptivity and pollen viability is based on three replicates. Basic statistics, such as trait means and variances and analysis of variance (ANOVA) were calculated using SPSS 10.

RESULTS

Phenological behaviour. The species starts sprouting from perennating organs immediately after the melting of snow during June (Table 2). Seed germination, though insignificant, occurs in the last week of June or in the first week of July. Initiation of floral bud formation was noticed a few days after sprouting and in most of the studied populations it was noticed that just sprouted individuals, whose leaves had not as yet attained normal green colour, bore a very small flowering spike initial. The flower development takes place in an acropetal fashion and each flower anthesce in about 10-12 days. Each flower remains open for about 8-10 days and the seed formation starts from the month of July and continues until October.

Vegetative and reproductive traits. The plant height varied significantly ($P \leq 0.000$) across populations with highest value (14.88±1.79 cm) in Harmukh range population while a minimum height of (6.05±0.23 cm) was recorded for individuals of Apharwat population. The leaf number per plant again showed a significant variation ($P \leq 0.000$) with the highest (6.53±0.63) in Sinthan population and the lowest (2.40±0.13) in Hangduph population. The number of spikes and flowers per plant showed a significant variation ($P \leq 0.004$) and ($P \leq 0.008$) respectively among the studied populations (Fig. 3). Although the overall percentage fruit- and seed-set was much low in all the investigated populations, yet it varied significantly ($P \leq 0.020$) and ($P \leq 0.020$) respectively among the studied population and both showed the highest values in Harmukh range population. Most of these traits values are highest for Harmukh range population because it is at a higher altitude with less vegetation (other species) and hence experiences less herbivory.

Table 2

Chronology of various phenophases in *Lagotis cashmeriana* based on 15 tagged individuals in each population studied in the Kashmir Himalaya

Features	Population					
	Apharwat (Gulmarg)	**Boaj Ki Lakad (Sonamarg)**	**Hangduph (Sonamarg)**	**Harmukh (Naranag)**	**Khillanmarg (Gulmarg)**	**Thajwas (Sonamarg)**
Initiation of sprouting of perennating organs	1st week of June	3rd week of June	3rd week of June	2nd week of June	4th week of June	4th week of June
No. of days during which sprouting continued	114	107	109	102	102	102
Initiation of seed germination	3rd week of June	1st week of July	1st week of July	3rd week of June	1st week of July	1st week of July
No. of days for which seed germination continues	62	50	52	63	48	45
End of sprouting phase	Last week of September	2nd week of October	2nd week of October	Last week of September	2nd week of October	2nd week of October
Initiation of floral bud formation	1st week of June	3rd week of June	3rd week of June	2nd week of June	4th week of June	4th week of June
No. of days for which floral bud formation continues	98	95	95	92	88	88
Initiation of flower anthesis	3rd week of June	1st week of July	1st week of July	4th week of June	2nd week of July	2nd week of July
Days taken by flower to anthesce	10-12 days	10-12 days	10-12 days	10-12 days	10-12 days	10-12 days
Pattern of floral anthesis in spike	Acropetal	Acropetal	Acropetal	Acropetal	Acropetal	Acropetal
No. of days for which a flower persists	~8-10 days	~8-10 days	~8-10 days	~8-10 days	~8-10 days	~8-10 days
Initiation of seed formation	1st week of July	3rd week of July	3rd week of July	2nd week of July	Last week of June	Last week of July
No. of days for which plant continues to produce seeds	80	70	75	65	68	64

End of sexual phase	2nd week of September	Last week of September	Last week of September	1st week of September	Last week of September	Last week of September
End of seed formation	3rd week of September	1st week of October	1st week of October	3rd week of September	1st week of October	1st week of October

Population density and fate of individuals. Apharwat population showed the highest (15.6 ind. m^{-2}±0.38) and Harmukh range the lowest (10.64 ind. m^{-2}±0.44) population density, hence showing significant ($P \leq 0.000$) variation across populations (Fig. 4). The number of vegetative and reproductive individuals varied significantly ($P \leq 0.000$) among the studied populations and the highest number of vegetative and reproductive individuals occurred in Apharwat and Khillanmarg populations, respectively. Mortality showed insignificantly ($P \leq 0.373$) variation across studied populations. From the recorded data it appears that only a limited number of individuals make the reproductive stage and still only a few successfully contribute further through seed production.

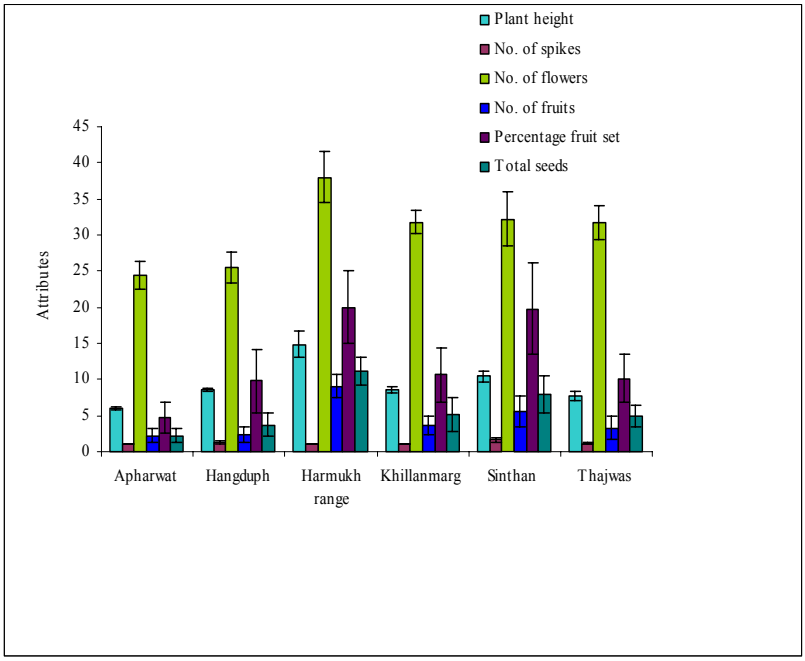

Fig. 3 – Vegetative and reproductive attributes in various populations of *Lagotis cashmeriana* in the Kashmir Himalaya.

Herbivory. Damage by herbivory across different populations frequently lead to death of sprouted individuals. The highest percentage (17.61±4.19) of

individuals was herbivored in the Booj Ki Lakad and lowest in Hangduph (13.04±2.96) populations. In particular, herbivory had a more significant effect on reproductive individuals and it was the highest (0.3±0.11) in Khillanmarg followed by (0.08±0.04) in Apharwat and (0.05±0.03) in Harmukh range and Hangduph populations. The number of vegetative and reproductive individuals damaged by herbivores varied insignificantly ($P \leq 0.281$) and ($P \leq 0.215$) respectively, across studied populations. Consequently, this herbivore damage makes this species pay in terms of reduced seed set and hence only a few new recruits.

Stigma receptivity. Flowers of this species exhibited both dichogamy as well as herkogamy. The stamens were epipetalous and only the anthers projected above the petal lobes. Carpel, with bicarpellary syncarpous ovary, was up to 1.5 cm above the anthers. On the day of anthesis the stigma was fully developed and receptive but bore only a few deposited and germinated (8.33%) pollen grains. The number of deposited pollen grains as well as the percentage of germinated pollens started increasing thereafter. The percentage of germinated pollen grains was 11.85 ± 6.66 one day after anthesis, 16.6 ± 1.2 two days after anthesis and the highest 23.23 ± 3.57 on third of anthesis. Subsequently, the percentage germination started falling and was about 3.50 ± 3.50 on the fifth day after anthesis. Hence the stigma receptivity gradually increased from the day of anthesis and reached its peak on the 3rd day of anthesis.

Breeding behaviour. Bagging experiments revealed that individuals of this species produced seeds only upon cross-pollination. No seeds were formed either under apomictic or autogamous conditions. Percentage fruit set was higher (30.52±5.07) in manually cross pollinated flowers than in emasculated flowers tagged for open pollination (20.2±1.91) and control (25.31±4.23) (Fig. 5). Also the percentage fruit set showed the same trend. The breeding experiments established that *Lagotis cashmeriana* species is an obligate out-crosser. The manually cross pollinated flowers probably got a sort of pollen supplementation and hence greater percentage fruit set as against other treatments, while the emasculation treatments may have lowered the seed set percentage also due to removal of pollen reward for insect visitors.

Insect visitation. Only one insect species (*Bumbus* sp.) of Aphidae was found foraging *Lagotis cashmeriana* flowers. The pollen load on front legs, thorax and abdomen of the insect was very high, while a limited number of pollen grains was observed on the hind legs. After landing on the lower petal lobe, it brushes its body against the bent stigma to enable itself to reach and collect pollen from the anthers on the upper petal lobes. We observed that on each visit the insect spent at the most 5-8 seconds on each flower and visited 2-3 flowering plants in one visit. The individuals of *Bumbus* sp. visited the flowers more frequently between 8 am to noon and then again from 3:30 pm till 6 pm. The overall visitation rate and insect visitation frequency was considerably low in all the three populations studied. The frequency of visitation increased with increase in the number of flowering individuals and the number of flowers in a population. The population at

Khillanmarg depicted relatively higher values in the traits, such as number of insect visits/hour (5.23±1.61), total number of flowers visited/hour (11.71±3.26), visitation rate/hour (0.02±0.01), and visitation frequency (0.04±0.01) than the corresponding values (4.25±1.43), (8.84±3.19), (0.01±0.00), (0.03±0.01) and (3.30±1.11), (8.33 ±3.15), (0.01±0.00), (0.02±0.00) for populations at Hangduph and Apharwat, respectively (Fig. 6).

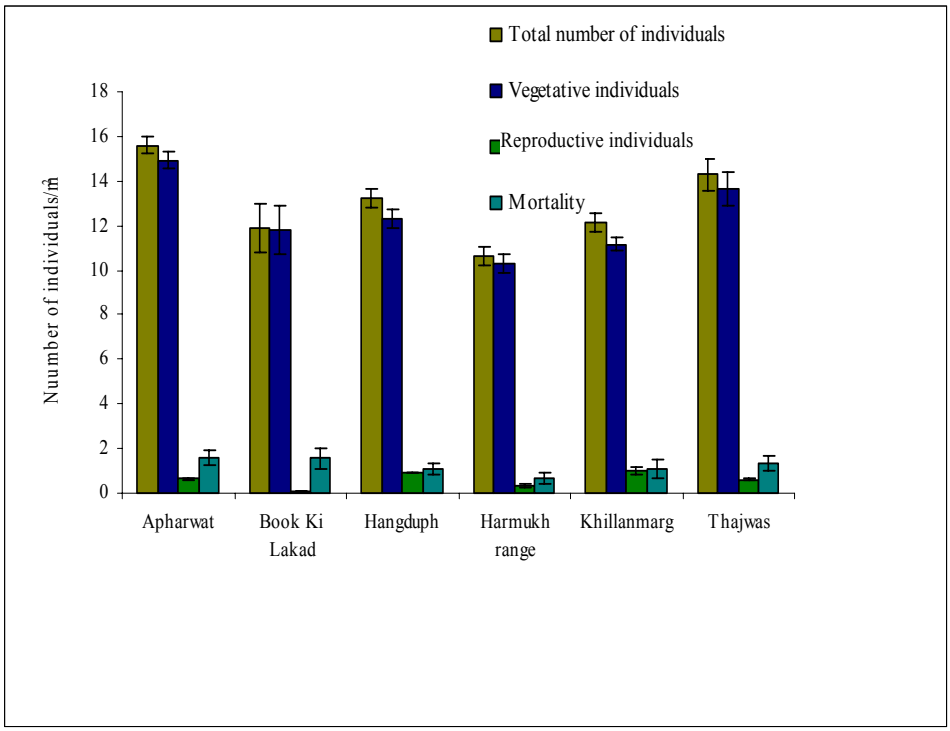

Fig. 4 – Comparative demographic details of *Lagotis cashmeriana* at various populations in the Kashmir Himalaya.

Pollen mother cell meiosis. The pollen mother cell meiosis in the *Lagotis cashmeriana* showed that the species is diploid with n = 11 and 2x = 2n = 22. The 22 chromosomes form 11 metaphasic bivalents (rings and rods). In the three populations, many PMCs exhibited meiotic abnormalities, such as irregular anaphasic separation, laggards and five poles. The irregular anaphasic segregation led to unequal separation of the chromosomes which rendered one pole with a greater number of chromosomes than the other. A good proportion of cells showed 2-3 laggards. A few pollen mother cells were with five poles instead of usual four poles. The fifth pole was formed towards the centre of a PMC. The PMCs with irregular anaphasic separation were in greater proportion in all the studied population followed by PMCs with laggards; the PMCs with five poles were less

frequent. The Khillanmarg population showed the highest percentage (58.88) of abnormal PMCs followed by Apharwat (54.24) and Thajwas (53.79) populations. The pollen viability was the highest in Thajwas (50.66±7.53) population and the lowest (28.33±2.91) in Khillanmarg population. Hence, abnormal pollen mother cell meiosis considerably reduces the pollen viability which in turn results into reduced seed set in the studied populations.

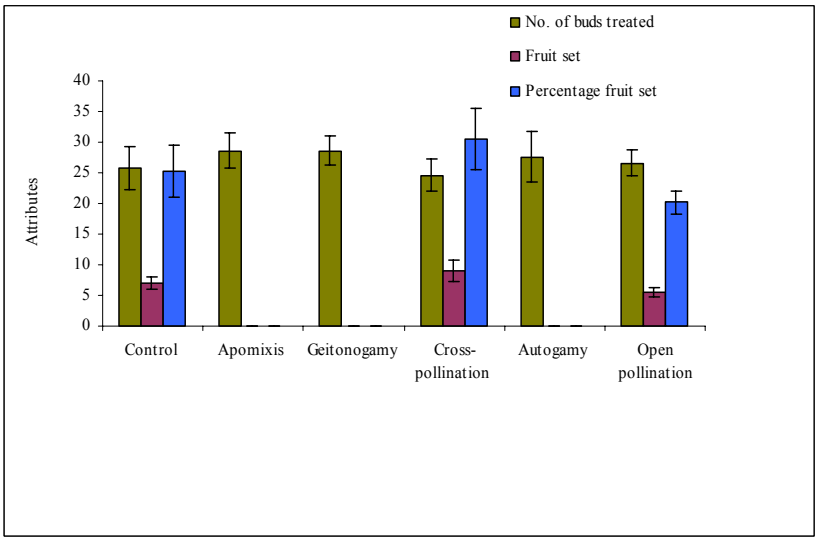

Fig. 5 – Breeding behaviour of *Lagotis cashmeriana* in the Kashmir Himalaya.

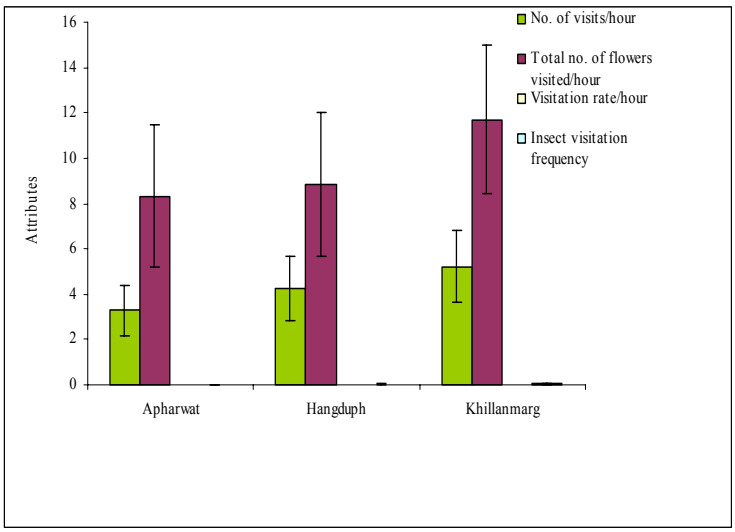

Fig. 6 – Insect visitation frequency at some populations of *Lagotis cashmeriana* in the Kashmir Himalaya.

DISCUSSION

The present study revealed that *Lagotis cashmeriana* is a barochorous species, with low survival and reproductive capacity of its individuals leading to occurrence of its small scattered populations in the Kashmir Himalaya, India. This narrow distribution makes the species very vulnerable to extirpation, as is the case with many other narrow endemics (Schemske *et al*. 1994; Kwit *et al*. 2004; Yates & Ladd 2004). *Lagotis cashmeriana* is represented by a small number of populations comprised of few reproductive individuals. Reproductive individuals of *L. cashmeriana* bear small, showy flowers. The stigmas are at least 0.7-1.5 cm above the anthers and the species is protogynous. Hence the species exhibits separation of sexual organs both in time as well as in space, i.e. it is dichogamous as well as herkogamous. This spatio-temporal separation of male and female reproductive functions is generally expected to promote out-crossing, either by reducing within-flower selfing (Darwin 1876; Muller 1883; Proctor & Yeo 1973; Faegri & van der Pijl 1979; Lloyd & Webb 1986; Webb & Lloyd 1986; Richards 1986) or between flower selfing (Harder & Barrett 1996; Holsinger 1996). The gene flow in this species is predominantly biparental, or of correlated paternity owing to least communication between its widely spaced populations. Degree of non-random matings in a population (self-fertilization, biparental inbreeding, or correlated paternity) directly affects rates of inbreeding (Ellstrand *et al.* 1978; Ritland 1984, 1989; Barrett & Kohn 1991).

Low pollinator service, as found in the present study, may influence sexual reproductive success (Jennersten, 1988) with attendant influence on the population density (Bawa 1990; Menges 1991; Aizen & Feinsinger 1994). Low visitation of flowers by *Bumbus* sp., the only pollinator of the species, is presumably due to small population density, limited and sparsely distributed reproductive individuals, which in turn decreases floral attractiveness and rewards to pollinators as reported by Sih & Baltus 1987, Byers 1995 and Kunin 1997 in such similar studies.

Further, this species depicts abnormal meiotic behaviour which results in low pollen viability as has been reported by many other workers as well (Pagliarini *et al.* 1998; Maataoui & Pichot 2001) in similar studies. The species bears two anthers and two ovules per flower. The pollen count and the percentage of germinated pollen grains, however, were considerably low even on the most receptive stigmas. Meiotic abnormalities/irregularities (such as, irregular anaphasic separation, laggards, more than four poles) were highly prevalent in pollen mother cells in the species. This reproductive bottleneck is further exacerbated by the occurrence of a limited number of reproductive individuals, low pollen count per anther, patchy distribution and wide separation of its populations and above all the occurrence of considerably low insect visitation frequency. *In vitro* pollen germination experiments also confirmed low percentage of pollen germination. Although a large number of flowers is borne by reproductive individuals, the fruit-

and seed set is considerably low. Furthermore, many fruits are totally unfilled/empty, or are with only a single seed instead of usual two. Herbivore induced damage of the individuals within the already sparse populations is another factor that is responsible for the current threat status of *L. cashmeriana* in the Kashmir Himalaya, India. Further the seeds of this species have deep (physical + physiological dormancy) and require prolonged chilling in conjuncture with pricking to accomplish germination (Dar *et al*. 2009).

The low pollen viability mostly as a consequence of abnormal pollen mother cell meiosis which ultimately manifests in the form of extremely scarce fruit and seed set is the most likely factor responsible for the present threat status of this species. Besides deep dormant seeds, a limited number of reproductive individuals in already sparse populations, significant herbivore-induced damage of both vegetative and reproductive individuals, and low insect visitation of cross-pollinated flowers are some of the key factors that contribute to its restricted distribution and depauparate populations. In view of the above stated factors, recovery activities should concentrate on protecting the existing refuge sites of this species in order to salvage it from the brink of extinction.

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