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INDUCED GENETIC VARIABILITY IN CHICKPEA (*CICER ARIETINUM* L.) I. FREQUENCY AND SPECTRUM OF CHLOROPHYLL MUTATIONS

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Abstract

A relative study of frequency and spectrum of chlorophyll mutations induced by physical (gamma rays) and chemical (EMS) mutagens in M_2 population was conducted in two desi (Pb2000, C44), one each of kabuli (Pb1) and desi x kabuli introgression line (CH 40/91) of chickpea (*Cicer arietinum* L.). The treatments included two doses each of gamma irradiation and concentrations of EMS. The overall frequencies and spectrum of four types of induced chlorophyll mutants were viridis (9.01%), followed by xantha (8.61%), chlorina (5.82%), albina (0.5%) and others (0.26%). EMS treatments were found to be more efficient and effective than gamma rays in all the varieties. It appeared that recombinant of desi x kabuli chickpea introgression genotype was more responsive to chemical and physical mutagens as compared to pure desi and kabuli chickpea genotypes. The study of induced genetic variability for frequency and spectrum of chlorophyll mutations on recombinant of desi x kabuli chickpea introgression genotype is the first report in chickpea.

Introduction

Chickpea (*Cicer arietinum* L.) is an annual, autogamous legume and the only cultivated species within the genus *Cicer*. Pakistan is the second major producer of chickpea (9.5%) after India (65%) followed by Turkey (6.7%) in the world (Anon., 2005). The most commonly followed breeding approach for the improvement of crop is usually recombination breeding. The existing chickpea germplasm indicates limited variability for improvement of economic traits. Mutation breeding is a powerful and effective tool in the hands of plant breeders especially for autogamous crops having narrow genetic base (Micke, 1988). Mutagenic agents have been used to induce useful phenotypic variations in plants for more than seven decades ago. During the past 70 years, more than 2,252 mutant varieties including cereals, oilseeds, pulses, vegetables, fruits, fibers and ornamentals have been officially released in 50 countries all over the world (Maluszynski *et al*., 2000). Although induced mutations have been undertaken in the past on some grain legumes, however limited attempts have been made on chickpea (Kharkwal, 2003; Haq *et al*., 2001; Haq *et al*., 2002; Cagirgan & Toker, 2004; Gaur & Gaur, 2002; Khan & Wani, 2005). The literature is scarce for the comprehensive and systematic studies of frequency and spectrum of chlorophyll mutations induced by a wide array of treatments of physical and chemical mutagens on distinctly diverse genotypes of chickpea.

Chlorophyll mutants are employed as markers for the evaluation of gene action of mutagenic factors in induced mutation studies (Gaul, 1964). The spectrum and frequency of chlorophyll mutations are assessed in M_2 population easily and is being used as a primary index of effectiveness of mutagens and mutability of the genotypes towards the mutagens which in turn would be useful to generate the wide array of desirable mutations in the treated population.

In the present study attempt has been made to understand the comparative response of physical and chemical mutagens on two desi, one kabuli and one desi x kabuli introgression genotype, with a view to determine the mutagen and treatment causing maximum chlorophyll mutations in M_2 population.

Materials and Methods

Seed source: The seed material used for this study comprised of 4 chickpea genotypes 2 desi genotypes (Pb2000 and C44), one kabuli genotype (Pb-1) and one recombinant of desi x kabuli introgression (CH41/91 or P40/91).

Pb2000: An outcome of a cross between local desi adapted varieties C87x C44/C21-6-2, very high yielding and tolerant to *Ascochyta* blight and *Fusarium* wilt was released for general cultivation in the year 2000 (Akhtar *et al.,* 2004).

C44: High yielding, local selection, tolerant to *Acochyta* blight and *Fusarium* wilt was released for general cultivation in 1994 (Akhtar *et al.,* 2003).

Pb1: Local small seeded, high yielding kabuli variety tolerant to blight and susceptible to *Fusarium* wilt was released for general cultivation in 1926 (Akhtar *et al.,* 2003).

CH40/91(P40/91): An introgression of desi x kabuli line/variety (CH2 x C44) and resistant to blight and wilt.

Treatments: Preliminary radiosensitivity studies (not included in the manuscript) were carried out in the lab and from the GR_{50} values, it appeared appropriate to use a dose range of 300-400 Gy gamma irradiation and 0.3-0.4% of EMS for Pb.2000; 500-600 Gy gamma irradiation and 0.3-0.4% of EMS for C44; 200-300 Gy gamma irradiation and 0.2-0.3% of EMS for Pb.1, and 200-300 Gy gamma irradiation and 0.2-0.3% of EMS for CH40/91, which were effective to reduce about 20-40% shoot and or root length, the plant growth and survival rate under laboratory and field conditions.

One thousand and five hundred seeds of each genotype were taken for each selected doses of treatment and seeds were given chemical mutagen treatment (EMS) in laboratory and physical treatment (Gamma irradiation) in the gamma cell at the Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan during October 2000. Moisture content of the seeds at the time of mutagenic treatment was brought to 12% by keeping them in a desiccator containing Calcium chloride. Prior to the mutagenic treatments, all genotypes were grown for one generation to ensure their homozygosity. The well-developed seeds of same age were selected and subjected to mutagenic treatments. Seeds were sown as M_1 generation and at maturity all the surviving plants were individually harvested.

Layout Plan: The material for the present study comprised of individually harvested M_1 plants from the selected dose collected from chickpea experimental area of Nuclear Institute for Agriculture and Biology (NIAB) at Faisalabad situated at Latitude 31^o 4167' N and longitude 73^0 0833'E at an altitude of 564 feet (183 meter above sea level at Karachi) in the Punjab province of Pakistan, during winter season 2001-2002. The number of surviving plants in C44 and Pb-1 were quite large (a range of 251 to 412

plants in C44 and 345 to 668 plants in Pb-1 respectively) in all the treatments, all the M_1 plants were collected for raising M_2 generation. But in Pb2000 and CH40/91 the number of surviving plants were less than 250 (except for 300 and 400Gy in Pb2000 and only 400Gy treatments in CH40/91 respectively). Seed samples from each M_1 plant was sown in a plant-to-row method in the field at Faisalabad during October 2001. The respective non-treated control was also planted after every tenth row for better comparison with normal variation.

Each row measuring three meter (20 plants) in length and a distance of 15 cm was maintained between plants and 30 cm between rows. The experimental soils were slightly sandy (pH 7.56), rich in organic matter content and high in N content. At the time of "Rauni", field was treated with Biflex (Termiticide) @ 2.5 liter ha^{-1} to protect crop from the attack of termites. The field was fertilized with 125 kg ha^{-1} of DAP prior to planting. The experiment was not given any irrigation except seasonal rainfall. The experimental area was hand weeded manually three times during cropping season. The treated and control populations were observed thoroughly in M_2 for lethal and non-lethal mutations from emergence till the age of four weeks after germination and were registered.

The frequency of chlorophyll mutations were determined and grouped by their types in M_2 generation by using modified classification of Lamprechet (1960) and Kharkwal (1998) on 10-20 day old seedlings. Different types of chlorophyll mutations observed in the present study could be grouped into lethal and non lethal types. The lethal group included albina and xantha, while viridis and chlorina were non lethal. The types of chlorophyll mutations observed were:

albina – a lethal mutation with entirely white leaves with no carotinoids, survived for about a week; **chlorina** – non lethal mutation, plant color was very light green or yellowish green which persisted throughout the growth period. They had normal viable flowering and fruiting, but had arrested growth; **xantha**- also lethal, plant color was yellow to whitish yellow with prevailing carotinoids, survived upto 2-3 leaf stages;

viridis – non lethal mutation, a viable mutant, leaves initially light/yellow green but gradually turn green color at seedling stage (a heterozygous group). **Others** – with no definite pattern of chlorophyll deformities.

The mutation frequency was computed on M_2 family basis (% of mutated progenies) and M_2 plant basis (% mutants) after confirming the true breeding behavior of M_2 variants in the M_3 generation as computed by Kharkwal (1999) in chickpea. Data was analyzed by using Microsoft Excel program.

Results and Discussions

Four main types of chlorophyll mutations, albina, xantha, chlorina, viridis and some others/chimeras (Table 1) were identified in four genotypes. Albina and xantha mutants died within 10 to 15 days after emergence. In few cases chlorina mutants did survive, however, all the viridis mutants survived and were very vigorously growing but had few branches, very week stem, low number of seeds and pods per plant. Certain other chlorophyll mutations (such as Maculata, Tigrina, Terminalis, Virescent, Alboviridis, albaxantha, alboviridis etc) were not observed in M_2 population. Albina type of mutants was rarely induced in all four chickpea genotypes and their number was much less (only 24 albina in four genotypes) than other three types of chlorophyll mutations (892). The spectrum of chlorophyll mutations was different in gamma irradiation and EMS treatments. In Pb2000 and CH40/91, albina mutant were least (1 in each genotype) in number in gamma irradiation as well as EMS treatment

while in C44 and Pb-1, more albina mutants were observed (13 in C44 and 9 in Pb-1). Haq (1990) reported four types of chlorophyll mutations in induced mutation studies and found least number of albina mutants in three kabuli genotypes (ILC482, ILC3279 and ILC6104). Out of 583 chlorophyll mutations, only 85 mutants (50 from gamma irradiation and 35 from EMS) were albina in three genotypes.

Table 1. Frequency and spectrum of chlorophyll mutants in M_2 generation of four chickpea genotypes.

Genotypes	Treatments	Total families	No. of M_2 plants	Relative frequency (%) of chlorophyll spectrum					
				Albina	Xantha	Chlorina	Viridis	Others	Total
Pb.2000									
	300Gy	254	3756	-	0.13	-	0.32	-	0.45
	400Gy	295	4254	-	0.40	0.05	0.33	0.02	0.80
	0.3%EMS	202	3587	0.03	0.89	0.56	0.84	-	2.32
	0.4% EMS	184	3319	-	0.57	0.72	0.60	-	1.89
	Total			**0.03**	**1.99**	**1.33**	**2.09**	**0.02**	**5.46**
C44									
	500Gy	257	4150	0.02	0.34	0.07	0.48	-	0.91
	600Gy	251	4272	-	0.21	0.35	0.19	-	0.75
	0.3%EMS	412	5506	-	0.49	0.22	0.51	0.02	1.24
	0.4% EMS	290	4138	0.29	0.87	0.34	0.80	0.14	2.44
	Total	**14916**		**0.31**	**1.91**	**0.98**	**1.98**	**0.16**	**5.34**
Pb.1									
	200Gy	367	5519	0.02	0.47	0.36	0.43	-	1.29
	300Gy	345	5133	-	0.56	0.23	0.47	-	1.26
	0.2%EMS	668	9433	0.06	0.54	0.37	0.48	-	1.45
	0.3%EMS	615	8100	0.02	0.32	0.20	0.32	0.02	0.89
	Total	**28185**		**0.1**	**1.89**	**1.16**	**1.7**	**0.02**	**4.87**
CH40/91									
	200Gy	252	3219	-	0.53	0.50	0.34	0.06	1.43
	300Gy	143	1678	0.06	0.30	0.54	0.95	-	1.85
	0.2%EMS	92	980	-	1.02	0.82	0.82	-	2.65
	0.3%EMS	112	1235	-	0.97	0.49	1.13	-	2.59
	Total		**7112**	**0.06**	**2.82**	**2.35**	**3.24**	**0.06**	**8.52**
	G. Total		**68279**	**0.5**	**8.61**	**5.82**	**9.01**	**0.26**	**24.20**

From the relative frequency and spectrum of chlorophyll mutations produced in each treatment (Table 1), it is evident that both gamma rays and EMS induced a wide spectrum of chlorophyll mutations in all the four chickpea genotypes. The genotype CH40/91 appeared to be more responsive (8.52%) towards the physical and chemical mutagens followed by Pb2000 (5.46%), C44 (5.34%) and Pb-1 (4.87%). The overall frequency of viridis (9.01%) mutants were relatively high in both mutagens (physical and chemical) followed by xantha (8.61%), chlorina (5.82%), albina (0.5%) and others (0.26%). Thus the trend of chlorophyll frequency was in the order viridis>xantha>chlorina>albina>others. Among the various induced chlorophyll mutations in four chickpea genotypes, the relative range of frequencies of four types of chlorophyll mutations was in the order viridis (0.32 to 0.84%), xantha (0.13 to 0.89%) and chlorina (0.05 to 0.72%) in Pb2000, viridis (0.19 to 0.80%), xantha (0.21 to 0.87%) and chlorina (0.07 to 0.35%) in C44, viridis (0.32 to 0.48%), xantha (0.32 to 0.56%), and chlorina (0.20 to 0.37%) in Pb-1 and viridis (0.34 to 1.13%), xantha (0.30 to 1.02%), and chlorina (0.49 to 0.82%) in CH40/91. On overall basis of three genotypes (two desi type Pb2000, C44 and one introgression genotype CH40/91), the general trend of relative frequency of different chlorophyll mutations can be represented as viridis> xantha> chlorina>albina and in kabuli genotype Pb-1 can be represented as xantha> viridis>

chlorina> albina. Similar pattern of chlorophyll mutations as observed in the present study in two desi and one introgression genotypes (viridis>xantha) has been observed by Toker & Cagirgan (2004), Ambarkar (1997) in chickpea, Suryawanshi, (2000) in urdbean and Solanki & Sharma (2001) in lentil. Similar trend as observed in kabuli genotype for different chlorophyll mutations were also reported by Sharma and Sharma, (1981), Solanki & Sharma, (2001) in lentil, Singh *et al.,* (1999) in Urdbean and Kalia *et al*., (1981) in chickpea. Different pattern of chlorophyll mutations were reported by Haq (1990) who observed maximum number of xantha mutants (172) followed by chlorina (166), viridis (160) and albina (85) in three kabuli chickpea genotypes. Our results are contrary to the findings of Haq, (1990). He reported that the xantha and chlorina mutants were predominant. But in the present studies, same trend of predominant occurrence of xantha mutant was observed only in kabuli genotype Pb1. The maximum induction of xantha mutations suggests that genes for xanthophylls are readily available for mutagenic action. Occurrence of chlorina mutants have been attributed to different causes such as impaired chlorophyll biosynthesis, further degradation of chlorophyll and bleaching due to deficiency of carotenoids (Bevines *et al.,* 1992). It means that the genetic differences in genotypes under reference for inducing chlorophyll mutation type have been observed and identified by many workers in Bengal gram (Nerkar & Mote, 1978) and lentil (Sharma & Sharma, 1981).

The data on chlorophyll mutation frequency on M_2 population basis as well as on M_2 family basis are presented in Table 2. It is evident from the overall data that higher doses of physical mutagen in two genotypes (400Gy in Pb2000 and 300 Gy in CH40/91) were more effective in inducing higher frequency of chlorophyll mutations and was found less effective in other two genotypes (C44 and Pb-1). In case of chemical mutagen (EMS), the lower concentrations were more effective in inducing higher frequency of chlorophyll mutations in three genotypes (Pb2000, Pb-1 and CH40/91) except in one desi genotype (C44), where an increase in the frequency of chlorophyll mutations with an increase in the doses of EMS was observed. The highest overall frequency of chlorophyll spectrum (2.66% and 2.59%) was observed in both the doses of EMS (0.2 and 0.3%) in introgression line CH40/91 followed by higher dose (0.4%) in desi line C44 (2.44%). However, overall effect of mutagens differed among four genotypes as the chemical mutagen (EMS) was more effective in inducing a higher frequency (15.47%) and broader spectrum of chlorophyll mutants than gamma rays (8.73%) (Table 3).

The induction of a particular chlorophyll mutation within relative proportion in both types of chickpea genotypes (desi and kabuli) provide an excellent example of parallelism in genetic variability as was first suggested by Vavilov. Ambarkar (1997) reported predominance of viridis among chlorophyll mutant types in chickpea. Sjodin (1962) reported that viridis was most common in *Vicia faba* and xantha was the next most common mutant type, whereas albina was very rare mutant as in most leguminosae. The viridis types were predominant than albina, xantha and chlorina types, irrespective of the cultivar in rice bean as reported by Prakash & Shambulingappa, (1999) and Prakash & Khanure, (2000). The reason for the appearance of greater number of viridis after xantha may be attributed to involvement of polygenes in the chlorophyll formation (Ahmad, 1996). Athwal *et al*., (1970) reported that albina constituted the largest single category of mutants observed in gamma ray treated population of one desi and one kabuli chickpea variety. Kharakwal *et al*., (1988) also reported predominance of albina among chlorophyll mutant types in chickpea. Chlorophyll development seems to be controlled by many genes located on several chromosomes (Goud, 1967) that could be adjacent to centromeres and proximal segments of the chromosome

(Swaminthan *et al*., 1964: 1965). Mutations in these chlorophyll genes may ultimately cause chlorophyll mutations. Swaminthan *et al*., (1962) and Ramulu (1970) suggested that differences in the mutation spectrum and rate in different genotypes may be due to difference in the location of genes in relation to the centromere. These varietal differences in the frequency of chlorophyll mutations indicate that number of genes controlling chlorophyll development may differ in different varieties of chickpea. Such a conclusion gets support from the earlier work that at least 250-300 loci for chlorophyll synthesis exist in barley (Ramulu, 1970). Gustafsson, (1963) reported 125-150 loci for albina, 125 loci for viridis and only 15-50 loci for other type of chlorophyll mutations. In the present studies relative differences in types of chlorophyll mutants can be taken as an indication that it is worthwhile to use more than one mutagen in a mutation studies, since it is confirmed that a particular desirable mutant arises much more rarely from treatment with one mutagen, but more frequently with others, thus considerably increasing the chances of selecting desired mutant(s).

Table 2. Frequency of chlorophyll mutants in M_2 generation of four chickpea genotypes.

Genotypes	Treatments	Frequency on M_2 family basis			Frequency on M_2 population basis		
		Total families	Segregating families	%	Total plants	Mutants	%
Pb.2000							
	300Gy	254	14	5.51	3756	17	0.45
	400Gy	295	21	7.12	4254	34	0.80
	0.3%EMS	202	52	25.74	3587	83	2.31
	0.4% EMS	184	38	20.65	3319	63	1.90
	Overall	**233.75**	**31.25**	**14.76**	**3729**	**49.25**	**1.37**
	Total	**935**	**125**	**59.02**	**14916**	**197**	**5.46**
C44							
	500Gy	257	29	11.28	4150	38	0.91
	600Gy	251	21	8.37	4272	32	0.75
	0.3%EMS	412	43	10.44	5506	68	1.24
	0.4% EMS	290	64	22.07	4138	101	2.44
	Overall	**302.5**	**39.25**	**13.04**	**4516.5**	**59.75**	**1.34**
	Total	**1210**	**157**	**52.16**	**18066**	**239**	**5.34**
Pb.1							
	200Gy	367	49	13.35	5519	71	1.28
	300Gy	345	46	13.33	5133	65	1.26
	0.2%EMS	668	124	18.56	9433	137	1.45
	0.3%EMS	615	51	7.32	8100	72	0.88
	Overall	**498.75**	**67.5**	**13.14**	**7046.25**	**86.25**	**1.23**
	Total	**1995**	**270**	**52.56**	**28185**	**345**	**4.87**
CH40/91							
	200Gy	252	29	11.51	3219	46	1.43
	300Gy	143	30	20.98	1678	31	1.85
	0.2%EMS	92	25	27.17	980	26	2.66
	0.3% EMS	112	20	17.86	1235	32	2.59
	Overall	**149.75**	**26**	**19.38**	**1778**	**33.75**	**2.13**
	Total	**599**	**104**	**77.52**	**7112**	**135**	**8.53**
	G.Total				**68279**	**916**	**24.20**

Table 3. Total (pooled) frequency and spectrum of different types of chlorophyll mutants induced in M_2 generation of chickpea genotypes.

Mutagen basis (mutagens pooled over varieties)		Relative frequency (%) of chlorophyll spectrum					
Mutagens	**Total no. of M_2 plants**	**Albina**	**Xantha**	**Chlorina**	**Viridis**	**Others**	**Total**
Gamma	31981	0.1	2.94	2.1	3.51	0.08	**8.73**
EMS	36298	0.4	5.67	3.72	5.50	0.18	**15.47**
Total	**68279**	**0.5**	**8.61**	**5.82**	**9.01**	**0.26**	**24.20**
Variety basis (varieties pooled over treatments)							
Pb2000	14926	0.03	1.99	1.33	2.09	0.02	**5.46**
C44	18066	0.31	1.91	0.98	1.98	0.16	**5.34**
Pb-1	28185	0.1	1.89	1.16	1.7	0.02	**4.87**
CH40/91	7112	0.06	2.82	2.35	3.24	0.06	**8.52**
Total	**68279**	**0.5**	**8.61**	**5.82**	**9.01**	**0.26**	**24.20**

On the other hand, Gustafsson (1963) believed that ionizing radiations produce high frequency of albina mutations while the chemical mutagens produce other types of chlorophyll mutations in cereal crops. But, in the present studies in legume crop i.e. chickpea, the frequency of four types of chlorophyll mutations was much higher in the EMS treatments in four genotypes than gamma irradiation. Some other studies have also reported that EMS induces a wide spectrum and high frequency of chlorophyll mutations as in mungbean (Singh *et al.,* 2005; Khan *et al.,* 2005) and barley (Gaul, 1964). Nerkar (1970) and Chekalin (1977) also observed occurrence of certain types of mutations more frequently than others in *Lathyrus sativus*. The comparative superiority of chemical mutagens over gamma rays producing a higher frequency and spectrum of chlorophyll mutations suggest that the chemical mutagens are more efficient in inducing mutations of genes needed for chlorophyll development. Swaminathan *et al*., (1962) proposed that such high frequency is due to the preferential action of EMS on chlorophyll development genes located near centromere. The results also indicate that in spite of poor efficiency of gamma ray in producing certain type of chlorophyll mutations, the chemical mutagen was more specific in inducing certain type of chlorophyll mutations. Higher frequency and a wider spectrum of chlorophyll mutants in chemical mutagen have been reported (Bhattacharya, 2003; Koli & Ramkrishna 2002; Sharma & Sharma, 1984; Marki & Bianu, 1970; Kawai, 1969).

So far as radiations are concerned, similar results have been reported in peas (Singh 1988) and lentil (Solanki & Sharma, 1994). However, Kharkwal (1998) reported dose dependent decrease in the frequency of chlorophyll mutations with gamma irradiation. For chemical mutagen higher frequency of chlorophyll mutations with low doses of mutagens was observed in Pb2000, Pb-1 and CH40/91 and the present investigation confirm the result of earlier study (Yadav, 1987). It seems that the strong mutagens reach their saturation point even at lower doses in the highly mutable genotypes and further increase in dose does not add to the mutation frequency. With increase in dose beyond a point, the strong mutagens become more toxic than the higher doses of relatively weaker mutagens. The present findings of differential effect of physical and chemical mutagens in inducing chlorophyll mutations are in agreement with earlier reports (Gustafsson, 1947, Sreekantaradhya & Madhavamenon, 1979).

Conclusion

Marked varietal differences were present in the expression of induction of chlorophyll mutations at different doses/concentrations of mutagens due to genetic differences existing among the four varieties. Among the mutagens, chemical mutagen (EMS) was most effective and potent in inducing the chlorophyll mutations than physical mutagen (gamma irradiation). On the overall basis of four genotypes, the viridis type chlorophyll mutations were most frequent and albina where least common. Among the genotypes, introgression line of desi x kabuli (CH90/91) was most responsive and kabuli type Pb-1 was less responsive for induced mutations. The study of induced genetic variability and frequency and spectrum of chlorophyll mutations on recombinant of desi x kabuli chickpea introgression genotype is first report in chickpea and confirmed the findings of Kaul & Bhan, (1977) that genetic differences even of a single gene, induce significant changes in mutagen sensitivity that influence not only the rate but also the spectrum of recoverable mutations. It is further suggested that as all the gamma radiation and EMS doses induced reasonable chlorophyll mutations, hence all these treatments could be used in mutation breeding programs for inducing viable mutations.

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