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Induction of Variability and Selection of Elite Lines for Specific Alkaloids from a Population of Induced Mutagenesis in Opium Poppy (*Papaver somniferum* L.)

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ABSTRACT

Induced mutations often produce abnormalities which cause morphological alterations in external form of plants including colour, shape, size etc. In the present study two white flowered varieties of opium poppy i.e. NBRI-1 and NBRI-5 were subjected to physical (gamma rays), chemical (ethyl methane sulphonate; EMS) and combined (gamma rays + EMS) mutagenesis to determine the effective doses of mutagens to create plants deformities and chlorophyll (chl) mutation. The study also includes selection of elite lines for specific alkaloids especially for thebaine and codeine. The frequency of chl mutations was maximum for the treatment 50 kRad (0.42%) followed by 40 kRad (0.24%) in NBRI-1 while frequency was highest for the treatment 50 kRad + 0.8% EMS (0.86%) followed by 50 kRad + 0.4% EMS (0.60%) and 50 kRad + 0.6% EMS (0.56%) in NBRI-5. Among the chlorophyll mutations, albino was the most frequently screened followed by xantha type at all doses. The variety NBRI-5 was more responsive to the mutagens than NBRI-1 for chl factor. A typical chl variant was observed in M_1 generation in the combined dose of 10 kRad + 0.4% EMS in plant no.9 of NBRI-5. The treatments 20 kRad in NBRI-1 and 30 kRad, 40 kRad and 10 kRad + 0.4% EMS in NBRI-5 showed high thebaine in both M_2 and M_3 generations.

Keywords: alkaloids, chlorophyll mutation, elite lines, mutagenesis, opium poppy

INTRODUCTION

Induced mutagenesis is one of the powerful tools to create genetic variability in plants and other living organism (Novak and Brunner 1992). Mutation can create novel and unique variations when natural variability does not provide the gene for desired traits (Velmurugan *et al.* 2010). Mutation breeding is an established method for affecting genes either by treating seeds or other plant parts through chemical and/or physical mutagens (Datta and Chakrabarty 2009). A number of varieties with desired traits have been developed through mutation breeding. Various classes of physical and chemical mutagens have diverged effects in their efficiency in inducing mutations. A combination of different mutagens, if their mutagen induction process is independent and capable of interaction, may increase the mutation frequency and can alter the mutation spectrum. Ionization radiations still remain most suitable agents for inducing genetic variability (Irfaq and Nawab 2003; Joseph *et al.* 2004; Tah 2006). Application of radiation has been most frequently used for induction of mutation resulting in direct development of 89% mutant varieties (Velmurugan *et al.* 2010). A number of chemicals have also been found equally and even many times more effective and efficient mutagens (Solanki and Phogat 2005; Rekha and Langer 2007; Ganapathy *et al.* 2008; Dhanavel *et al.* 2008; Basu *et al.* 2008). Induced mutations often produce abnormalities which cause morphological alterations in external form of plants including colour, shape, size, etc. These plant deformities are treated as macromutants or visible mutants (Khan *et al.* 2009). Chlorophyll (chl) mutations are generally used for assessment of genetic effects of mutagenic treatments (Lal *et al.* 2009). This type of mutation is very important to study dose sensitivity, effectiveness and efficiency of mutagen and their doses (Thilagavathi and Mullainathan 2009). The manifestation of chl mutation not only depends upon property of the genotype, but also on the nature of the mutagens applied. These plant deformities and chl mutation also helps to study the developmental and physiological processes occurring in the crop. The present study was undertaken to determine the effective doses of mutagens in opium poppy to obtain plants with deformities and chl mutation caused due to induction of mutagens followed by the selection of elite lines for specific alkaloids especially for thebaine and codeine. Thebaine is used as starting material for the production of 14-hydroxymorphinans, such as oxycodone, naloxone, naltrexone, naltrexone methonobromide, nalbuphine and nalmefene having the ability to be used as analgesics and narcotic antagonists while codeine is considered as a pro-drug since it is metabolized *in vivo* to the primary active compounds morphine and codeine-6-glucuronide.

MATERIALS AND METHODS

Experimental design

Two white flowered, high-yielding varieties of opium poppy (*Papaver somniferum* L.) viz. NBRI-5 and NBRI-1 was the plant material used for the present study. NBRI-1 was developed through rigorous selection and NBRI-5 was developed by hybridization using two distinct pure homozygous inbreds BR007 x BR008 followed by selection after selection. NBRI-1 was subjected only to physical (gamma rays) mutagenesis while NBRI-5 was given physical (gamma rays), chemical (ethyl methane sulphonate; EMS) and combined treatments (gamma rays + EMS). The source of gamma rays was Co^{60} from gamma chamber 9000 with a dose rate of 63 sec/kR while EMS was procured from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India; Catalogue. no. 052820). The experimental materials were divided into three groups. In group I seeds of both the varieties were treated with different doses of gamma rays (γ rays) ranging from 10 to 60 kRad.

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Table 1 Frequency of chlorophyll mutations in M_2 generation (M_1 progeny basis) in varieties NBRI-1 and NBRI-5 of *Papaver somniferum* L. with respect to treatments.

Treatments	Number of progenies studied	Chlorophyll mutation		Treatments	Number of progenies studied	Viability of mutation	
		Number of segregating progenies	Mutation/100 M_1 progenies			Number of segregating progenies	Mutation/100 M_1 progenies
NBRI-1 (Gamma rays)							
kR10	15	0	0.00	kR10	15	1	6.67
kR20	15	0	0.00	kR20	15	3	20.00
kR30	15	0	0.00	kR30	15	2	13.33
kR40	15	1	6.67	kR40	15	3	20.00
kR50	15	3	20.00	kR50	15	4	26.67
NBRI-5							
Gamma rays							
kR10	15	1	6.67	kR10	15	2	13.33
kR20	15	0	0.00	kR20	15	4	26.67
kR30	15	2	13.33	kR30	15	3	20.00
kR40	15	0	0.00	kR40	15	5	33.33
kR50	15	2	13.33	kR50	15	7	46.67
EMS							
0.2%	15	0	0.00	0.2%	15	0	0.00
0.4%	15	2	13.33	0.4%	15	0	0.00
0.6%	15	4	26.67	0.6%	15	2	13.33
0.8%	15	5	33.33	0.8%	15	3	20.00
Gamma rays + EMS							
kR10+0.2%EMS	15	1	6.67	kR10+0.2%EMS	15	2	13.33
kR10+0.4%EMS	15	4	26.67	kR10+0.4%EMS	15	3	20.00
kR10+0.6%EMS	15	7	46.67	kR10+0.6%EMS	15	3	20.00
kR10+0.8%EMS	15	2	13.33	kR10+0.8%EMS	15	4	26.67
kR20+0.2%EMS	15	2	13.33	kR20+0.2%EMS	15	1	6.67
kR20+0.4%EMS	15	4	26.67	kR20+0.4%EMS	15	5	33.33
kR20+0.6%EMS	15	5	33.33	kR20+0.6%EMS	15	6	40.00
kR20+0.8%EMS	15	3	20.00	kR20+0.8%EMS	15	5	33.33
kR30+0.2%EMS	15	3	20.00	kR30+0.2%EMS	15	2	13.33
kR30+0.4%EMS	15	4	26.67	kR30+0.4%EMS	15	6	40.00
kR30+0.6%EMS	15	4	26.67	kR30+0.6%EMS	15	7	46.67
kR30+0.8%EMS	15	5	33.33	kR30+0.8%EMS	15	8	53.33
kR40+0.2%EMS	15	3	20.00	kR40+0.2%EMS	15	2	13.33
kR40+0.4%EMS	15	4	26.67	kR40+0.4%EMS	15	5	33.33
kR40+0.6%EMS	15	5	33.33	kR40+0.6%EMS	15	6	40.00
kR40+0.8%EMS	15	5	33.33	kR40+0.8%EMS	15	7	46.67
kR50+0.2%EMS	15	4	26.67	kR50+0.2%EMS	15	3	20.00
kR50+0.4%EMS	15	5	33.33	kR50+0.4%EMS	15	4	26.67
kR50+0.6%EMS	15	6	40.00	kR50+0.6%EMS	15	5	33.33
kR50+0.8%EMS	15	5	33.33	kR50+0.8%EMS	15	5	33.33

In Group II, seeds of NBRI-5 were presoaked for 12 h in distilled water and later treated with EMS solution prepared in phosphate buffer at pH = 7.0 with the different concentrations of 0.2, 0.4, 0.6 and 0.8% for 6 h and then washed in running tap water for 2 h. In group III, seeds of NBRI-5were treated with combined similar doses of both physical (10 to 60 kRad) and chemical mutagens (0.2, 0.4, 0.6 and 0.8% EMS). The treated seeds of all three mutagenesis treatments were sown in first week of November to study M_1 generation. Usually after a week, seeds started germination and seedlings were appeared. The flowering of plants were started in middle of the February and selected plants were selfed to get M_2 seeds which were harvested in first week of April. The M_2 seeds were further advanced in next year to raise the M_3 generation.

Recording data

Chl mutants were studied between the 10^{th} and 15^{th} day after sowing and were classified according to the system of Gustafsson (1940). 5517 M_1 plant progenies and 80383 seedlings were studied for chl mutations in the M_2 generation. The frequency of chl mutation was estimated both as percent of M_2 segregating families (M_1 plant progeny basis) and also on M_2 seedling basis.

The deformities were noticed as leaf abnormality (like partially developed leaf), stem abnormality (twin stems), flower abnormality (5 petals instead of 4 regular ones or fringed petals) and capsule abnormality (bilobe/multilobed capsule or twin capsule) in M_1 generation. These abnormalities were treated as macromutants or visible mutants. The deformities were taken as percentage over total population.

RESULTS

Chlorophyll mutants

Chl mutations in M_1 plants of both the varieties were studied in all the 34 doses of mutagen treatments. In M_1 generation, the frequency of chl mutation based on number of segregating progenies was observed only in two treatments i.e. 50 kRad (20.00%) and 40 kRad (6.67%) in NBRI-1 while in NBRI-5 involving all the doses of 3 mutagenesis showed maximum frequency of chl mutation in treatment 10 kRad + 0.6% EMS (46.67%) followed by 50 kRad + 0.6% EMS (40.00%) (**Table 1**). A large M_2 population (1280 to 2904) of each treatment was studied for chl mutation. The frequency of chl mutations was maximum for the treatment 50 kRad (0.42%) followed by 40 kRad (0.24%) in NBRI-1 while frequency of chl mutation was highest for the treatment 50 kRad + 0.8% EMS (0.86%) followed by 50 kRad + 0.4% EMS (0.60%) and 50 kRad + 0.6% EMS (0.56%) in NBRI-5 (**Table 2**). The chl mutations observed in M_2 generation were mainly albino, xantha, albino-xantha and xantha-alba types.

Albino was the most frequent occurring type followed by xantha in all the doses. In NBRI-1, three types of chl mutations i.e. albino, xantha and xantha-alba were noticed in dose 50 kRad. The combined doses 50 kRad + 0.8% EMS, 50 kRad + 0.6% EMS, 40 kRad + 0.6% EMS and 20 kRad + 0.8%EMS in NBRI-5 showed a wide spectrum with all the four kinds of chl mutation, whereas the doses 10

Table 2 Spectrum of chlorophyll mutants expressed in percentage in M_2 generation in variety NBRI-1 and NBRI-5 of *Papaver somniferum* L. with respect to treatments.

Treatment	Mutant Seedling	Albino	Albino-xantha	Xantha-alba	Xantha
NBRI-1 (Gamma rays)					
kR10	0.0	0.0	0.0	0.0	0.0
kR20	0.0	0.0	0.0	0.0	0.0
kR30	0.0	0.0	0.0	0.0	0.0
kR40	5.0	100.0	0.0	0.0	0.0
kR50	8.0	50.0	0.0	37.5	12.5
NBRI-5 (Gamma rays)					
kR10	1.0	0.0	0.0	100.0	
kR20	0.0	0.0	0.0	0.0	0.0
kR30	4.0	25.0	0.0	0.0	75.0
kR40	0.0	0.0	0.0	0.0	0.0
kR50	5.0	40.0	40.0	0.0	20.0
NBRI-5 (EMS)					
0.2%EMS	0.0	0.0	0.0	0.0	0.0
0.4%EMS	4.0	25.0	0.0	25.0	50.0
0.6%EMS	6.0	66.7	0.0	33.3	0.0
0.8%EMS	7.0	57.1	14.3	0.0	28.6
NBRI-5 (Gamma rays + EMS)					
kR10+0.2%EMS	2.0	50.0	0.0	50.0	0.0
kR10+0.4%EMS	12.0	58.3	1.67	0.0	25.0
kR10+0.6%EMS	11.0	45.4	18.2	36.4	0.0
kR10+0.8%EMS	6.0	83.3	0.0	0.0	16.7
kR20+0.2%EMS	1.0	100.0	0.0	0.0	0.0
kR20+0.4%EMS	3.0	66.7	0.0	33.3	0.0
kR20+0.6%EMS	5.0	40.0	0.0	0.0	60.0
kR20+0.8%EMS	8.0	62.5	12.5	12.5	12.5
kR30+0.2%EMS	3.0	66.7	0.0	0.0	33.3
kR30+0.4%EMS	6.0	66.7	0.0	33.3	0.0
kR30+0.6%EMS	7.0	57.1	14.3	0.0	28.6
kR30+0.8%EMS	9.0	44.4	0.0	33.3	22.2
kR40+0.2%EMS	4.0	25.0	50.0	0.0	25.0
kR40+0.4%EMS	5.0	40.0	20.0	20.0	20.0
kR40+0.6%EMS	7.0	57.1	0.0	28.6	14.3
kR40+0.8%EMS	8.0	50.0	0.0	25.0	25.0
kR50+0.2%EMS	5.0	60.0	0.0	40.0	0.0
kR50+0.4%EMS	9.0	66.7	0.0	33.3	0.0
kR50+0.6%EMS	8.0	37.5	25.0	25.0	12.5
kR50+0.8%EMS	11.0	36.4	27.3	18.2	18.2

kRad, 20 kRad, 30 kRad in NBRI-1 and doses 20 kRad, 40 kRad and 0.2% EMS in NBRI-5 did not show any chl mutation. A typical chl variant was observed in M_1 generation in the combined dose of 10 kRad + 0.4% EMS in plant no.9 of NBRI-5. The plant was variegated vertically with nearly half green and half chrome yellow (**Fig. 1A**), which was considered as a variant with an impression of macro mutation and was compared for yield and alkaloid content in its progeny with a control.

Plant anomalies

Viable mutations like hairy peduncle (**Fig. 1B**), plants with large capsule (**Fig. 1C**), plants having more than six capsules (**Fig. 1D**) and pink stigmatic rays (**Fig. 1E**) were observed in all the doses of both the varieties. It was noticed that 50 kRad was most effective dose for inducing viable mutation in both the varieties in M_1 generation while on M_2 seedlings basis, frequency of viable mutation ranged from 0.67% (20 kRad) to 1.05% (50 kRad) in NBRI-1 and from 0.29% (0.2% EMS) to 1.59% (50 kRad) in NBRI-5 (**Table 3**). The maximum number of M_1 segregating progenies for viable mutation were observed in 50 kRad (26.67%) in NBRI-1 (**Table 1**) followed by 20 kRad and 40 kRad, which were up to 20.00%. Whereas, in NBRI-5 for all the three mutagenesis, maximum mutation frequency for viable mutations were noticed in doses 40 kRad + 0.8%EMS, 30 kRad + 0.6% EMS and 50 kRad (46.67%) (**Table 1**).

The total frequency of visible mutations at M_1 plant progeny basis (Msp) was calculated by pooling the number of progenies segregating for chl as well as viable mutations (**Table 4**). In NBRI-1 mutation events/100 M_1 progenies (Msp) was maximum for dose 50 kRad (46.67%) followed by 40 kRad (26.67%) while, in NBRI-5, the dose 30 kRad + 0.8% EMS showed maximum Msp (86.67%) followed by 40 kRad + 0.8% EMS (80.00%). In M_2 generation of NBRI-1, pooled frequency of chl and visible mutation (Msd) ranged from 0.00% to 1.47% (**Table 4**) in γ ray and from 0.29% to 1.59% in all the treatments of three mutagenesis of NBRI-5 (**Table 4**). The highest frequency in NBRI-1 was 1.47% for the dose 50 kRad followed by 1.15% for 40 kRad, while highest frequency in NBRI-5 was 1.59% for the treatment 50 kRad followed by 1.42% for 10 kRad + 0.6% EMS.

The macrovariations like elongated deformed capsule and few dwarf variants were observed in M_1. In deformed capsule no seed formation had taken place though the formation of latex was quite normal while dwarf variants gave normal seed yield in M_1 generation but revert back to normal plant height in subsequent generations. Further, whole M_2 population was thoroughly examined for plant abnormalities and visible macrovariations. Plant abnormalities comprised of partially developed leaves (**Fig. 1F**), twin stems, deformed capsule developed from partially developed ovary (**Fig. 1G**), fringed petals in comparison to normal smooth petal, five petalled flower in comparison to four petalled one's, persistent sepals and bilobed capsule (**Figs. 1H, 1I**) and variegated capsule or plant (**Fig. 1A**). The maximum frequency of abnormalities i.e. 28.33% was similar in both the varieties but least was 6.67% in NBRI-1 and 8.33% in NBRI-5 (**Table 5**) for gamma rays treatments. Similarly, the total deformities in NRBI-5 ranged from 8.33 to 18.33% for EMS and 1.33 to 41.67% in combined doses (**Table 5**). The abnormalities also showed an increasing trend with ascending doses of the mutagens. It was interesting to notice that nearly all the deformed plants were highly sterile (sterility >85%), lethal and not heritable to M_2 generations.

Identification of elite lines

In M_2 generation of NBRI-1 the plant no. 9/2 (14.27%) of 10 kRad and 5/3 of 20 kRad had high morphine content, plant nos. 9/2 (6.08%) of 10 kRad and 5/2 and 5/3 (5.49%) of 20 kRad had high codeine and plant no. 5/3 (4.75%) of 20 kRad had high thebaine. In NBRI-5 plant nos. 6/3 (14.09%) and 7/2 (15.54%) of 40 kRad, 6/1 (15.31%) and 9/2 (13.87%) of 10 kRad + 0.2% EMS and 4/3 (21.87%) of 20 kRad + 0.4% EMS had high morphine, plant no. 4/1 (7.43%) of 0.6% EMS and 6/1 (7.63%) of 10 kRad + 0.2% EMS had high codeine, plant nos. 3/1 (8.64%) of 30 kRad and 6/3 (10.72%) and 7/2 (7.61%) of 40 kRad had high thebaine and plant no. 9/5 (7.61%) and 10/2 (18.17%) of 30 kRad + 0.4% EMS had high narcotine (**Table 6**). In M_3, further improvement upto 28.64% in plant no. 9/2/2 of 10 kRad followed by 9/2/1 (25.89%) and plant no. 5/3/1 (25.57%) followed by 5/3/2 (21.92%) in 20 kRad were observed for morphine in variety NBRI 1. In NBRI-5 plant nos. 6/3 (14.09%) and 7/2 (15.54%) of 40 kRad, 6/1 (15.31%) and 9/2 (13.87%) of 10 kRad + 0.2% EMS and 4/3 (21.87%) of 20 kRad + 0.4% EMS had high morphine in M_2 and plant nos. 6/3/3 (29.37%) and 7/2/2 (26.62%) of 40 kRad, 6/1/2 (24.98%) and 9/2/2 (24.35%) of 10 kRad + 0.2% EMS had high morphine in M_3 (**Table 6**). In NBRI-1, plant nos. 9/2 (6.08%) of 10 kRad and 5/2 and 5/3 (5.49%) of 20 kRad in M_2 had high codeine. In NBRI-1, plant no. 5/3 of 20 kRad showed 4.75% thebaine in M_2 and when progressed to M_3 generation showed high value of thebaine in its progenies (plant nos. 5/3/1, 5/3/2 and 5/3/3 showed 6.64, 8.65 and 3.41% thebaine, respectively) (**Table 6**). In NBRI-5, thebaine was high in plant nos. 3/1 (8.64%) of 30 kRad and 6/3 (10.72%) and 7/2 (7.61%) of 40 kRad which progressed to M_3, showed similar high values of thebaine in their progenies. In NBRI-5, the plant nos. 9/5 (7.61%) and 10/2 (18.17%) of 30 kRad + 0.4% EMS showed enhance-

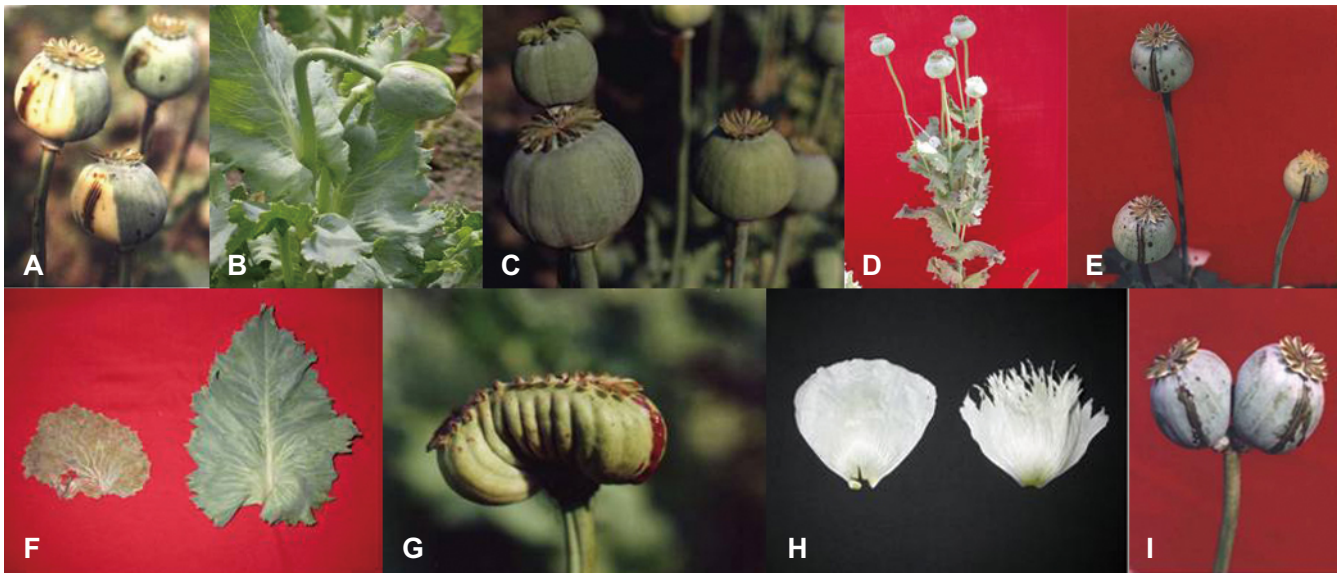

Fig. 1 (**A**) Variegated capsule; (**B**) Peduncle with hairs; (**C**) Large capsule; (**D**) Plant with six capsules; (**E**) Capsule with pink stigmatic rays. (**F**) Partially developed leaf; (**G**) Deformed capsule; (**H**) Fringed petals; (**I**) Double capsule.

Table 3 Mutation frequency in M_2 seedlings raised from treatments in *Papaver somniferum* L.

Treatment	Number studied		Mutation percent		
	M_1 plant progenies	M_2 seedlings	Chlorophyll mutation (M_2 seedlings)	Viable mutation (M_2 seedlings)	Total visible mutation (M_2 seedlings)
NBRI-1 (Gamma rays)					
Control	229	3435	0.00	0.00	0.00
kR10	162	2769	0.00	0.00	0.00
kR20	160	2350	0.00	0.67	0.67
kR30	154	2064	0.00	0.74	0.74
kR40	136	1909	0.24	0.91	1.15
kR50	141	1621	0.42	1.05	1.47
NBRI-5 (Gamma rays)					
Control	221	3630	0.000	0.00	0.00
kR10	182	3121	0.032	0.69	0.72
kR20	178	2529	0.000	0.81	0.81
kR30	172	2186	0.183	0.92	1.10
kR40	159	1978	0.000	1.07	1.07
kR50	153	1679	0.290	1.29	1.59
NBRI-5 (EMS)					
0.2%EMS	208	2690	0.00	0.29	0.29
0.4%EMS	143	2365	0.17	0.58	1.75
0.6%EMS	131	1924	0.31	0.75	1.06
0.8%EMS	121	1533	0.46	0.82	1.28
NBRI-5 (Gamma rays + EMS)					
kR10+0.2%EMS	197	2904	0.07	0.71	0.78
kR10+0.4%EMS	184	2739	0.44	0.10	0.54
kR10+0.6%EMS	166	2502	0.44	0.98	1.42
kR10+0.8%EMS	162	2468	0.24	0.68	0.92
kR20+0.2%EMS	189	2715	0.04	0.73	0.77
kR20+0.4%EMS	169	2589	0.12	0.84	0.96
kR20+0.6%EMS	153	2399	0.21	0.97	1.18
kR20+0.8%EMS	152	2294	0.35	0.10	0.45
kR30+0.2%EMS	170	2142	0.14	0.65	0.79
kR30+0.4%EMS	155	2300	0.26	0.71	0.96
kR30+0.6%EMS	137	2063	0.34	0.82	1.16
kR30+0.8%EMS	136	2035	0.44	0.87	1.31
kR40+0.2%EMS	158	2055	0.19	0.77	0.97
kR40+0.4%EMS	154	1927	0.26	0.85	1.11
kR40+0.6%EMS	136	1894	0.37	0.96	1.33
kR40+0.8%EMS	134	1732	0.46	0.10	0.57
kR50+0.2%EMS	108	1627	0.31	0.99	1.29
kR50+0.4%EMS	72	1499	0.60	0.10	0.71
kR50+0.6%EMS	71	1436	0.56	0.16	0.67
kR50+0.8%EMS	64	1280	0.86	0.12	0.98

ment in M_2 for narcotine and further confirmed improvement when progressed to M_3. The plant nos. 9/5/1, 9/5/2 and 9/5/3 showed 8.47, 16.09 and 14.05% narcotine, respectively in M_2. In M_2, the selected lines did not show papaverine, but few individual in M_3 i.e. 9/2/2 of 10 kRad and 3/2/3 of 30 kRad showed 4.35 and 5.03% papaverine, respectively in NBRI-1 while plant no.8/1/1 of 30 kRad + 0.4% EMS showed 2.06% papaverine in NBRI-5.

Table 4 Mutagenic effectiveness and efficiency of the different mutagenic treatments in the varieties NBRI-1 and NBRI-5 of *Papaver somniferum* L.

Treatments	Injury (I :%)	Fertility (S :%)	Mutation events/100M_1 progenies (Msp)	Mutation events/100M_2 seedlings (Msd)	Mutagenic effectiveness (Msp/tc,kR)	Mutagenic efficiency		
						Msp/I	Msp/S	Msd/S
NBRI-1(Gamma rays)								
kR10	18.07	9.34	6.67	0.00	0.67	0.369	0.714	0.00
kR20	26.51	14.26	20.00	0.67	1	0.755	1.403	0.047
kR30	38.55	17.67	13.33	0.74	0.44	0.346	0.754	0.042
kR40	46.99	53.11	26.67	1.15	0.67	0.568	0.502	0.022
kR50	46.99	56.83	46.67	1.47	0.93	0.9932	0.321	0.026
NBRI-5 (Gamma rays)								
kR10	28.57	9.62	20.00	0.72	2.00	0.700	2.08	0.075
kR20	40.66	13.33	26.67	0.81	1.33	0.656	2.00	0.061
kR30	49.45	17.34	33.33	1.10	1.11	0.674	1.923	0.064
kR40	49.45	20.54	33.33	1.07	0.83	0.674	1.62	0.052
kR50	60.44	37.88	60.00	1.59	1.20	0.9927	1.58	0.042
NBRI-5 (EMS)								
0.2%	41.76	9.87	0.00	0.29	0.00	0.00	0.00	0.029
0.4%	46.15	27.36	13.33	1.75	5.55	0.29	0.49	0.027
0.6%	69.23	28.67	40.00	1.06	11.11	0.58	1.39	0.037
0.8%	68.13	42.02	53.33	1.28	11.11	0.78	1.27	0.030
NBRI-5 (Gamma rays + EMS)								
kR10+0.2%EMS	13.19	12.12	20.00	0.78	16.67	1.52	1.65	0.064
kR10+0.4%EMS	8.79	13.73	46.67	0.54	19.45	5.31	3.39	0.039
kR10+0.6%EMS	30.77	32.67	66.67	1.42	18.52	2.17	2.04	0.043
kR10+0.8%EMS	34.07	33.47	40.00	0.92	8.33	1.17	1.19	0.028
kR20+0.2%EMS	26.37	29.06	20.00	0.77	16.67	0.76	0.69	0.026
kR20+0.4%EMS	30.77	32.06	60.00	0.96	25	1.95	1.87	0.029
kR20+0.6%EMS	30.77	15.33	73.33	1.18	20.37	2.38	4.78	0.077
kR20+0.8%EMS	28.57	43.49	53.33	0.45	11.11	1.87	1.23	0.010
kR30+0.2%EMS	41.76	32.67	33.33	0.79	27.78	0.79	1.02	0.024
kR30+0.4%EMS	36.26	24.75	66.67	0.96	27.78	1.84	2.69	0.039
kR30+0.6%EMS	38.46	39.48	73.34	1.16	20.37	1.91	1.86	0.029
kR30+0.8%EMS	39.56	60.12	86.66	1.31	18.05	2.19	1.44	0.022
kR40+0.2%EMS	38.46	58.02	33.33	0.97	27.78	0.87	0.57	0.017
kR40+0.4%EMS	38.46	60.02	60.00	1.11	25	1.56	0.99	0.018
kR40+0.6%EMS	46.15	67.74	73.33	1.33	20.37	1.59	1.08	0.019
kR40+0.8%EMS	45.06	27.26	80.00	0.57	16.67	1.78	2.93	0.021
kR50+0.2%EMS	47.25	76.75	46.67	1.29	38.89	0.99	0.61	0.017
kR50+0.4%EMS	50.55	66.23	60.00	0.71	25.00	1.19	0.91	0.011
kR50+0.6%EMS	58.24	83.57	73.33	0.67	20.37	1.26	0.88	0.008
kR50+0.8%EMS	62.64	85.17	66.66	0.98	13.89	1.06	0.78	0.011

* Mutagenic effectiveness estimated on the basis of variable mutagen EMS.
I: Percent seedling height reduction over control.
S: Percent Reduction of Fertility in M_1 over control.
tc: The product of treatment time x initial concentration of the mutagen.
kR: Radiation dose in kilo rads.

DISCUSSION

Scope of chlorophyll mutation in plants

Gustafsson (1940) was the pioneer worker who estimated chl mutation in barley and classified the mutants according to the presence or absence of chl and variegations i.e., albino (white, lethal, no chl or carotenoids), xantha (yellow to yellowish white, lethal, carotenoids present but chlorophyll absent), albino-xantha (white to yellow lethal), xantha-alba (yellowish white, lethal), albino-viridis (uniform light yellow green colour of leaves, viable), marginata (different colour at margins), chlorina (uniform green colour with white on tips, viable) and striata (longitudinal strips of different colours, viable). Chl mutations are one of the reliable indices for assessment of genetic effects of mutagenic treatments (Lal *et al.* 2009). Chl mutation is broadly a morphological changes generally considered as the secondary effect of physiological disturbances (Spanol *et al.* 2003). Although not much is known about the origin or reason of formation of these mutations however, some suggestions about their existence are available in literature. According to Kaplan (1954), chromosomal aberrations caused by mutagen treatments of seed are responsible for induction of these mutations and loss of photosynthetic competence includes breakdown of proteins and destruction of membrane by lipid degradation (Spanol *et al.* 2003). A number of studies were done to isolate chl mutants in various crops e.g., Mahana and Singh (1982) in *Physalis ixocarpa,* Kumar et al. (2001) in chilli pepper, Khan *et al.* (2005) in *Cicer arietinum*, Lal *et al.* (2009) in black gram, Abdullah *et al.* (2009) in *Curcuma alismatifolia*, Khan and Tyagi (2009) in *Glycine max* (L.) Merrill and Mohamed *et al.* (2011) in *Physalis ixocarpa* Brot.

Chloprohyll mutation and opium poppy

1. With respect to varieties

The typical chl mutations induced through different doses of mutagens in the two varieties were albino, albino-xantha, xantha-alba and xantha types, which suggested that there was a differential response of the two varieties (NBRI-1 and NBRI-5) of opium poppy to γ rays. Similar findings were also noticed by Pillai *et al.* (1993) and Shadakshari *et al.* (2001) in *Oryza sativa* L.; Paul and Singh (2002) in *Lens culinaris* Medik and Ramesh and Kumar (2005) in barley mutants. For chemical and combined mutagenesis all the treatments showed chl mutation except in dose 0.2% EMS in NBRI-5. It suggested that NBRI-5 was more responsive to the mutagens than NBRI-1 for chl factor. High frequency of chl mutations in different treatments of three mutagens in NBRI-5 also confirmed similar findings. The result showed that manifestation of chl mutation not only depends upon

Table 5 Effect of mutagens in NBRI-1 and NBRI-5 of *Papaver somniferum* L. with respect to deformities %.

Treatments	Leaf abnormality	Stem abnormality	Flower abnormality	Capsule abnormality	Total
NBRI-1 (Gamma rays)					
kR10	1.67	1.67	1.67	1.67	6.67
kR20	3.33	0	1.67	3.33	8.33
kR30	3.33	1.67	1.67	3.33	10.00
kR40	6.67	3.33	5.00	5.00	20.00
kR50	10.00	5.00	6.67	6.67	28.33
NBRI-5 (Gamma rays)					
kR10	1.67	3.33	0.00	3.33	8.33
kR20	3.33	3.33	1.67	3.33	11.67
kR30	3.33	5.00	3.33	5.00	16.67
kR40	5.00	6.67	3.33	5.00	20.00
kR50	8.33	8.33	5.00	6.67	28.33
NBRI-5 (EMS)					
0.2%EMS	1.67	1.67	1.67	3.33	8.33
0.4%EMS	3.33	1.67	1.67	3.33	10.00
0.6%EMS	3.33	3.33	3.33	5.00	15.00
0.8%EMS	5.00	3.33	3.33	6.67	18.33
NBRI-5 (Gamma rays + EMS)					
kR10+0.2%EMS	1.67	3.33	1.67	1.67	8.33
kR10+0.4%EMS	1.67	3.33	3.33	3.33	11.67
kR10+0.6%EMS	3.33	5.00	3.33	5.00	16.67
kR10+0.8%EMS	3.33	6.67	5.00	5.00	20.00
kR20+0.2%EMS	1.67	3.33	3.33	1.67	10.00
kR20+0.4%EMS	3.33	5.00	6.67	3.33	18.33
kR20+0.6%EMS	6.67	6.67	6.67	6.67	26.67
kR20+0.8%EMS	8.33	8.33	8.33	8.33	33.33
kR30+0.2%EMS	1.67	3.33	3.33	5.00	13.33
kR30+0.4%EMS	3.33	6.67	6.67	6.67	23.33
kR30+0.6%EMS	6.67	8.33	8.33	8.33	31.67
kR30+0.8%EMS	10.00	10.00	10.00	8.33	38.33
kR40+0.2%EMS	5.00	5.00	5.00	3.33	18.33
kR40+0.4%EMS	5.00	6.67	6.67	6.67	25.00
kR40+0.6%EMS	6.67	8.33	8.33	8.33	31.67
kR40+0.8%EMS	10.00	10.00	8.33	10.00	38.33
kR50+0.2%EMS	5.00	5.00	5.00	5.00	20.00
kR50+0.4%EMS	8.33	8.33	6.67	8.33	31.67
kR50+0.6%EMS	10.00	10.00	6.67	10.00	36.67
kR50+0.8%EMS	11.67	11.67	8.33	10.00	41.67

the property of the genotype, but also on the nature of the mutagens applied (Boranayaka *et al.* 2010).

2. With respect to doses

If only treatments are to be taken irrespective of the varieties, combined treatment was most effective in obtaining chl mutation followed by EMS. In the present study three EMS treatments (0.4, 0.6 and 0.8%) out of four and four (10 kRad, 30 kRad, 40 kRad and 50 kRad) out of five γ rays treatments showed chl mutation (Lal *et al.* 2009; Boranayaka *et al.* 2010). Singh *et al.* (2001) noticed similar effectiveness of EMS over γ rays in *Abelmoschus esculentus* (L.) Mocnch and also suggested that the frequency of chl mutation was dose dependent. Even El-Shouny *et al.* (2001) noticed dose-dependant tendency of mutagens for chl mutation in *Triticale*. The analysis of data on frequency of chl mutations of M_1 plant basis (i.e. on the basis of number of M_1 plant progenies showed segregation) showed that the combined mutagen has effectiveness without having differentiation among its dosages and on M_2 seedlings basis it was clearly appeared that combined mutagen doses of 50 kRad + 0.8% EMS and 50 kRad + 0.6% EMS were potent doses for NBRI-5 and 50 kRad for NBRI-1. The M_2 results also clearly indicated that gamma rays, EMS and combined mutagens affect differentially on the spectrum of chl mutations. The absence of mutants in M_1 generation but their appearance in M_2 generation suggested that the mutants obtained in M_2 generation were governed through recessive genes (Boranayaka *et al.* 2010).

In the present study, a large extent of chl mutations were induced through combined doses (50 kRad + 0.8% EMS, 50 kRad + 0.4% EMS and 50 kRad + 0.6% EMS) in NBRI-5. The treatments 50 kRad + 0.4% EMS and 40 kRad + 0.8% EMS of NBRI-5 showed high frequency of chromosomal aberration. The chl mutation was observed in the dose 50 kRad in NBRI-1, but frequency of chromosomal aberration was high in 40 kRad. It suggested that some other mechanism also play in the induction of chl mutations. Waghmare and Mehra (2001) suggested that chl mutations might be caused by a single point mutation or multi-mutations, which resulted segregation of different types of chl mutations in single family.

3. Frequency of viable mutants

Total frequency of visible mutations (i.e. total frequency of chl and viable mutations) at both M_1 plant progeny basis (Msp) and M_2 seedling basis (Msp) was observed high in NBRI-5 than NBRI-1. It suggests that probably the operations of diplontic selection in M_1 somatic tissues and haplontic selection in the gametes of M_1 plants were more rigorous in NBRI-5 than NBRI-1 to cause mutation expression (Swaminathan 1970). Nevertheless, possibility of genotypic difference between two varieties in terms of their variations in mutability of gene loci cannot be ignored. On the basis of relative effects of different mutagen treatments, it clearly emerges that for inducing high visible mutations, the treatments 30 kRad + 0.8% EMS and 50 kRad were relatively highly effective in NBRI-5, while, the doses 50 kRad and 40 kRad were relatively highly effective doses for NBRI-1 for inducing chl mutation.

Table 6 Elite lines (mutants) for alkaloids induced by different doses in M_2 and M_3 generations of two *Papaver somniferum* L. varieties, NBRI-1 and NBRI-5.

Plant no.		Plant number	Morphine %	Codeine %	Thebaine%	Narcotine%	Papaverine%
NBRI-1							
kR10	M_2	9/2	14.27	6.08	1.78	7.42	0.00
	M_3	9/2/1	25.89	2.80	1.84	11.85	0.23
		9/2/2	28.64	5.34	4.83	16.62	4.35
		9/2/3	13.25	5.00	3.37	8.82	0.56
kR20	M_2	5/2	14.11	5.49	4.75	7.72	0.00
	M_3	5/2/1	23.13	5.55	4.97	7.73	0.00
		5/2/2	16.45	4.12	3.47	7.00	0.00
		5/2/3	15.96	4.09	3.34	6.07	0.00
	M_2	5/3	14.12	5.49	4.75	7.72	0.00
	M_3	5/3/1	25.57	3.99	**6.64**	6.95	0.00
		5/3/2	21.92	4.01	**8.65**	7.02	0.00
		5/3/3	19.20	4.24	**3.41**	6.86	0.00
kR30	M_2	3/2	14.29	4.69	3.68	7.29	0.00
	M_3	3/2/1	10.44	6.16	5.17	9.36	0.00
		3/2/2	10.71	3.75	3.07	5.24	2.16
		3/2/3	15.35	4.35	4.72	5.99	5.03
NBRI-5							
kR30	M_2	3/1	20.45	5.17	8.64	13.25	0.00
	M_3	3/1/1	10.89	3.06	**6.19**	7.21	0.00
		3/1/2	12.19	3.18	**5.51**	7.24	0.00
		3/1/3	11.73	3.04	**5.52**	7.30	0.00
kR40	M_2	6/1	13.37	3.78	8.35	3.58	0.00
	M_3	6/1/3	16.13	4.27	3.05	4.80	0.00
		6/1/4	14.83	4.48	6.63	4.89	0.00
		6/1/6	15.98	4.17	3.09	4.83	0.00
	M_2	6/3	14.09	4.12	**10.72**	2.69	0.00
	M_3	6/3/3	29.37	4.78	**11.69**	0.00	0.00
		6/3/4	17.69	4.16	**7.10**	4.91	0.00
		6/3/6	17.96	4.53	**3.69**	4.82	0.00
	M_2	7/2	15.54	6.62	**7.61**	10.19	0.00
	M_3	7/2/1	25.94	5.82	**8.67**	5.84	0.00
		7/2/2	26.62	5.71	6.42	4.99	0.00
		7/2/3	25.09	5.12	6.33	2.88	0.00
0.2%EMS	M_2	8/3	16.15	6.73	3.86	10.86	0.00
	M_3	8/3/1	12.24	6.24	2.19	6.69	0.00
		8/2/2	12.15	5.54	2.15	6.52	0.50
		8/2/3	12.71	5.34	2.32	6.42	0.00
0.4%EMS	M_2	4/4	15.83	5.09	3.81	7.59	0.00
	M_3	4/4/1	15.24	5.08	2.22	12.50	0.21
		4/4/2	13.43	3.72	1.76	8.17	0.00
		4/4/3	13.24	3.72	1.94	8.21	0.00
0.6%EMS	M_2	4/1	11.63	**7.43**	0.99	7.19	0.00
	M_3	4/1/1	12.09	**6.61**	1.06	5.16	0.00
		4/1/2	11.55	**5.48**	1.22	4.55	0.00
		4/1/3	10.28	**5.37**	1.15	4.63	0.00
kR10+0.2%EMS	M_2	6/1	15.31	**7.63**	5.47	11.62	0.00
	M_3	6/1/1	27.16	**5.25**	4.65	10.67	0.00
		6/1/2	24.98	4.41	4.33	8.81	0.00
		6/1/3	21.46	3.98	2.85	7.89	0.00
	M_2	9/2	13.87	6.12	5.79	9.56	0.00
	M_3	9/2/1	21.47	6.89	3.32	13.55	0.00
		9/2/2	24.35	6.19	2.39	14.79	0.00
		9/2/6	16.59	4.09	2.38	7.86	0.00
kR10+0.4%EMS	M_2	9/1	14.63	6.62	5.09	10.83	0.00
	M_3	9/1/1	13.44	4.87	4.29	**16.16**	0.00
		9/1/2	14.05	3.19	2.80	7.56	0.00
		9/1/3	12.79	3.26	2.89	7.57	0.00
kR20+0.4%EMS	M_2	4/3	**21.87**	3.99	1.55	12.77	0.00
	M_3	4/3/1	**21.33**	6.31	3.94	13.15	0.00
		4/3/2	**16.22**	4.65	3.31	8.17	0.00
		4/3/3	**15.94**	5.15	3.27	7.98	0.00
kR20+0.6%EMS	M_2	6/1	12.25	5.12	5.84	0.00	0.00
	M_3	6/1/1	21.67	6.98	7.89	12.66	0.00
		6/1/2	24.77	4.65	3.72	10.29	0.39
		6/1/3	26.09	4.04	3.99	10.24	0.00
kR20+0.8%EMS	M_2	10/1	17.58	**8.84**	4.85	11.39	0.00
	M_3	10/1/1	25.79	**4.79**	2.84	8.56	0.00
		10/1/2	23.55	4.02	3.05	9.84	0.48
		10/1/3	26.61	5.11	3.87	11.92	0.00

Table 6 (Cont.)

Plant no.		Plant number	Morphine %	Codeine %	Thebaine %	Narcotine %	Papaverine %
NBRI-5 (Cont.)							
kR30+0.4%EMS	M_2	8/1	18.62	6.15	3.28	**16.43**	0.00
	M_3	8/1/1	17.09	3.48	2.12	7.44	**2.06**
		8/1/2	15.67	2.89	2.31	7.22	0.87
		8/1/3	15.65	2.89	2.31	7.46	0.31
	M_2	9/1	13.49	5.91	2.08	14.48	0.00
	M_3	9/1/1	21.57	4.14	2.29	11.09	0.00
		9/1/2	21.84	4.25	2.28	11.37	0.00
		9/1/3	15.18	3.38	2.28	7.41	0.00
	M_2	9/5	13.28	4.90	2.98	7.61	0.00
	M_3	9/5/1	18.46	2.63	2.17	**8.47**	0.00
		9/5/2	26.39	6.68	4.94	**16.09**	0.00
		9/5/3	28.16	4.97	3.26	14.05	0.00
	M_2	10/2	16.69	**5.89**	4.25	**18.17**	0.00
	M_3	10/2/1	21.57	**7.38**	3.49	**15.02**	0.00
		10/2/2	16.86	4.17	3.17	9.16	0.00
		10/2/3	10.53	4.28	3.15	9.42	0.00

Selections of elite lines

Keeping in mind, worldwide ever increasing demand of alkaloids, the elite families and lines with high alkaloid was considered for selection and development of mutant lines. Elite lines for high morphine were found in the treatment 10 kRad in both M_2 and M_3 generations of NBRI -1. The plant number 9/2 of 10 kRad showed morphine up to 14.27% in M_2 and 15.89% (in 9/2/1), 28.64% (9/2/2), and 13.25% (9/3/3) in M_3 so, 10 kRad was considered an effective dose to obtain elite family for high morphine in NBRI-1. In NBRI-5, the plant no. 4/3 (morphine 21.81%) of the treatment 20 kRad + 0.4% EMS when progressed from M_2 to M_3 showed that is 50% of its progenies i.e. 4/3/1, 4/3/2 and 4/3/3 in M_3 had high morphine i.e. 21.33, 16.22 and 15.94%, respectively. The development and stabilization of such high morphine lines could be an impetus to meet out the world demand of morphine in pharmacopoeal purposes. The dose 20 kRad of NBRI-1 and 10 kRad + 0.2% EMS of NBRI-5 possessed high codeine in M_3 progenies. Beside this, treatment 0.6% EMS exhibited high codeine in M_3 generation similar as M_2 population.

In recent years, thebaine is one of the most important alkaloid of opium latex due to its worldwide demand in pharmaceutical industries as being source for the production of non-addictive analgesic (Hevel *et al.* 2001; Streetdrugs 2004). The mutant lines developed with high thebaine and low morphine could be advantageous to meet out the world demand. It is evident from the results that the treatments 20 kRad in NBRI-1 and 30 kRad, 40 kRad and 10 kRad + 0.4% EMS in NBRI-5, showed high thebaine in both M_2 and M_3 generations. Moreover, M_3 results showed that 80% of the subfamilies have high thebaine similar to their M_2 families. These remarkable micro-mutational effects gave a promising value for codeine and thebaine. These obtained mutant lines can further be utilized in future for enhancement of thebaine through hybridization.

The dose 30 kRad + 0.4% EMS in NBRI-5 was the most effective dose for narcotine enhancement which could be considered elite family for selection. But, in NBRI-1, none of the families or subfamilies showed enhanced effect when progressed from M_2 to M_3 generation. Only few individual had high value, which could not be taken as a representative of the whole family. It was also evident that enhancement of thebaine was at lower doses of mutagen used in combination while narcotine was at higher doses. This was mainly due to the difference in the metabolic pathway of formation of these two alkaloids (Prajapati *et al.* 2002; Shukla *et al.* 2006). Thus, any micromutational changes at different gene loci, causes enhancement of these two alkaloids differentially. Since, papaverine was generally irregular having nil to less value in the individual opium samples, only few individuals in M_3 showed papaverine 4.35% in plant number 9/2/2 in the dose 10 kRad, 5.03% in plant number 3/2/3 in 30 kRad of NBRI-1 and 2.06% in plant no. 8/1/1 of the dose 30 kRad + 0.4% EMS in NBRI-5.

In the present study, selection of elite M_2 families having superior quantitative or qualitative traits were exercised on both non-segregating and segregating M_2 families involving normal looking plants for each treatment based on the earlier studies on pea and lentil (Sharma 1986). The M_3 sub-families raised from individual plant of each elite M_2 family to confirm their stability in M_3 showed very narrow difference among them which suggested that early generation selection at both family and individual plant level is highly dependable in opium poppy. Comparative studies of selection in M_2 and M_3 generations revealed in many cases that the two generations might not be different in respect of selection response (Scossiroli 1968). Tickoo and Jain (1979) and Solanki and Sharma (2001) concluded that promising progenies could be identified with high degree of confidence in M_2 on the basis of mean and variance. The concept of early generation selection has further been probed into and elaborated by Sharma (1986) in leguminous crops viz. pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.)

Apart from demonstrating high reliability and efficiency in early generation (M_2) selection, it has been suggested that the efficiency of early generation selection varies with character i.e. some characters do not show greater response to selection in the advance generation. The observed very high efficiency of M_2 generation over M_3 in terms of contribution to total micro-mutation in the material showed that the metric character like alkaloid content (morphine, codeine, thebaine, narcotine and even papaverine) was endowed with the property of responses in early generation. It is important to state that though selection has not been exercised in M_1, except random culling of the plants, still major portion of transgressed micro-mutation lie only within the selected group of M_1 plant. For this two factors are likely to be responsible. Firstly, the population size in the selected group being more than in the unselected group, so the probability of inclusion of the elite mutants into the former in large number is obvious. Secondly, chance factor may have greatly favoured selected group for inclusion of the elite within it.

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