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EMS induced mutagenesis in green gram for small seeded bruchid resistant genotype

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ABSTRACT

Present investigation was conducted in green gram variety PM5. It is a high yielding, bold seeded as well as highly resistant to bruchid, a devastating coleopteran storage pest. The seeds of that variety were exposed to near LD_{50} concentration of EMS to induce mutation. In comparison to the untreated PM5 plants, different types of phenotypic variations were observed in M_1 generation. In M_2 generation, many putative mutants were identified. Small seededness in few M_2 plants was the most significant finding in the present study. It is noteworthy to mention that, except one plant, rest of those M_2 plants showed decrease in bruchid resistance as revealed by the M_3 seeds feeding assay.

Keywords: Bruchid resistance, EMS, green gram, small seeded putative mutants

After the discoveries of Muller and Stadler, a large amount of genetic variability has been induced by various mutagens and contributed to modern plant breeding. For the past five decades the induced mutation had played a major role in the development of superior plant varieties especially in cereals and pulses. One of the chief advantages of mutation breeding applied to this crop that it can give rise to many different alleles with different degree of trait modifications (Chopra, 2005). Mutation induction can be done on the plants by mutagenic treatment of certain materials of plant reproductive organs such as seeds, stem cutting, pollen, root rhizome, tissue culture and others. One of the important alkylating agents like EMS has recently received much attention as the most effective mutagenic agent in higher plants known today. Studies revealed that EMS is an effective mutagen and has been used to induce genetic variability in a number of crop plants (Jabeen and Mirza, 2002; Kumar and Rai, 2005).

Green gram (*Vigna radiata* L. Wilczek) is one of the most important pulse crops in India. The seeds are excellent source of easily digestible protein (25%) of low flatulence. Sprouted seeds of green gram synthesize vitamin C, and show increased level of riboflavin and thiamine during germination. The present study reports EMS induced mutagenesis of the elite green gram cultivar Pant Mung 5, which was released in 2002; this variety yields 12 to 15 q ha^{-1} (Singh and Khulbe, 2009). Apart from the high yielding trait, this cultivar possesses many other specialties like earliness, bold seededness, and resistance to soil salinity as well as mungbean yellow mosaic virus. More ever, in laboratory experiment, we found PM5 has highly resistant (around 87%) to the devastating coleopteran storage pest bruchid (*Callosobruchus chinensis* L.). Though it is a very popular cultivar throughout India, the people of West Bengal do not prefer it as *daal;* rather it is highly consumed as tadka (curry) due to its bold seededness. Hence, reduction in seed size in PM5 variety could be an economically important trait if we consider West Bengal scenario.

MATERIALS AND METHODS

Preparation of buffer solution: Solution A was prepared by dissolving 27.8 g of sodium dihydrogen phosphate in 1000 ml distilled water and solution B was prepared by dissolving 53.65 g of disodium hydrogen phosphate in 1000 ml distilled water. 390 ml of solution A and 610 ml of solution B were mixed to make 1000 ml phosphate buffer by maintaining the pH 7.

EMS treatment and Determination of LD_{50}: Three hundred thirty selected seeds of PM5 varietys were pre-soaked in sterile distilled water for 6 h before application of chemical mutagen. The chemical mutagen used in the present study was EMS. It is an alkylating agent and its half life at 30°C is 26 hours (SIGMA Leaflet). Freshly prepared EMS solution with the different range of concentration *viz*. 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0% were prepared in phosphate buffer solution by maintaining pH 7.0. These seeds were treated for 4 h at a constant room temperature with intermittent shaking with. The seeds were then thoroughly washed in running tap water for 1 h to remove the adherent chemical mutagen. Both the treated and the untreated

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control seeds were sown in the glass wares immediately after chemical mutagen treatment.

Raising of M_1 generation: After calculation of LD_{50}, 900 seeds of PM5 were treated with specific concentration (near LD_{50}) of EMS. Both treated and untreated (Control) Seeds were sown on 15th February 2018 at District Seed Farm, AB block, Kalyani, BCKV to raise M_1 generation. Proper spacing and recommended packages of practices were followed to raise a good crop stand. Harvesting of seeds was performed from each of the single plant from the M_1 generation.

Raising of M_2 generation: To raise M_2 generation, each single plant seeds of M_1 generation were sown on 19th February, 2019 at the Instructional Farm, Jaguli, BCKV. Harvesting was done properly from each M_2 plants.

Test of bruchid resistance: The bruchid species *C. chinensis* L. was used for phenotypic screening of PM5 genotypes as well as selected putative M_2 mutants PM5 seeds .The insects were reared on susceptible green gram seeds keeping in the glass jars of 12 inches height following the procedure of Sarkar and Bhattacharyya (2014). The insect rearing glass jars were kept in an incubator at 30^0C temperature and 70% relative humidity to maintain stock culture and always fresh seeds were fed to the newly emerged adults for maintaining favourable environment along with continuous insect supply. 50 fresh seeds were kept in a small plastic container separately. Freshly emerged adults of bruchid were taken out from stock culture and male and female insects were chosen on the basis of their antennae type. Five pairs of freshly emerging adult beetles were released into each plastic container and closed with perforated lid for laying of eggs over the induced PM5 seeds following the procedure suggested by Sarkar *et al*. (2011). All the containers were kept in incubator maintaining 30 degree temperature and 70% relative humidity for 20 days. After 20 days, the number of damaged seeds were counted and recorded properly. The damaged seeds were considered as the susceptible one and undamaged seeds as resistant one due to inability of the bruchid larva to feed it and as a result no adult emergence occurred (Sarkar and Bhattacharyya, 2015).

Statistical analysis: MS EXCEL was utilized for estimation of average, standard deviation, standard error of mean. Dendrogram was prepared for classification of 11 putative M_2 mutants along with control plant on the basis of pods per plant, seed per pod, 100 seed weight, single plant yield, pod length, mature pod colour, seed lusture, and seed shape. Squared Euclidian distance between putative mutants was calculated from the standardized data matrix by Unweighted Pair group Method using Arithmetic Averages (UPGMA) method, subsequently the 11 putative M_2 mutants were grouped into different clusters using Statistical analysis system (SAS). Student t-test was conducted for five putative tall M_2 mutants.

RESULTS AND DISCUSSION

LD_{50} calculation: PM5 variety exhibited gradual increase in germination percentage up to 0.5% EMS concentration. Thereafter, germination percentage was reduced with the increase in the concentration of the EMS solution. The data obtained during calculation of LD_{50} is presented in the Table 1. In some EMS concentrations (such as 0.2, 0.3, 0.4, and 0.5%), germination percentage of seeds was higher in comparison to that from control (without EMS). Calculated LD_{50} for PM5 was 0.72% as EMS solution (Figure 2).

Study of M_1 generation: We treated 900 fresh and healthy PM5 seeds with 0.7% EMS solution following the procedure as discussed in materials and method section; another fresh and healthy 100 PM5 seeds were taken as control. A total of 81 plants out of 100 control PM5 seeds germinated and 79 plants out of them survived till the end. On the other hand, 473 M_1 seedlings germinated from 900 seeds (treated with 0.7% EMS solution) and finally, 468 M_1 plants survived till the end. Different types of variations in morphology and behaviour *viz*. purple leaf veins and purple stem, viviparous germination, hypersensitive response in leaves, chimeric leaves, tallness, absence of flower, and deep green leaves were noticed by comparing with the control PM5 plants. The photographs of plants as well as seeds are presented as Figure 3 (a & b).

It was found that one plant had much taller height (53.1 cm) than the control plants those generally had average height of 30.7 cm. This trait might be a dominant mutation or there could be some environmental influences also. The plant was marked for confirmation of the inheritance pattern of tallness in the M_2 generation. It lodged during the pod developmental stage although it bore less number of pods (7 pods) than the control plant. Another single plant was observed among the M_1 plants that possessed purple colour in the leaf veins and stems. This trait also might be dominant. For evaluation in the M_2 generation this particular red plant was also marked. Presence of anthocyanin generally causes the purple colour and this trait might confer the resistance against biotic stresses as that particular red plant shows comparatively robust growth. Apart from those two plants, one viviparous germinated plant was found. Though the seeds get germinated being attached to the plant, seeds cannot be harvested. Another one non-

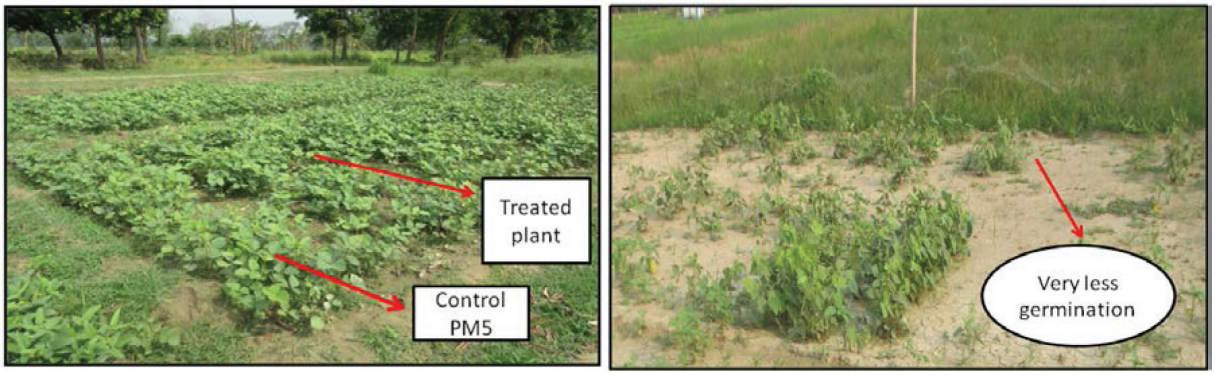

Figure 1(a) M_1 field **(b) M_2 field**

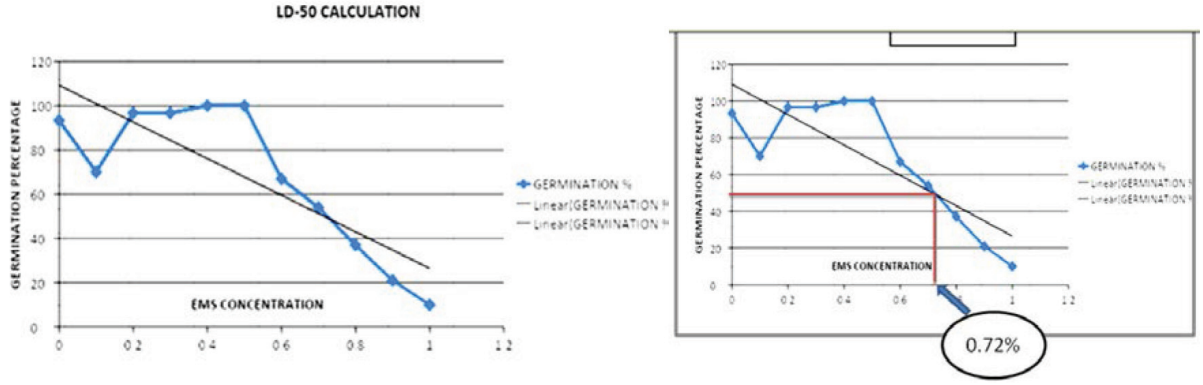

Fig. 2: Calculation of LD_{50}

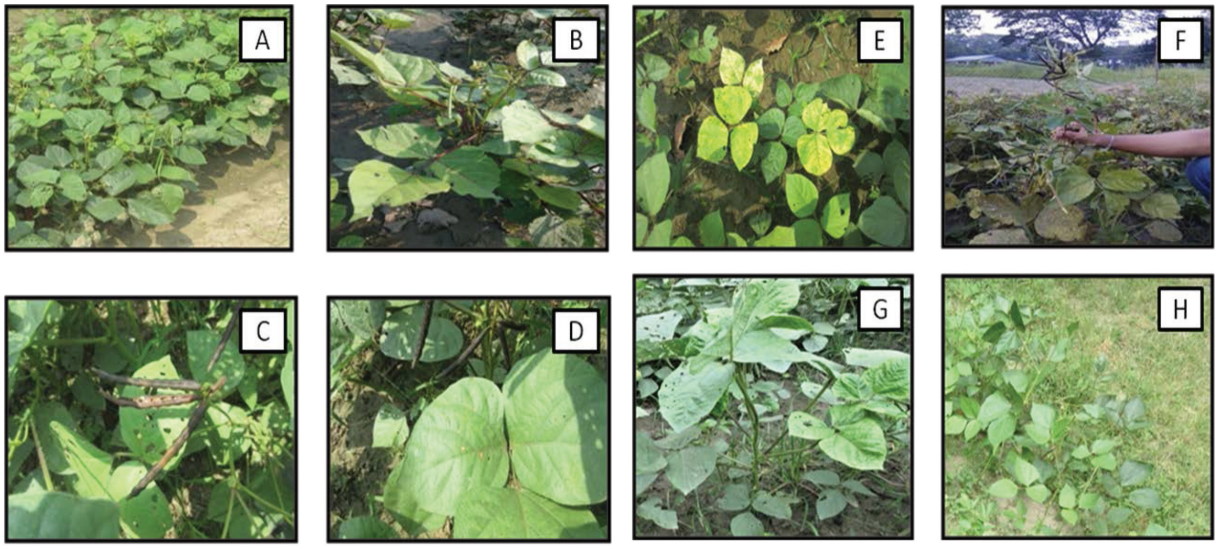

Fig. 3(a): Some mutants identified in M_1 generation

flowering mutant plant was found. This could be useful in forage green gram breeding, but harvesting of M_2 seeds was not possible as it did not flower till the harvesting of the rest plants. Along with this, one hypersensitive mutant, one chimeric mutant and one deep chlorophyll mutant were also observed. Harvesting of pods was conducted from each of the other M_1 plants separately. The pods were stored with proper labelling.

Study of M_2 generation**:** It was found that all the M_1 plants had bore less number of pods than the control plants (as visually screened) and minimum of three or six pods were also harvested from many of them. We finally harvested pods from 466 M_1 plants separately and picked up two intact pods each from the 466 lots; seeds were separated and sown in the field to raise M_2 generation. To maintain the uniformity, approximately

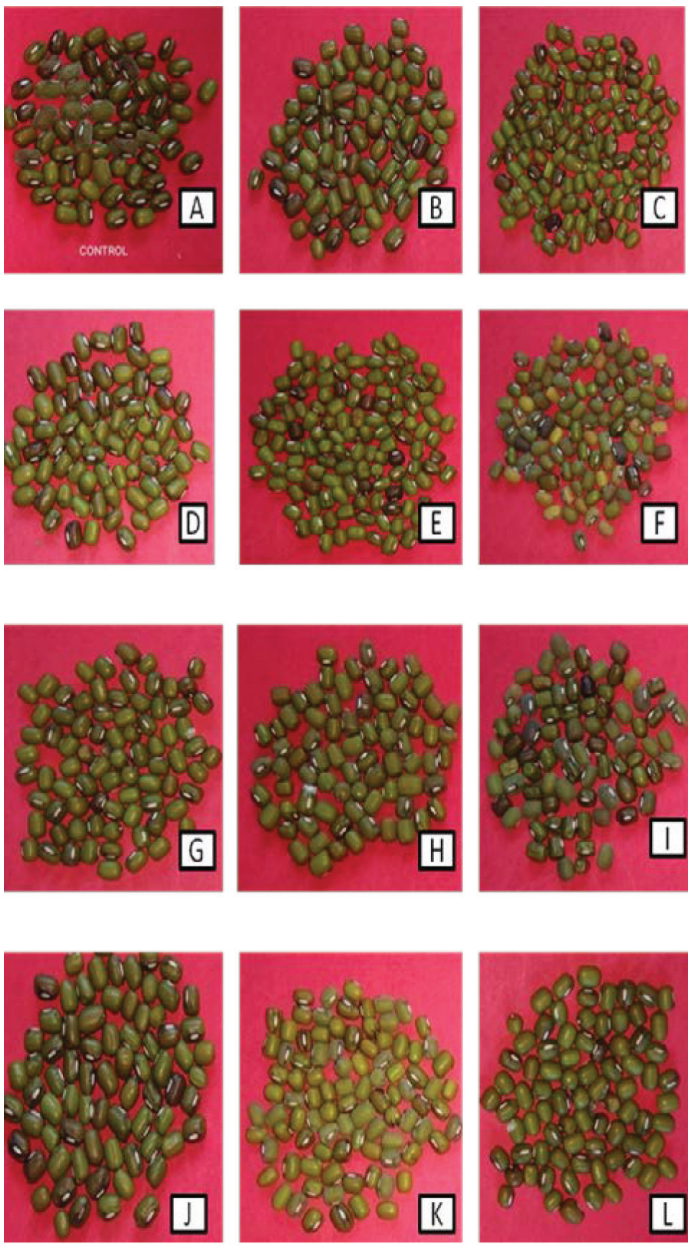

Fig. 3 (b): Seeds of putative small seeded mutants obtained from M_2 plants

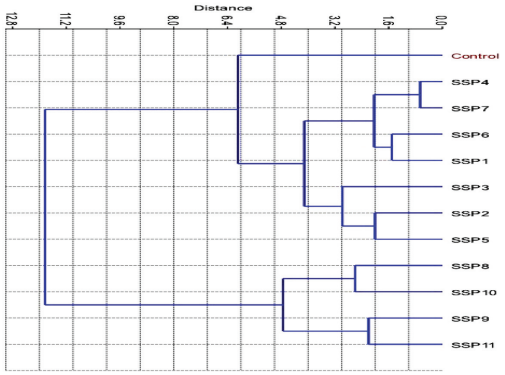

Fig. (4): Dedrogram of mutants

Table 1: Effect of different concentrations of EMS on germination

Concentration of EMS (%)	Germination (%)
0	93.33
0.1	70
0.2	96.67
0.3	96.67
0.4	100
0.5	100
0.6	67
0.7	54
0.8	37
0.9	21
1.0	10

Table 2: Student t test for 5 putative tall mutants

	Tallness		
	Control plants	Mutant plants	
	27.2	53.1	
	31.6	54.2	t value: 11.67
	25.7	52	df: 4
	30.1	61.5	
	32.3	52.6	
Mean	29.38	54.62	
SD	2.840246	3.939162	
Variance	8.067	15.517	

twenty seeds were sown from each of the M_1 seed lot for growing various M_2 families. To study the inheritance pattern of the height and purple pigmentation, we gave special emphasis for the seeds harvested from the tall variant and the anthocyanin variant i.e. the red plant. Few PM5 seeds were also sown to have control plants. The germination percentage was drastically reduced in the M_2 seeds (34.52%) and the number of plants was much lower than that of the sown seeds. Two M_2 families comprising of respectively 11 and 18 plants were survived from the seed lots sown from the tall and the red M_1. Among the M_2 families, excluding those special two, different types of putative mutants with variations in morphology and behaviour *viz*. late maturity, synchronous maturity, upward pod, chimeric leaves were noticed as compared to the control PM5 plants. Two synchronously maturing plants, three plants with upward pod orientation, one chimeric leaf plant and three late matured plants were observed. The synchronous maturity is one of the desirable traits in green gram. Hence this trait should be evaluated during M_3 generation. These two synchronously matured M_2 plants bore smaller number of pods than the control plant but the seed size was bold like the control PM5. Three plants out of 12 were found with purple veins in leaf and stem in the M_2 family. This trait might be a dominant mutation as it was observed in M_1 and also observed in these three M_2 progenies of the red plant. Rest 9 plants had similar leaf and vein colour as control plant. There were 11 plants comprising one M_2 family and they were the progenies of the tall M_1 plant as previously mentioned; out of them five plants were tall (with 53.1, 54.2, 52, 61.5, and 52.6 cm), like that of the M_1 plant (53.1 cm). The control plant generally had average height of 30.54 cm. This trait might be a dominant mutation as it was observed also in M_1. Rest six plants in the same M_2 family exhibited almost similar height like the control PM5. A t-test was conducted to find out whether there was any significant difference between those five plants with their control for the tallness trait. They were also bold seeded and low yielder as observed in our study.

Table 2 shows much higher calculated t-value (11.67) than that of the tabulated value (2.776) and thus we can say that there is significant difference between control plants with the five taller putative mutants. The M_3 seeds were harvested from each of the M_2 plants separately and stored with proper labelling. M_3 seeds were harvested from around 3200 M_2 plants into separate packets. Out of them 18 and 11 M_3 seed packets were from the M_2 plants those were the progenies of the red and tall M_1 plants, respectively. PM5 bears bolder seeds and the seeds showed bruchid resistance. 11 packets with small seeded pods were found. All the seeds from those 11 packets were separated and marked them as SSP1 to SSP11. Hundred seed weight from each of the 11 derivatives was measured following DUS test for green

Table 3: Morphological features of pod, seed and yield attributing traits for the small seeded putative mutants

Small seeded putative mutants	Morphological features of pods and seeds					Yield attributing traits			
	Mature pod colour	Pod size (Average pod length in cm)	Seed coat colour	Seed lustre	Seed shape	100 seed weight (g)	Seed yield (g)	Pods plant^{-1}	Seeds pod^{-1}
SSP 1	Black	Short (7.63)	Green	Shiny	Drum	2.86	6.33	21	10.99
SSP 2	Black	Short (7.02)	Green	Shiny	Drum	2.73	4.96	18	10.44
SSP 3	Black	Medium (8.36)	Green	Shiny	Drum	2.81	4.72	16	10.66
SSP 4	Black	Short (6.36)	Green	Shiny	Drum	2.89	5.76	21	9.66
SSP 5	Black	Short (7.6)	Green	Shiny	Drum	2.58	5.39	19	11.66
SSP 6	Black	Short (7.42)	Green	Shiny	Drum	2.81	6.8	22	11
SSP 7	Black	Short (6.86)	Green	Shiny	Drum	2.91	5.49	21	10
SSP 8	Black	Short (6.52)	Green	Shiny	Drum	2.51	7.22	32	9.6
SSP 9	Black	Short (7.2)	Green	Shiny	Drum	2.96	8.58	29	10.2
SSP 10	Black	Short (7.3)	Green	Shiny	Drum	2.68	8.2	34	10
SSP 11	Black	Short (6.72)	Green	Shiny	Drum	2.42	6.77	28	10
Control (PM5)	Black	Medium (8.36)	Green	Shiny	Drum	5.12	9.01	16	11.2
Mean						2.94	6.6025	23.08333	10.45083
STDEV						0.706657	1.428299	6.141636	0.652373
SEM						0.203994	0.412314	1.772938	0.188324

Table 4: Bruchid feeding assay in PM5 seeds

Replication	Total no. of fresh seed	Toatal no. of seed over which eggs were laid	No. of intact seeds	No. of damaged seeds	Percentage of resistance	Mean percentage of resistance
1	50	50	44	6	88	
2	50	50	42	8	84	87.33
3	50	50	45	5	90	

gram (PPV and FRA, 2007). It was observed that, among these 11, three M_3 seed packets were harvested from the three M_2 plants those were the progenies of the red M_1 plant and marked them as SSP1, SSP2 and SSP3. Mature pod colour, pod curvature, pod length, seed coat colour, seed lustre and seed shape was recorded on the basis of DUS test on green gram (PPV & FRA, 2007), in comparison with the control PM5 pods and seeds; yield attributing traits of them were also recorded (Table 3).

Based on the hundred seed weight it also can be concluded that these 11 lots of M_3 seeds fell under the small category. From the table it is noticed that the PM5 derivative SSP3 bore medium sized pod like PM5. Rest of them bore short sized pods. Seed coat colour in each case was green along with shiny lustre and drum shape as like the control PM5 seeds. Hence on the basis of morphological preference of seeds, these eleven PM5 derivatives are similar to the PM5 seeds, and therefore they could be readily acceptable although evaluation in the M_3 generation has to be performed. Due to its bold

Table 5. Screening of green gram small seeded putative mutant against Bruchid in M_2 generation

Small seeded putative mutants	Total no. of fresh seed used	Total no. of seed over which eggs were laid	No. of intact seeds	No. of damaged seeds	Percentage of resistance
SSP 1	50	50	44	6	88
SSP2	50	50	38	12	76
SSP3	50	50	37	13	74
SSP4	50	50	37	13	74
SSP 5	50	50	40	10	80
SSP6	50	50	33	17	66
SSP 7	50	50	26	24	52
SSP 8	50	50	38	12	76
SSP 9	50	50	34	16	68
SSP 10	50	50	36	14	72
SSP 11	50	50	29	21	58
Control	50	50	43	7	86

seededness, PM5 exhibited higher value in seed yield and hundred seed weight than the derivatives. On the basis of SEm value, it was found that, all the derivatives (except SSP3) showed higher number of pods as compared to control. They could be recommended for future breeding. In case of seeds per pod data, only one plant (SSP5) showed higher value as compared to the control seed. This could be better pre-breeding material for the green gram breeding programme.

Dendrogram represents classification/clustering of 11 small seeded putative mutant along with control plant (PM5) on nine different characters (pod per plant, seed per pod, 100 seed weight, single plant yield, pod length, mature pod colour, pod curvature, seed lustre, and seed shape) based on Squared Eudidian distance matrix (Figure 4).

From the dendrogram, we can conclude that all the putative mutants classified into three different clusters. Cluster having SSP9 and SSP11 were most diverse to control genotype. Control genotype had least distance with SSP4 putative mutant. Hence, it is least diverse to control one. According to the figure the dendrogram shows all the putative mutants were different from each other based on the character considered. Sarkar and Bhattacharyya (2014) found significant and positive correlation between small seed and bruchid resistance. Although PM5 is a bold seeded genotype but the seeds contradictorily exhibited higher bruchid resistance (87.33%) in this study.

CONCLUSION

Small size green gram is very much popular in West Bengal and in our experiment, we are able to produce 11 small seeded putative mutants and among them some are high yielder. Those mutants could be recommended for future breeding. It was also found that two putative mutant (SSP1 and SSP5) exhibited higher resistance, 88% and 80% respectively and could be regarded as bruchid resistant as like as the PM5 seeds.

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