Supaeromaculata Lines in Pea (*Pisum sativum* L.) Obtained Following Mutagenesis

Nadka Naidenova and Roumiana Vassilevska-Ivanova*

Institute of Genetics, Bulgarian Academy of Sciences, Plovdivsko shossee Str., Sofia-1113, Bulgaria

Abstract: Six pea (Pisum sativum L.) mutant lines characterized by more extensive expression of the patches of silver flecking (aeromaculata) were isolated following mutagenesis. Line III/122 demonstrated the strongest expression of the supaeromaculata phenotype; the silver flecks covered large area of leaf surface. The phenotype of 1/221, 2/123, 3/13, 2/1413 and X/3 was expressed clearly but quite weaker than that of III/122 line. The mutants have shown to have single-gene recessive inheritance. Allelism test between mutants and line III/122 (preliminary known to bear the aero1-10 allele) shows that they are not allelic. The mutants 1/221 and 2/1413 were allelic but the alleles were not identical. Therefore, we suggested that the induced mutations are controlled by five different recessive genes. Crosses between mutants produced plants with much stronger supaeromaculata expression than their corresponding mutants. Following selection of some homozygous plants, two new supaeromaculata phenotypes were developed. The pea lines described here are generally considered to represent a diversity of useful materials for research on plant physiology and biochemistry.

Key Words: Mutants, pea, Pisum sativum L., aeromaculata, supaeromaculata, Inheritance.

INTRODUCTION

Irregular gray-green patches of silver flecking (aeromaculata) caused by subepidermal air spaces are common in wild-type pea leaves. This phenotype is known as supaeromaculata [1, 2]. The aeromaculata mutation is characterized by increased silver flecking on leaflets and stipules. Since it is easily induced a set of mutant lines is developed [1, 3-5]. A number of genes that controlled the extent of flecking on the pea leaf are described. The dominant *Fl* gene posses a series of alleles such as Fl^w, Fl^v, *Fl* and *fl*; among them the Fl^w causes the most extensive silver flecking. The *fl* (*flecking*) mutation results in lost of flecking [1, 6]. Pea plants bearing the dominant *Arg* (*Argenteum*) gene have conspicuous grey-green silvery leaves that contrasts sharply with the green of normal plants [7]. Phenotypically, the *Arg* plants distinguished from the *Fl* plants by the degree of silver flecking. The *Fl* gene limited the expression while the *Arg* gene resulted in the expansion of the mutation giving the whole plant grey-green silvery cast leaves [2, 8]. The *Aero1* (*Aeromaculata1*) and *Aero2* (*Aeromaculata2*) genes promote the more extensive areas of flecking (aeromaculata) than in wild-type pea leaves and supaeromaculata phenotype [9, 10]. This phenotype is not uncommon and at least ten *aero1* alleles are known [5, 9]. In contrast, only one *aero2-1* allele has been reported by Murfet and Taylor [10]. These supaeromaculata phenotypes have been included in a number of studies on peas, because the loose epidermis allows fairly easy manipulation of the leaf for absorption studies, and the like. Also, they might be of interest in the light-microscopic studies of host-parasite interaction, in the studies of nutrients and pesticides and also in the physiological and biochemical studies [11-13].

The present work is a portion of a pea research program with the objective of producing and evaluating new germplasm lines isolated after chemical and physical mutagenesis providing useful materials for plant breeding. Several biologically interesting mutants in pea have been realized with improvements including increased yield, lodging resistance (afila leaf trait), larger seeds, increased protein content and modified maturity [14-16]. Also, in pea (*Pisum sativum* L.), mutational approach has been widely exploited in basic research, in particular in the field of seed biology and plant architecture [17]. Detailed characterization of some novel functional legume genes renders pea an ideal candidate for a genomic approach, as TILLING (targeting induced local lesions in genomes), for example [18]. Developing pea collection by mutagenizing *P. sativum* cultivars could be used for both forward and reverse genetics studies, establishing an associated TILLING platform and phenotype database [18].

The observation of aeromaculata mutations is limited and it concerns only a small part of the available mutant lines in different pea collections. Therefore, our objectives for this article are (1) to describe the mutant phenotype of supaeromaculata lines developed at the Institute of Genetics, BAS, (2) to determine the inheritance of the mutant trait, and (3) to evaluate seed productivity of these lines in comparison to wild type.

MATERIALS AND METHODS

Six supaeromaculata lines -1/221, 2/123, 2/1413, 3/13, III/122 and X/3 were used for this work. All the lines were obtained after different type of mutagenic treatment of dry seeds of five pea cultivars (Table **1**). The mutants were iden-

*Address correspondence to this author at the Institute of Genetics, Bulgarian Academy of Sciences, Plovdivsko Shossee Str., Sofia-1113, Bulgaria; E-mail: ru_vas_bg@yahoo.com

1874-8406/09

tified in M_2 and only X/3 was identified in M_3 generation. The mutant phenotype was confirmed in subsequent generations. The pea material was raised in a field at Sofia during

Table 1. Origin of the Induced Mutant Lines

Line	Cultivar	Type of Treatment
1/221	Auralia	100 Gy γ-rays
1/123	Borek	150 Gy γ-rays
2/1413	Borek	100 Gy γ-rays
3/13	9-76	150 Gy γ-rays
III/122*	Virtus	50 Gy γ-rays + DS (0.2 %)
X/3*	Krasnoufimsky	50 Gy γ-rays

*-Seeds kindly provided by Dr. M. Vassileva; DS-dietylsulphate.

March - July of 2005 - 2007. The field had sandy loam soil. Plants were seeded in the field in two rows 2.0 m long spaced 20 cm apart, with two replications. Standard agronomic practices included application of fertilizers, herbicides and insecticides. Plant height (cm), number of pods, number and weight (g) of seeds per plant and thousand seeds weight (g) (TSW) were recorded of 30 random plants from each genotype for a 3 - year period of time. The inheritance data were obtained after reciprocal crosses between mutant lines and parental cultivars. An allelism test between lines was performed as well. Goodness-of- fit of the observed ratio to theoretical ratio was determined by chi-square test [19].

RESULTS AND DISCUSSION

Phenotypic Characteristics

The most obvious feature of mutant lines includes the more extensive expression of the patches of silver flecking on the leaves (aeromaculata), thus characterizing them as supaeromaculata phenotype [1, 2]. There are some very marked similarities as well as differences in silver flecking which enable some of the lines to be grouped together. With regard to the extent of phenotypic expression of the mutant trait, the material can be subdivided into four groups: a/ III/122; b/ 2/123 and 3/13; c/ 2/1413 and X/3 and d/ 1/221.

Supaeromaculata phenotype of III/122 mutant line is clearly visualized by the presence of very extensive expression of the patches of silver flecking on the leaves which covered almost the entirely leaf surface, thus resulting in a silvery appearance (Fig. **1**). III/122 may be easily distinguished from the initial line (cultivar Virtus) and from the other mutant lines described here. Increased flecking occurred still in young seedlings on the second leaf (node 4) but it is more pronounced on the leaflets and stipules at node 5-6. The mutant phenotype remains stable and well visible at various developmental steps during the course of ontogeny till the plant maturity. In addition, flowering in III/122 was strongly promoted in terms of node of flower initiation. The onset of flowering begins earlier (usually within the range of 9 to 11 nodes) than this of the initial cultivar Virtus (14-18 nodes). Also, the mutant plants had reduced stature and formed lower total number of nodes than Virtus (13-17 nodes vs. 18-24). On initial cultivar plants, normally only one lateral branch is produced (rarely two) while III/122 developed one to three basal branches. The branching

Fig. (1). Leaves of cv Auralia and mutant lines III/122, 2/123, 3/13 and 1/221 (from left to right).

resulted in increased number of pods and seeds per plant (Table **2**).

Phenotypically, the lines 2/123 and 3/13 were very similar with their extensive silver flecking on the leaves (Fig. **1**); the grey spots are small and scattered on the whole surface of leaflets and stipules. In younger plants, the increased flecking was clearly evident at node 5 (3-rd expanded leaf) and they are keeping this appearance till the first flower node. After that, the expression of the mutant trait decreased and at the higher nodes it is less extensive. Supaeromaculata phenotype of both lines was much weaker than phenotype of III/122. In addition, a new specific trait characterizing the leaflets appeared in line 2/123; after nodes 6-8 the central veins formed small tendrils (Fig. **2**). Both lines revealed lower seed production; 2/123 had reduced plant height and thicker stem (Table **2**).

The lines 2/1413 and X/3 were characterized by extensive silver flecking which is result from small spots scattered on the leaf surface; they are well visible on third expanded leaf (node 5). The mutant trait is clearly evident on the whole leaflets and stipules and its expression remains stable during ontogenesis. Both supaeromaculata lines were also characterized by changed leaf colour; thus, the leaves of 2/1413 are yellowish-green, a result from chlorophyll mutation *chlorotica* that occurred still at germination stage and remains distinguished during whole vegetation. At early germination stage, the young plants of the line X/3 were very bright yellow. After 3-4 weeks this color changed, so the leaves at lower nodes became light-green while these at the upper nodes became yellowish-green. The line 2/1413 had higher stature with good seed production but the seeds are small with low TSW (Table **2**). The onset of flowering of the line is delayed within 7 to 10 days when compared to the cultivar Borek. Line X/3 had shorter stature than cv Krasnoufimsky, good seed production and higher TSW (Table **2**).

The line 1/221 was characterized by extensive silver flecking only on the stipules surface (Fig. **1**). The mutant trait occurred at node 5 (3-rd expanded leaf) and remains visible during whole ontogenesis. The plants were higher with lower seed production than the initial cultivar Auralia (Table **2**).

Genetic Analysis

Genetic behavior of the induced supaeromaculata mutation was studied by making reciprocal crosses between the mutant lines and the parental cultivars. Table **3** gives the results of crosses. It was observed that all F_1 plants had wild-type phenotype. F_2 from the crosses involved lines 1/221, 2/123, 3/13 and III/122 segregated into wild type and mutant phenotype in 3: 1 ratio (Table **3**). The results indicated that the supaeromaculata mutation was controlled by a single recessive gene. The crosses between 2/1413 and X/3 lines and their initial cultivars Borek and Krasnoufimsky produced F_2 progeny that segregated into 9:3:3:1 ratio, thus revealing the independent inheritance of both mutant traits. These data indicated that two different recessive genes were affected (Table **3**). A diallel crosses between lines were made to elucidate their identity, as well. The crosses between 1/221 and 2/1413 produced F_1 with the mutant phenotype of 1/221 (extensive silver flecking only on the stipules); F_2 segregated into four classes since the line 2/1413 possesses two mutant traits; in terms of flecking the plants segregated into two classes [126 (1/221):34 (2/1413), χ^2=1.20, P >0.25], i. e. the mutant alleles are not identical. In the all other crosses the F_1 plants had normal phenotype which suggests that different recessive genes controlled silver flecking on the leaves. F_2 from crosses between phenotypic similar mutants 2/123 and 3/13 segregated into 9:6:1 ratio (137 normal plants: 76 mutants: 11 double recessive plants, χ^2 = 2.36, P >0.25). The double recessive plants revealed very extensive silver flecking as the spots are large and scattered on the

Table 2. Productivity Elements of the Investigated Mutant Lines

Genotype	Plant Height, cm x ± SE	No of Pods x ± SE	Seeds/Plant		TSW, g
			Number x ± SE	Weight, g x ± SE	
cv Auralia	61.0 ± 1.10	7.8 ± 0.15	34.6 ± 0.74	9.3 ± 0.2	269.1
1/221	66.3 ± 0.99***	6.8 ± 0.16***	26.8 ± 0.62***	6.85 ± 0.17***	254.9
cv Borek	52.6 ± 0.97	8.0 ± 0.16	36.6 ± 0.78	9.95 ± 0.25	271.1
2/123	42.3 ± 0.85***	6.3 ± 0.17***	26.7 ± 0.65***	6.95 ± 0.2***	258.8
2/1413	59.8 ± 0.77***	7.6 ± 0.15	34.8 ± 0.63	6.84 ± 0.14***	196.7
cv 9-76	49.3 ± 0.68	7.0 ± 0.15	33.1 ± 0.77	9.01 ± 0.18	272.4
3/13	47.5 ± 1.00	5.8 ± 0.12***	26.5 ± 0.65***	7.4 ± 0.19***	279.8
cv Virtus	60.7 ± 0.81	6.8 ± 0.22	26.5 ± 0.82	6.02 ± 0.19	227.4
III/122	30.8 ± 0.51***	9.1 ± 0.38***	32.0 ± 1.26**	8.56 ± 0.35***	276.5
cv Krasno	97.7 ± 2.11	8.0 ± 0.21	30.7 ± 0.79	5.41 ± 0.16	176.1
X/3	66.2 ± 2.38***	8.4 ± 0.26	26.2 ± 0.97***	5.18 ± 0.14	199.3

x-mean value for 3 years; TSW-thousand seeds weight; **, ***-P< 0.01 and 0.001, respectively, based on *t*-test; cv Krasno-cv Krasnoufimsky.

Fig. (2). Leaves of cv Borek (left) and mutant line 2/123 (right).

Table 3. Results for F_1 and F_2 of Crosses between Mutant Lines (M) and Parental Cultivars (WT)

Cross	F_1	F_2 Segregation		χ^2	P
		WT	M		
				3:1	
1/221 x Auralia	WT	209	58	1.53	0.25 ÷ 0.10
2/123 x Borek	WT	375	117	0.39	0.75 ÷ 0.50
3/13 x 9-76	WT	307	91	0.97	0.50 ÷ 0.25
III/122 x Virtus	WT	219	71	0.04	0.90 ÷ 0.75
				9:3:3:1	
2/1413 x Borek	WT	287 (WT) : 75 (normal color, more silver flecks): 45 (chlorotica color, normal flecking) : 25 (M)		24.56	< 0.001
X/3 x Krasno	WT	90 (WT) : 23 (normal color, more silver flecks) : 22 (chlorotica color, normal flecking) : 12 (M)		3.38	0.50 ÷ 0.25

cv Krasno=Krasnoufimsky.

whole leaf surface. F_2 progenies from crosses between 1/221 with 2/123, 3/13 and III/122 lines segregated into 9:3:3:1 ratio. The plants from each one line may be easily recognized; the double recessive plants were clearly visualized by their strong phenotypic expression, especially these obtained with III/122 line. After crosses between 1/221 and X/3, F_2 progeny segregated into 96 normal plants: 35 normal green, strongly flecking, 13 (1/221): 17 (chlorotica, nearly normal flecking): 10 (X/3), (χ^2 = 1.71, P >0.75) which fits closely a 9:3:1:2:1 ratio. F_2 from crosses between 2/123 and III/122 segregated into 9:3:3:1, i. e. 163 (normal plants):57 (III/122):43 (2/123):13 (strongly spotting), χ^2 = 3.44, P >0.25; between 3/13 and III/122 into 122 (normal plants) :36 (III/122) :36 (3/13): 10 (strongly spotting), χ^2 =1.31, P >0.50. Six segregation classes with different frequency were observed in F_2 after crossing between 2/1413 and X/3 lines or after involving them in the crosses with lines 2/123, 3/13 and III/122. The segregation ratio in each cross was changeable with enhanced χ^2 value due to the significant plant deficiency in some classes.

In the current study, line III/122 demonstrated the strongest expression of the supaeromaculata phenotype, earlier flowering and maturity. It was established that it carries supaeromaculata mutation allelic to type *aero1-1* allele. III/122 is referred as *aero1-10* and has weaker expression than *aero1-1* [9]. Sidorova and Uzhintseva [5] reported for several supaeromaculata mutants that were allelic but the alleles were not identical. The authors concluded that the gene *Aero1* is represented by a multiallelic series. Later, Taylor and Murfet [9] numbered these alleles from *aero1-2* to *aero1-9*. Also, the authors [10] reported for a new *Aero2* gene in one supaeromaculata mutant whose phenotype expression was weaker both from *aero1-10* and *aero1-1*; that mutant was not allelic with *aero1* and possesses an *aero2-1* allele. In our study, the crosses between III/122 line and the rest other lines gave F_1 plants with normal leaf flecking, thus indicating that supaeromaculata phenotype most probably is controlled by other unknown genes. Visually, the mutant phenotype of 1/221, 2/123, 3/13, 2/1413 and X/3 was expressed clearly but quite weaker than that of III/122 line (*aero1-10*) and also from *aero1-1* and *aero2-1* phenotypes described by Murfet and Taylor [10].

All the six lines described here revealed specific phenotypic features. For example, in 1/221, supaeromaculata mutation affected only the stipules while in 2/123 and 3/13 it appears both on stipules and leaflets at node about first flower; in 2/1413 and X/3, the silver flecking was spread on the leaves of whole plant. It was established that only 1/221 line had supaeromaculata mutation allelic to 2/1413 line but both alleles are not identical. In the rest other lines, mutation is controlled by four different recessive genes. Hence, it may suggest, that five genes are affected by aeromaculata mutation, in presented here lines.

Crosses between mutant lines resulted in segregated plants revealing much stronger supaeromaculata expression than their corresponding lines. For selection of homozygous progeny, part of these plants was reproduced in F_3-F_5. Based on this approach, two lines were obtained: III/240H from cross III/122 x 1/221 and 2/1430H from cross 2/123 x 3/13. Both lines represented new supaeromaculata phenotypes (Fig. **3**). For example, the plants of III/240H have completely gray-green, silvery leaves due to the large and fused flecking covered almost whole leaf surface; these spots occurred still at node 3(first expanded leaf). Among all investigated plant materials, the III/240H has the most pronounced phenotypic expression (Fig. **3**) and visually it is similar to the dominant

Fig. (3). Leaves of lines III/240H (left) and 2/1430H (right).

Arg (Argentum) mutant described by Marx [7, 8]. In line 2/1430H, the grey spots on the leaves were spread on almost whole leaf surface (Fig. **3**) and visually the line certainly had features in common and closest affinity, with the pea *aero2-1* mutant reported by Murfet and Taylor [10].

In conclusion, our results indicate that the silver flecking on the pea leaves is controlled by different from *Aero1* and *Aero2* genes. Therefore, it would be of interest to investigate the aeromaculata mutation using allelism tests with more mutants. The pea lines described here, especially III/240H and 2/1430H are generally considered to represent a diversity of useful materials for research on plant physiology and biochemistry.

REFERENCES

[1] Blixt S. Studies in induced mutations in Peas. VI. Mutations in seed-colour, flower- colour, maculum-colour, pod-colour, and grey spotting of leaves. Agric Hort Genet 1962; 20: 95-100.

[2] Marx GA. Linkage of an aeromaculata mutant on chromosome 1. Pisum Newsl 1986; 18: 42-4.

[3] Blixt S, Erenberg L, Gelin D. Studies of induced mutations in Peas. XVI. Effect of duration of treatment with ethyleneimine and ethylmethansulphonate. Agric Hort Genet 1966; 24: 111-27.

[4] Vassileva M. Studies on the sensitivity to different physical and chemical mutagenic factors and the mutation process in peas (*Pisum sativum* L.). D Sci Thesis, Sofia 1980; (in Bulgarian).

[5] Sidorova KK, Uzhintseva LP. Analysis of induced *supaeromaculata* mutations in pea. Genetika 1995; 31(8): 1177-9 (in Russian).

[6] Blixt S. Mutation genetics in Pisum. Agric Hort Genet 1972; 30(1-4): 1-293.

[7] Marx GA. Argenteum: a mutant under nuclear and experimental control. Pisum Newsl 1978; 10: 34-7.

[8] Marx GA. Argenteum (*Arg*) mutant of Pisum. J Heredity 1982; 73: 413-20.

[9] Tailor SA, Murfet IC. A supaeromaculata mutation affects heterochrony in pea. Physiol Plant 2003; 117: 100-7.

[10] Murfet IC, Taylor SA. The aero2 (aeromaculata2) mutation in pea increases leaf flecking and complexity but, unlike aero1, does not promote flowering. Pisum Genet 2004; 36: 14-9.

[11] Hoch HC, Pratt C, Marx GA. Subepidermal air spaces: basis for the phenotypic expression of the *Argenteum* mutant of *Pisum*. Am J Bot 1980; 67(6): 905-11.

[12] Jewer PC, Incoll LD, Shaw J. Stomatal response of *Argenteum*-a mutant of *Pisum sativum* L. with readily detachable leaf epidermis. Planta 1982; 155(2): 146-53.

[13] Smith S, Weyers JDB, Jewers PC, Höglund HO. Purified guard cell protoplasts from the leaf epidermis of the *Argenteum* mutant of *Pisum sativum*. Pisum Newsl 1990; 22: 55-8.

[14] Swiecicki WK. Mutant cultivars of legumes in Poland. In: Plant Mutation Breeding for Crop Improvement, Proceedings of a Symposium, International Atomic Agency, vol. 1, Vienna, Austria 1991; pp. 419-25.

[15] Naidenova N, Vassilevska-Ivanova R. Lodging resistant pea line derived after mutagenic treatment. Compt Rend ABS 2006; 59 (3): 317-20.

[16] Vassilevska-Ivanova R, Naidenova N. Assessment of the stability and adaptability of waxbloom and waxless pea (*Pisum sativum* L.) mutant lines. Sci Hort 2006; 109: 15-20.

[17] Demason DA, Chawla R. Auxin/gibberillin interactions in pea leaf morphogenesis. Bot J Linn Soc 2006; 150: 45-59.

[18] Dalmais M, Schmidt J, Le Signor C, *et al*. UTILLdb, a *Pisum sativum in silico* forward and reverse genetics tool. Genome Biol 2008; 9: R43doi:10.1186/gb-2008-9-2-r43.

[19] Rokickij P. Biologichaskaja statistika. Minsk, Vuicheichaja skola 1967 (in Russian).

Received: December 18, 2008 Revised: February 4, 2009 Accepted: March 3, 2009