CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

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Summary

Tomatoes are one of the most important crops in the world because of their wide world contribution to human nutrition. Tomato is self-pollinated culture, but a low rate of natural cross-pollination could occur. The co-existence between the conventional, organic and GMO tomato in the future agriculture need application of suitable tools for restriction of pollen flow.

In practice there is no cytoplasmic male sterile system still applied that's why nuclear male sterility is used in tomato seeds production.

In Co-extra project (WP1) both types of male sterility, cytoplasmic and nuclear are used in order to evaluate the existence of pollen flow under tomato field condition, and the stability of nuclear male sterility with practical application. The result obtained will allow formulation of practical recommendations for the farmers and seed producers related to the co-existence between the conventional and GM tomato production.

The stability of **sporogenious and functional nuclear** male sterile lines was investigated under different environmental conditions: South Bulgaria, East Bulgaria and in the green house conditions. Our preliminary observations show that both sterility are stable during the period of tomato seed production (from June 1st ± 5days till July 10th ± 5 days). After this period instabilities in the relation to the sterility trait of the investigated sterile genotypes were observed. It was shown that the environmental conditions could influence the stability of both types of nuclear male sterile lines. This funding could not affect the undesirable gene flow since in practice after the period of seed production the apexes of the plants are cut. This prevents the formation of new inflorescences and speed up the development of the fruits resulting after hand emasculation (if necessary) and pollination. Molecular approaches – RAPD and SSR are in progress to confirm the results.

Cytoplasmic male sterile line developed in AgroBioInstitute resulted from interspecific hybridization between wild tomatos species were used to assess the distance of pollen flow under filed conditions. Cytoplasm male sterile plants as pollen recipient and pollen traps were situated at different distances from the pollen donor. The existence of the vital pollen grins was determined on the pollen traps situated at the distances of 0.8m from the pollen source. At the same time no fruits set were formed on the sterile plants. This discrepancy could be explained with the lack of coincidence of the flowering time of the sterile and fertile plants due to the unfavorable climatic conditions. The experiment is going to be repeated. Molecular studies are in progress in order to genotype the CMS line and the pollinator *L. pennellii*.

1 Introduction

Tomato is predominantly a self-pollinated crop. Natural cross-pollination may occur with percentage ranging from 0.075 to 12% depending on environmental conditions and variety characteristics like stigma position, habitus etc.(Tomassoli et al. 2003). Planting designs, distances, as well as the presence and incidence of pollinating insects and microclimatic conditions during pollination can also influence of the percentage of cross-pollination.

In research done to test the rate of out crossing of cultivated tomato in USA it was demonstrated very low rate between 0-5% (Rick 1992, Scoot 1992). Natural cross-pollination of processing tomatoes in commercial fields in California has been reported to occur at the even lower rates of 0.0007 to 0.4080% (Groenwegen 1990). Iradi and Barba (2002) assessed the flow of pollen from transgenic tomato plants at two different field trials in Italy.

The authors did not detect cross – pollination events from both places. In 2003 Tomassoli and co-workers reported the transgene escape through natural cross - pollination from transgenic tomato carrying coat protein gene from cucumber mosaic virus (CMV), released in an experimental field for evaluating their resistance to CMV. The authors assessed rates of hybridization 0.056% in the fresh market tomato cultivar INB and 3.3% in the typical Italian cultivar San Marzano. Therefore identification of a tool for pollen flow restriction could help farmers to ensure co-existence between conventional and GM tomato plantings in the future.

Male sterility is a phenomenon provoking disturbance in pollen development or pollen release. There are two type of male sterility – cytoplasmic male sterility (CMS) and nuclear male sterility. Cytoplasmic male sterility results from rearrangements in mitochondrial DNA and is a well-studied cause of sterility in other systems, but does not naturally occur in *Lycopersicon* species (Gorman and McCormick 1997). Nuclear male sterility is of wild occurrence in tomato. There are tree types of nuclear male sterility (Kaul 1988): functional, structural and sporogenious. Functional mutants produce viable pollen but have defects in flower structure that prevent effective pollen release. Structural mutants completely lack the pollen-bearing organs, or stamens, or have defects in stamen form so severe that pollen is only rarely produced. Sporogenious mutants produce no or scarcity of viable pollen.

Both types of male sterility cytoplsmic and nuclear are object of our investigations aimed pollen flow study and stability of the sterility trait in respect of the different environments.

2 Cytoplasmic male sterility

The cytoplasmic male sterile line CMS – *pennellii* was produced as a hybrid between *L. peruvianum* and autotetraployd *L. pennellii*. After several backcross with the pollinating plant the hybrid in BC3P2 generation was completely sterile (Vulkova-Achkova 1980). The anthers of CMS – *pennellii* are 3mm in length and do not contain fertile pollen. CMS – *pennellii* and *L. pennellii* are morphologically identical and do not have any reproductive barriers.
During the first project year CMS – *pennellii* as pollen acceptor and *L. pennellii* as pollinator donor were planted in order to evaluate the pollen flow under field conditions. Two approaches were used:

- Assessment of the fruit formation on the recipient sterile plants

- Assessment of pollen transfer by pollen traps.

Because of the unfavorable climatic conditions the flowering period of pollen donor and pollen acceptor plants was in discrepancies. As a result no fruit set were detected on the sterile plants.

Pollen flow was assessed by pollen traps, situated on each row of the CMS plants at 0.8 m, 1.6 m, 2.4 m and 3.2 m distances from the pollen donor in all four directions. The number of trapped pollen grains was determined directly on the traps by a light microscope at magnification of 100X, using 1% acetocarmine staining procedure.

Our investigations showed presence of trapped *L. pennellii* pollen grains at 0.8 m distance from the source, situated northwest corresponding to the predominant wind direction.

The uses of this type of pollen traps can be successfully applied in studies of pollen flow control and can be implemented in tomato pollen mitigation studies. Even within the small scale of our experiment, it proved to be a good indicator of distribution of pollen for this important cultivated species.

Because of the existing incompatibility between the cultivated tomato *L. esculentum* and its wild relatives the transfer of CMS trait on *L. esculentum* and its practical application is still under study. The strategy for developing CMS in the cultivated tomato is discussed in the revue paper "Towards cytoplasmic male sterility in cultivated tomato" (Stoeva P. et al 2007).
In the frame of Co-extra project our studies are concentrated on the identification of molecular markers suitable for the application of a paternity test of the CMS – *pennellii* line. RAPD and SSR markers are used in order to distinguish CMS line from the pollinator *L. pennellii.*

3 Nuclear male sterility

During the second year project two nuclear male sterility lines with practical application in tomato seed production in Bulgaria and in some other EU countries like Czech Republic and Moldova were included. It would be important to note that during the last 15 years about 80% of the tomato hybrid varieties developed in Bulgaria is based on functional sterility conferred by the *ps 2* gene.

Functional male sterility is an important trait for the production of hybrid seeds. Among the genes coding for functional male sterility in tomato is the positional sterility gene *ps-2*. *ps-2* is monogenic recessive gene, confers non-dehiscent anthers and is one of the most suitable types of nuclear male sterility for practical uses in tomato (Gorguet B,et al. 2006). The ps-2 sterility is due to structural alterations in the zone of anther dehiscence. It was found that the endothecial cells in this zone were poorly developed and the power necessary for anther rupture could not be provided, that resulted in indehiscent anthers (Atanassova 1999). A number of studies on *ps*2 sterile lines provided evidence that occasionally the retention of the pollen was not totally consistent and as a result undesirable self pollination was observed (Philouze 1978, Atanassova and Georgiev, 1986). This problem was resolved by developing the line St 993 – ps2, which possessed a short style and the emasculation, can be applied without forceps (Georgiev and Atanassova, 1981; Atanassova and Georgiev 1986). Comparative study on hybrid seed yield provided evidence that the significantly higher hybrid seed yield obtained when using ps2 lines as female parents might be considered as main advantage in using this type of sterility in hybrid seed production (Atanassova B. 1999).

Because of the practical application of the functional sterility in tomato seed production in Bulgaria the following tasks were initiated:

- Assessment of the stability of the ps-2 type of sterility under different environmental conditions.
- Assessment of the capacity of ps-2 sterility as pollen flow sources

The line ps-2 possesses marker gene potato leaf (c). Usually the male sterile seed parents possess marker genes (potato leaf (c) or absence of anthocyanin) that facilitate the determination of the percentage of hybridity of the seed produced, as this characteristic is ranked of primary importance for hybrid seed.

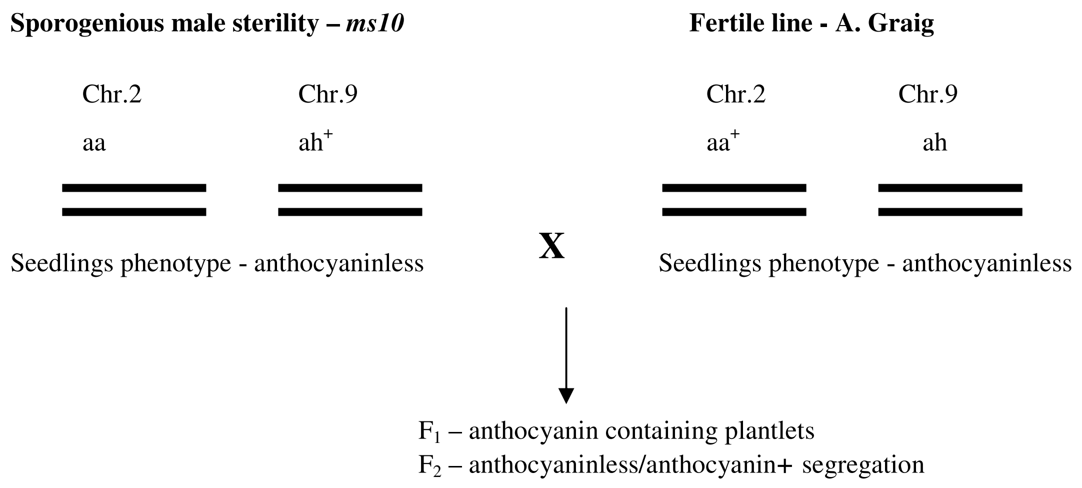
Sporogenious male sterile (M *ms 10 aa*)

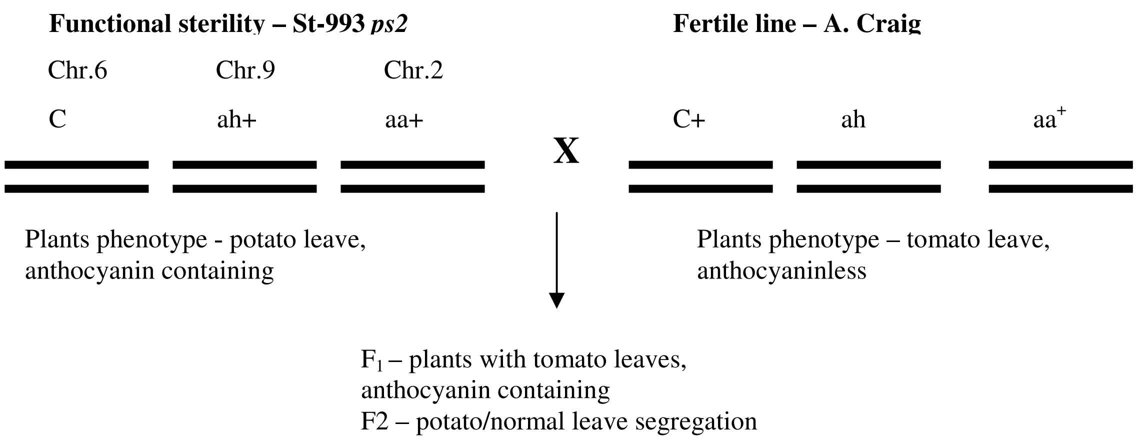
This type of sterility is controlled by gene male sterile 10 (*ms 10*) and has no viable pollen. The male sterile (*ms)* and stamenless (*sl*) mutants seem to be the most applicable in breeding programs mainly because of their complete male sterility and accessible stigma (Gorman and McCormick 1997). The line M *ms 10 aa* is maintained as a population of sterile and fertile plants. This disadvantage is eliminated by developing *ms10* ^{35} *aa* genotypes in which is possible to asses the sterile plants, as they are anthocyaninless and easy to be distinguished since early developmental stages. Gene *ms 10* is closely linked to gene *aa*. Because of anther deformation, some flowers exhibit exerted stigma, i.e., accessible for pollination without emasculation.

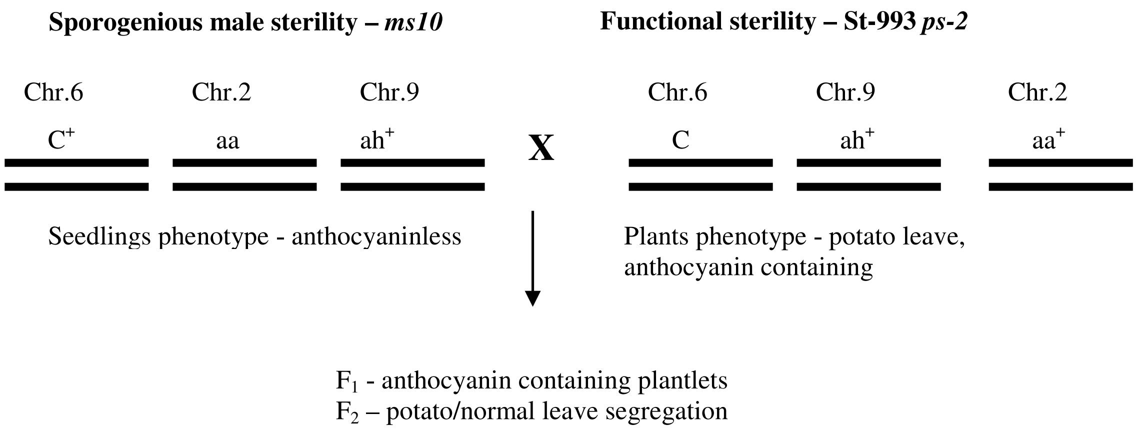
Ailsa Criag *ah*
The line is completely fertile possessing recessive marker gene *ah* (anthocyaninless of Hoffmann). The line was included in the study in order to investigate the eventual occurrence of cross pollination between the three lines.

Scheme: Morphological markers application

The genotypes used possessed marker genes which are not allelic and permit easy evaluation of the seed resulting from self or cross pollination.







Field trials

Two field trials were organized – one in the South of Bulgaria, near Stamboliiski town and one in the region of the Sofia plane – the East part of Bulgaria, at $130m^2$. The trial under plastic tunnel conditions was organized in the experimental field of ABI near by the city of Sofia at $30m^2$.

In Bulgaria the period of hybrid seed production lasts approximately 40-50 days, (from June 1st ± 5days – July 10th ± 5 days), the date of beginning and ending of the process depending mainly on the climatic conditions of the region. In practice, after the end of the process of seed production the apex of the plants have to be cut in order to avoid the formation of new inflorescences and to speed up the development of the fruits resulting from hand emasculation (if necessary) and pollination. In order to get larger information on gene flow and the stability of the sterility genotype we examined the sterile plants until September 10th. The results for both periods are presented separately: examination of the stability of the sterility during the period of seed production (June 1st - July 20th) and after the period of seed production (July 20th – September 10th).

Periodically, number of flowers and flowers bud per plant were counted. On Jully 20th number of fruits set per plant was evaluated and after maturity these fruits were collected and investigated for presence of seeds. If present, seeds from each plant and each fruit were individually collected for further determination of their genotype (that could be resulting from self – or cross fertilization). The same procedure was repeated during the period July 20th – September 10th

The seeds collected were planted and their genotype was determined based on the marker genes possessed by each one of the three lines. Molecular studies are in progress in order to evaluate the pollen flow source, and to confirm cross or self pollination nature of F_1 progeny.

4 Results

4.1 Stability of the sterility during the period of hybrid seed production (June 1st - July 20th)

During the period of hybrid seed production the performance of the sterility in both lines, possessing two different genes controlling male sterility can be determined as stable, regardless of the region (tabl.1-3). The mean temperatures during the period of observation in Stamboliiski region, South of Bulgaria were significantly higher than those in Sofia region. For example for the forth decade (June 21-30) the temperatures measured were 26.8 ºC and 22.1ºC respectively. It has to be underlined that the performance of the sterile lines is of great importance for the South of Bulgaria - Stamboliiski region, as it is the region of hybrid seed production in Bulgaria.

During the reported period both male sterile lines formed seedless, parthenocarpic fruits. The percentage obtained being significantly high in the line M *ms 10*. A number of studies provided evidence that due to unfavorable climatic conditions, including low temperatures during anthesis, tomato plants set seedless fruits, their percentage varying depending on the genotype. It would be important to note that in Sofia, under field conditions more than 20% of the fruits of the fertile line Ailsa Craig *ah*, set until the end of June were parthenocarpic, as well.

Table1. Stability of *ms 10* and *ps 2* sterile lines after the end of seed production period under the conditions of South Bulgaria

Location – experimental fields - Stamboliiski, South Bulgaria					
Mean temperatures during the five decades of observations					
23.5±2.6 C° May 21-31	16.0±2.1 C° June 1-10	21.6±4.7 C° June 11-20	26.8±1.9 C° June 21-30	21.8±2.4 C° July 1-10	23.1±1.6 C° July 11-20
Genotype	Number of plants	Number of flowers	Number of parthenocarpic (seedless) fruits	% (number of part. fruits/total number of flowers)	Number of fruits containing seeds
M *ms 10*	39	1721	404	23.0	0
St 993 *ps 2*	36	7038	88	1.2	0
A. Craig *ah* – fertile line	36	964	0		964

Table 2. Stability of *ms 10* and *ps 2* sterile lines after the end of seed production period under the conditions of plastic tunnel

Location – Kubratovo, near Sofia, plastic tunnel					
Mean temperatures during the five decades of observations					
20.1±4. 1 C° May 21-31	13.9±1.9 C° June 1-10	17.9±4.2 C° June 11-20	22.1±3.8 C° June 21-30	18.9±2.0 C° July 1-10	19.6±1.5 C° July 11-20
Genotype	Number of plants	Number of flowers	Number of parthenocarpic (seedless) fruits	% (number of part. fruits/total number of fruits)	Number of fruits containing seeds
M *ms 10*	26	611	168	25.0	0
St 993 *ps 2*	18	1647	25	1.5	0
A. Craig *ah* – fertile line	20	724	34		690

Table 3. Stability of *ms 10* and *ps 2* sterile lines after the end of seed production period under the conditions of East Bulgaria

Location – experimental fields – Sofia, open fields					
Mean temperatures during the five decades of observations					
20.1±4. 1 C° May 21-31	13.9±1.9 C° June 1-10	17.9±4.2 C° June 11-20	22.1±3.8 C° June 21-30	18.9±2.0 C° July 1-10	19.6±1.5 C° July 11-20
Genotype	Number of plants	Number of flowers	Number of parthenocarpic	% (number of part.	Number of fruits

			(seedless) fruits	fruits/total number of flowers)	containing seeds
M *ms 10*	50	1071	109	10.2	0
St 993 *ps 2*	50	2370	125	5.3	0
A. Craig *ah* – fertile line	50	985•	256	26.0	347•

•Percentage of fruit set in Ailsa Craig – 61.0%

4.2 Stability of the sterility after the period of hybrid seed production (July 21st - September 10th)

After the period of hybrid seed production instabilities in the performance of the sterile genotypes were observed. Results from the field trials in Sofia are not presented since due to the hail storm on June 30th no observations were carried out until July 10th .

Both sterile genotypes formed not only parthenocarpic, but also fruits containing seeds. The number of fruits with seeds formed on line *ps 2* were 19 (from 7 plants) from the Kubratovo trial and 16 (from 10 plants) from the Stamboliiski trial. The percentage of fruit set calculated per total number of flowers is 1.9 % in Kubratovo and 0.3% in Stamboliiski respectively (tabl.4,5). The data collected on the base of phenotypic markers performance, show that all seeds harvest and tested in this line, resulted from self-pollination. Studies on the expressivity of *ps 2* gene have shown that as a main cause for anther dehiscence could be considered the high temperatures during the end of July and during the month of August. Molecular studies are in progress (RAPD and SSR markers are applied) in order to confirm the self-pollination origin of the collected seeds.

The number of fruits with seeds obtained in *ms10* line were 3 (formed on 3 plants) planted in Stamboliiski and 6 (formed on 4 plants) planted in Kubratovo. The percentage of fruit set is 0.2% and 1.4% respectively calculated per total number of flowers (tables 4 and 5). All seeds harvest from the Stamboliiski region resulted from cross-pollination. The occurrence of cross-pollination in *ms 10* is probably due to the exerted stigma of the flowers that is easily accessible for wind blown pollen or pollen transported by insects from other flowers (fig.1).
In Kubratovo the percentage of cross - pollination detected for *ms10* line is 90.6%. Under this type of environmental conditions (plastic tunnel) only one fruit with 8 seeds resulting from self-pollination was obtained. The percentage of self-pollination is 9.3 evaluated on the basis of the total seed number obtained. This percentage is 5.8, calculated on the basis of the sum of seeds obtained from both locations Kubratovo and Stamboliiski (tables 4 and 5).

Molecular studies are in progress in order to evaluate the genotypes of all F_1 plants resulting from cross pollination from both locations. RAPD and SSR markers are applied.

Table 4. Stability of *ms 10* and *ps 2* sterile lines after the end of seed production period under the conditions of South Bulgaria.

Location – experimental fields - Stamboliiski, South Bulgaria					
Mean temperatures during the five decades of observations					
23.1±1.6 C° July 11-20	26.1±1.5 C° July 21-31	24.9±3.5 C° August 1-10	26.9±0.9 C° August 11-20	22.8.±2.4 C° August 21-31	20.8.±2.7 C° September 1-10

Genotype	Number of plants	Number of flowers	Number of parthenocarpic (seedless) fruits	% (number of part. fruits/total number of flowers)	Number of fruits containing seeds	% (number of fruits with seeds /total number of flowers)	Total number of seeds	Number of fruits and number of seeds resulting from selfing	% Seeds resulting from selfing/ total number of seeds	Number of fruits and number of seeds resulting from cross pollination	% Seeds resulting from cross pollination/ total number of seeds
M *ms 10*	39	1412	54	3.80	3	0.20	50	0	0	3 / 50	100.00
St 993 *ps 2*	36	5042	137	2.70	16	0.30	312	16 /312	100.00	0	0
A. Craig *ah* – fertile line	36	426	0		426				100.00		

Table 5. Stability of *ms 10* and *ps 2* sterile lines after the end of seed production period under the conditions of plastic tunnel.

Location – Kubratovo, near Sofia, plastic tunnel											
Mean temperatures during the five decades of observations											
19.6±1.5 C° July 11-20		21.7±2.0 C° July 21-31		20.5±3.4 C° August 1-10		22.2±1.7 C° August 11-20		20.4±2.3 C° August 21-31		19.2±1.6 C° September 1-10	
Genotype	Number of plants	Number of flowers	Number of parthenocarpic (seedless) fruits	% (number of part. fruits/ total number of flowers)	Number of fruits containing seeds	% (number of fruits with seeds /total number of flowers	Total number of seeds	Number of fruits and number of seeds resulting from selfing	% Seeds resulting from selfing/ total number of seeds	Number of fruits and number of seeds resulting from cross pollination	% Seeds resulting from cross pollination/ total number of seeds
M *ms 10*	26	432	110	25.0	6	1.4	86	1 / 8	9.3	5 / 78	90.6
St 993 *ps 2*	18	984	416	42.0	19	1.9	236	19/ 236	100.00	0	0
A. Cra	20	495	24	4.80	471	96.20	--	---	100.00	0	0

ig *ah* – fertile line											

Fig.1 – Exerted stigma on *ms10* male sterility tomato line

5 Conclusion:

Application of male sterile seed parent in tomato seed production reducing the time and cost associated with hand emasculation. Several requirements must be met by male sterile plants that are to be used in breeding programs, as:

- The plants must be completely male sterile;
- The female fertility must be normal;
- The male sterile trait must be totally recessive

According to the above mention points and our preliminary results it could be concluded that the sporogenous type of sterility is preferable as an approach to limit pollen flow because of the absence of pollen and scarcity of sterility restoration. During the following season the large scale filed trials will be organized in both locations - South and East Bulgaria, in order to evaluate the rate of the fertility reverse.

Functional male sterility (*ps*-2) is preferred by many breeders and seed producers for a number of reasons as easy maintenance of the sterile lines, high hybrid seed productivity etc.
During the following season we are planning to investigate the percentage of anther dehiscence in *ps* 2 genotype under the environments of the South and East Bulgaria. The duration of the pollen viability originating from *ps*-2 in comparison to the fertile line will be assessed. The capacity of *ps-2* line as pollen donor will be evaluated by studies of the segregation in F_2 progeny resulting by cross - pollination on ms10 line.

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