Journal of Agricultural Science; Vol. 8, No. 2; 2016
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Development and Fertility Restoration of CMS Eggplant Lines Carrying the Cytoplasm of *Solanum violaceum*

Konstantinos S. Krommydas[1], Zisis Tzikalios[1], Panagiotis Madesis[2], Fotios A. Bletsos[3], Athanasios Mavromatis[1] & Demetrios G. Roupakias[1]

[1] Laboratory of Plant Breeding, Department of Field Crops and Ecology, School of Agriculture, Faculty of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, Thessaloniki, Greece

[2] Institute of Applied Biosciences, CERTH, Thermi, Thessaloniki, Greece

[3] Department of Vegetables, Hellenic Agricultural Organization "Demeter", Thermi, Thessaloniki, Greece

Correspondence: Konstantinos S. Krommydas, Laboratory of Plant Breeding, Department of Field Crops and Ecology, School of Agriculture, Faculty of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, Thessaloniki, Greece. Tel: 30-6947-0064. E-mail: kos_krom@hotmail.com

Received: November 9, 2015 Accepted: December 8, 2015 Online Published: January 15, 2016

doi:10.5539/jas.v8n2p10 URL: http://dx.doi.org/10.5539/jas.v8n2p10

Abstract

A functional cytoplasmic male sterile (CMS) eggplant line carrying the cytoplasm of *Solanum violaceum* was developed in the past, but the fertility restoring genes (*Rf*-genes) were not identified. This work aimed to produce the CMS lines of three Hellenic eggplant cultivars (viz., 'Langada', 'Emi' and 'Tsakoniki') using the cytoplasm of *S. violaceum* and study the inheritance of the *Rf*-genes. The respective CMS eggplant lines were developed by the backcross method and examined for their fertility parameters. The results demonstrated that female fertility was not affected by the cytoplasm of *S. violaceum*. In contrast, the occurrence of three male fertility phenotypes (male sterile, male fertile and potentially male fertile) indicated that male fertility was affected by nuclear/cytoplasmic interactions. Male sterile plants were characterized by indehiscent anthers, low pollen viability and abnormal anther morphology. Male fertile plants formed dehiscent anthers with high pollen viability and normal morphology. Potentially male fertile plants initially formed dehiscent anthers, but in later stages formed exclusively indehiscent anthers. Male fertile plants were obtained in the advanced backcross populations of CMS 'Tsakoniki', but not in CMS 'Langada' and CMS 'Emi'. The genetic analysis of fertility restoration indicated that male fertility in the genetic background of cv. 'Tsakoniki' is controlled by one essential genetic locus, affected by a secondary modifying locus. Molecular analysis of cp-DNA and mt-DNA in the CMS lines indicated maternal inheritance of the cytoplasm organelles. Our findings demonstrate that the genotype of the eggplant parent can affect the expression of CMS as well as fertility restoration.

Keywords: alloplasmy, anther morphology, *Rf*-genes, *Solanum melongena*

1. Introduction

Eggplant (*Solanum melongena* L., *Solanaceae*) is an Old World vegetable crop of significant economic importance and its fruits are worldwide marketed for fresh consumption or processed. Today, commercial F_1 hybrids are widely used in eggplant cultivation because they exhibit heterosis in several agronomical traits including earliness and yield (Kakizaki, 1931; Rodríguez, Prohens, & Nuez, 2008; Sambandam, 1962). In the 2015 European catalogue of registered varieties, more than 75.0% of the eggplant entries were F_1 hybrids (European Commission, 2015). Since the eggplant flower is androgynous, emasculation and hand pollination are required in hybrid seed production, resulting in a higher seed cost. A strategy to reduce that cost is to use eggplant male sterile lines as female parents in hybrid seed production. In these lines flower emasculation is not necessary and pollination can be achieved manually or with insect pollinators.

In eggplant, a number of genic (Chauhan, 1984; Nuttall, 1963; Phatak, Liu, Jaworski, & Sultanbawa, 1991), cytoplasmic (Fang, Mao, & Xie, 1985; Hasnunnahar, Khan, & Isshiki, 2012; Isshiki & Kawajiri, 2002; Khan & Isshiki, 2011) and genetically engineered (Cao, Huang, Chen, & Lei, 2010; Toppino & Kooiker, 2011) male sterility systems were developed. Genic male sterility has a limited practical application because of its complex mode of maintenance and utilization (Budar & Pelletier, 2001). Besides, there is some evidence that expression

of genic male sterility in eggplant is affected by the environmental conditions (Hazra, Roy, & Choudhury, 2008). In addition, the cultivation of genetic engineered crops for human consumption is not widely accepted in many countries and has received mixed public acceptance, especially in the EU (Costa-Font, Gil, & Traill, 2008). On the other hand, the maintenance of CMS and their utilization in hybrid seed production is preferable because of the maternal inheritance of the male sterile character and the mode of fertility restoration (Budar & Pelletier, 2001). Therefore, the development of reliable CMS systems accompanied by genes that restore fertility (*Rf*-genes) is an efficient way to reduce the cost of eggplant hybrid seed.

Previous studies demonstrated that a number of wild species related to eggplant can serve as cytoplasm donors in the development of eggplant CMS lines of the alloplasmic type. Alloplasmic eggplant lines were obtained by substituting the eggplant cytoplasm with the cytoplasms of *S. gilo*, *S. violaceum*, *S. virginianum*, *S. kurzii*, *S. integrifolium*, *S. anguivi* and *S. grandifolium* (Fang et al., 1985; Hasnunnahar et al., 2012; Isshiki & Kawajiri, 2002; Khan & Isshiki, 2008, 2009, 2010, 2011; Saito et al., 2009).

The identification and incorporation of the appropriate *Rf*-genes in the male parent is essential for the effective utilization of an eggplant CMS line in a commercial hybridization system. To the best of our knowledge, the *Rf*-genes are available only in the pollen-non formation eggplant CMS lines carrying the cytoplasms of *S. grandifolium*, *S. integrifolium* and *S. anguivi* (Hasnunnahar et al., 2012; Khan & Isshiki, 2010, 2011; Saito et al., 2009). Taking into account that long term utilization of limited cytoplasm sources in eggplant cultivation has the potential risk of genetic vulnerability against new pathogens and pests (Khan & Isshiki, 2008), the identification of the respective *Rf*-genes in the rest of CMS eggplant lines could be beneficial for both practical and research purposes.

The aim of the present study was: firstly, to develop and study the CMS lines of the Hellenic eggplant cultivars ‘Langada’, ‘Emi’ and ‘Tsakoniki’ by utilizing the cytoplasm of *S. violaceum*, and secondly, to study the inheritance of the *Rf*-genes for this type of CMS.

2. Materials and Methods

2.1 Plant Material

Three Hellenic eggplant cultivars viz., ‘Langada’ (L), ‘Emi’ (E) and ‘Tsakoniki’ (T), were used as recurrent parents (nucleus donors) and the wild species *Solanum violaceum* (V) was used as maternal parent (cytoplasm donor) for the development of the respective eggplant CMS lines (Figure 1). The aforementioned cultivars were selected because they are of significant economic importance and are highly appreciated in the local market. Briefly, interspecific crosses were made using the wild species as the female parent and the eggplant cultivars as the male parents. The resulting interspecific hybrids viz., F_1(VxL), F_1(VxE) and F_1(VxT) were continuously backcrossed to their respective eggplant parent and five backcross generations were produced for each hybrid. Progenies obtained after self-pollination of individual male fertile plants were also cultivated and used in the genetic analysis of fertility restoration. The experiments were carried out following standard cultural practices in the experimental fields of Hellenic Agricultural Organization ‘Demeter’ (formerly, National Agricultural Research Foundation) located in Thermi, Thessaloniki.

2.2 Female Fertility

Female fertility of the backcross progenies was assessed by investigating the seed germination rate, percentage of successful backcrosses and number of seeds per fruit. Backcross seed germination rate was examined in eight plants of each backcross population, with the exception of BC_1 where one sample of 200 seeds was used per population. One hundred seeds per plant were sown in plastic pots in a heated glasshouse and the germination rates were recorded four weeks later. For investigating the percentage of successful backcrosses, three to five flowers per plant were emasculated and hand pollinated with fresh eggplant pollen and the percentage of successful backcrosses was calculated in each population. In addition, each year 12 flowers of each eggplant cultivar were emasculated and manually self-pollinated using pollen of the same cultivar. Average number of seeds per fruit was examined in eight randomly selected plants of each backcross population by counting the number of seeds per fruit.

2.3 Male Fertility

Male fertility was evaluated by pollen release ability, anther morphology and pollen viability examination in the first 10 flowers per plant. Pollen release ability was investigated by observing the terminal pore end of the anther in freshly opened flowers under a stereomicroscope. The plant material was classified as male fertile (MF), potentially male fertile (PMF) or male sterile (MS) according to the ability of the anther to release its pollen at the stage of anthesis. The observed frequencies of each class were recorded and chi-square (χ^2) goodness-of-fit

test was used for the statistical analysis. Since, the presence of three fertility phenotypes, including a potentially male fertile one, indicated the action of a major genetic locus plus at least one modifying locus, the observed frequencies were compared to the appropriate expected frequencies.

For the examination of anther morphology, anthers from 3 freshly opened flowers per plant were visually observed and classified as normal (having typical size and appearance), petaloid (transformed to petal like structures) or atrophic (having reduced size and dark brown color) and the observed frequency of each class was recorded. Pollen viability was estimated by the acetocarmine staining method. Concisely, anthers of the plant material were squashed in a 2% acetocarmine solution and placed under a light microscope (Qureshi, Khan, Arshad, Rashid, & Ahmad, 2009). More than 300 pollen grains per plant were observed with three replications and the percentage of viable (stained) pollen grains was calculated. Abnormal anthers were excluded from pollen release ability and pollen viability examination because it was preliminary confirmed that they did not contain pollen grains.

2.4 Cytoplasm Inheritance

The inheritance of the cytoplasm in the three CMS eggplant lines was confirmed by chloroplast (cp) and mitochondrial (mt) DNA analysis. Total DNA was isolated from fresh leaves by using the CTAB method (Doyle & Doyle, 1987) from 3 individuals of the following plant material: *S. violaceum*, cv. 'Langada', cv. 'Emi', cv. 'Tsakoniki', MS plants from populations $BC_4(V\times L)^6$, $BC_4(V\times E)^{14}$ and $BC_4(V\times T)^{24a}$ and MS and MF plants from population $BC_3(V\times T)S_1^{26}$ (Figure 1). High resolution melt (HRM) analysis and sequencing of the chloroplast *trn*L intron were employed in order to study the inheritance of cp-DNA. The normalized HRM curves of *trn*L amplicons were generated from the examined plants by using the Ph-*trn*L oligonucleotide set following the procedure described by Madesis, Ganopoulos, Argiriou, and Tsaftaris (2012) and used to discriminate the different genotypes. In addition, the chloroplast *trn*L intron was sequenced by using the set of primers F:CGAAATCGGTAGACGCTACG and R:GGGGATAGAGGGACTTGAAC in both directions and the obtained sequences were compared. Mitochondrial DNA was analyzed by RFLP analysis according to Khan & Isshiki (2008). Briefly, PCR amplified fragments of nad7/3-4 region and V7 region of mitochondrial small ribosomal subunit RNA (srRNA) gene were digested with *Alu*I and *Scr*FI, respectively. The resulting DNA restriction fragments were separated on a 1.5% agarose gel containing ethidium bromide and photographs were taken under UV illumination.

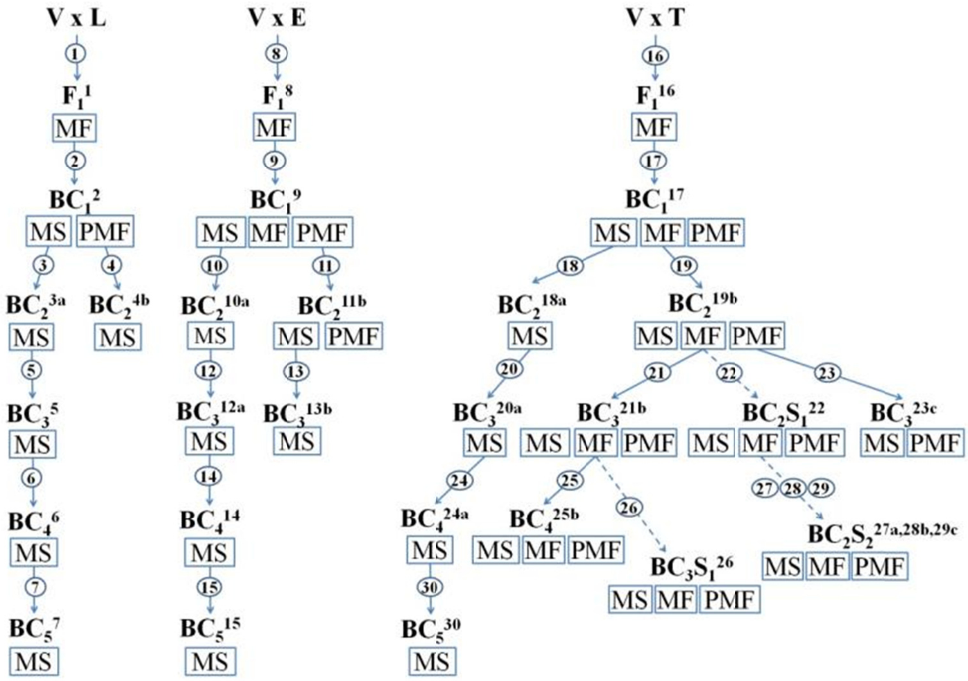

Figure 1. Flowchart outlining the development of the CMS lines of eggplant cultivars 'Langada' (L), 'Emi' (E) and 'Tsakoniki' (T) with the cytoplasm of *S. violaceum* (V)

Note. Solid lines indicate backcrossing of individual plants of the former population to the eggplant parent and dotted lines indicate selfing. Numbers in circles indicate the cross number from which the following population was obtained. Superscripted numbers indicate the cross number from which each population was obtained; letters (a, b and c) indicate origin from different maternal plants of the former population. Male sterile, male fertile and potentially male fertile phenotypes observed after backcrossing or selfing are designated as MS, MF and PMF, respectively.

3. Results

3.1 Development of CMS Eggplant Lines

The flowchart outlining the development of the CMS lines is presented in Figure 1. In general, the plant material consisted of healthy green plants with normal development; however, a number of dwarf and chlorotic plantlets occurred in all BC_1 populations and some chlorotic plantlets were also observed in the population $BC_2(V\times T)S_1^{22}$ (data not shown). Such plants could not be transplanted in the experimental field due to delayed growth and were subsequently excluded from fertility analyses.

3.2 Female Fertility

The three eggplant cultivars easily set fruits after artificial self-pollination and these fruits contained many seeds that germinated in a percentage more than 90.0% (Table 1). The percentage of successful backcrosses, number of seeds per fruit and seed germination rates were low to moderate in the interspecific hybrids and the early BC generations, but gradually increased in their advanced BC counterparts (Table 1). In the BC_5 populations the average seed germination was greater than 90.0%, the successful backcrosses ranged from 77.1 to 94.7% and the average number of seeds per fruit ranged from 876.5 to 1147.8 (Table 1).

Table 1. Seed germination (in percent), fruit setting after backcrossing and number of seeds per fruit in three eggplant cultivars, their interspecific hybrids with *Solanum violaceum* (V) and the respective backcross populations

Populations examined[z]	Seed germination		Backcrosses				
	(%)	N[y]	No. of plants backcrossed	No. of backcrosses attempted	Successful backcrosses (%)	No. of seeds per fruit	N
cv. 'Langada'(L)	93.5 ± 0.7[x]	6	15	75	89.3	1232.3 ± 64.3	12
cv. 'Emi'(E)	93.8 ± 0.8	6	14	71	91.6	1024.0 ± 39.8	12
cv. 'Tsakoniki'(T)	94.5 ± 1.0	6	14	70	91.4	1512.5 ± 48.4	12
F_1(V×L)[1]	-[w]	-	7	65	67.7	84.7 ± 4.0	8
BC_1(V×L)[2]	17.5	1	16	80	46.3	161.5 ± 29.9	8
BC_2(V×L)[3a,4b]	77.5 ± 3.9	8	22	110	55.5	234.7 ± 24.0	8
BC_3(V×L)[5]	83.9 ± 3.1	8	21	105	82.9	592.2 ± 32.9	8
BC_4(V×L)[6]	88.6 ± 2.2	8	28	140	88.6	656.3 ± 42.2	8
BC_5(V×L)[7]	92.3 ± 1.2	8	14	70	77.1	763.2 ± 52.8	8
F_1(V×E)[8]	-	-	2	15	53.3	77.5 ± 5.2	8
BC_1(V×E)[9]	50.0	1	25	140	66.4	145.8 ± 18.5	8
BC_2(V×E)[10a,11b]	70.1 ± 4.1	8	30	165	52.1	202.8 ± 17.3	8
BC_3(V×E)[12a]	65.4 ± 3.1	8	15	75	66.7	371.3 ± 31.6	8
BC_4(V×E)[14]	85.1 ± 2.1	8	18	90	75.6	780.8 ± 43.0	8
BC_5(V×E)[15]	91.0 ± 0.9	8	16	80	80.0	876.5 ± 48.0	8
F_1(V×T)[16]	-	-	2	16	75.0	92.5 ± 4.0	8
BC_1(V×T)[17]	35.0	1	29	145	37.2	203.3 ± 35.8	8
BC_2(V×T)[19b]	67.9 ± 3.5	8	28	140	59.3	351.8 ± 30.4	8
BC_3(V×T)[20a,21b]	72.9 ± 2.6	8	45	225	74.7	715.3 ± 54.7	8
BC_4(V×T)[24a]	91.9 ± 0.9	8	21	105	81.9	1018.7 ± 72.9	8
BC_5(V×T)[30]	93.5 ± 0.8	8	15	75	94.7	1203.7 ± 58.5	8

Note. [z]Superscripted numbers indicate the cross from which the examined population was obtained and correspond to the cross numbers given in Figure 1; letters (a and b) indicate origin from different maternal plants of the former population; [y]N = sample size; [x]Means±standard errors. For the eggplant cultivars means of six years are presented; [w]Not investigated.

3.3 Pollen Release Ability

Pollen release ability was normal in the eggplant cultivars, *S. violaceum* and their corresponding interspecific hybrids. Observations under the stereomicroscope showed that the terminal pore of the anther dehisced at anthesis and released pollen (Figure 2a). On the contrary, in the BC_1 populations three distinct classes of plants were identified with respect to the pollen release ability: (I) Pollen releasing class: plants that formed exclusively dehiscent anthers and set several fruits following self-pollination (Figure 2c); (II) Pollen non-releasing class: plants that formed exclusively indehiscent anthers and did not set fruits following self-pollination (Figure 2b); (III) Potentially pollen releasing class: plants that originally were of the pollen releasing type, but later (usually one month after flowering) formed anthers of the pollen non-releasing type. Such plants set a few seeded fruits after self-pollination of the first flowers, while the rest of the flowers were aborted. In addition, formation of seedless parthenocarpic fruit occasionally occurred in the pollen non-releasing and potentially pollen releasing progenies derived from the hybrid F_1(V×E) (data not shown). Since anther non dehiscence was the main reason of male sterility, the plant material was classified based on pollen release ability as male fertile (MF), male sterile (MS) and potentially male fertile (PMF).

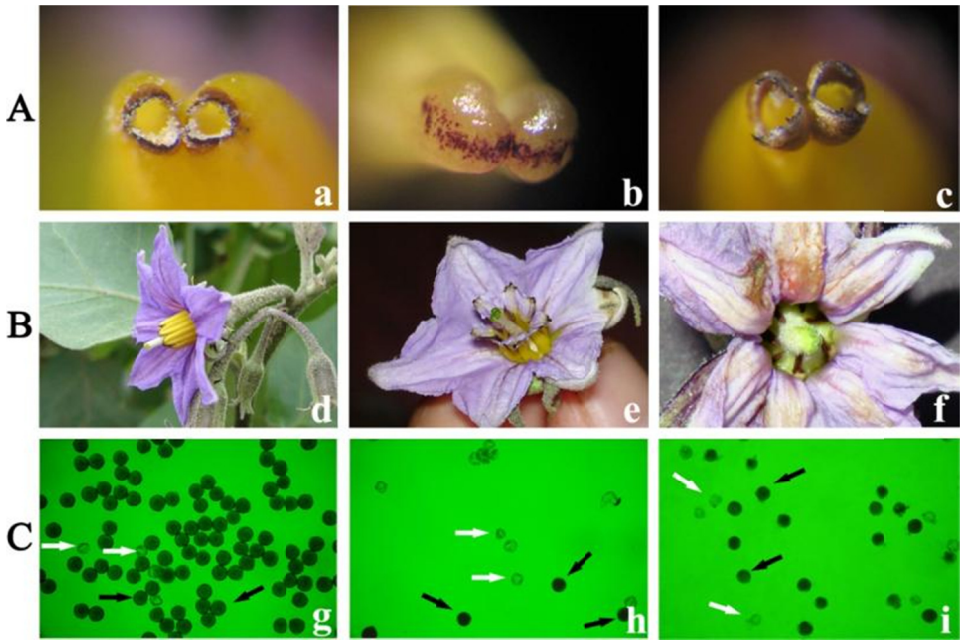

Figure 2. Anther dehiscence (A), anther morphology (B) and pollen viability (C) in eggplant, male sterile (MS) and male fertile (MF) plants carrying the cytoplasm of *S. violaceum*

Note. Dehisced anther pore in eggplant cultivar 'Tsakoniki' (a), non-dehisced anther pore in a MS $BC_1(V\times T)$ plant (b) and dehisced anther pore in a MF $BC_4(V\times T)$ plant (c). Normal anther morphology in a MS $BC_1(V\times E)$ plant (d), petaloid anthers in a MS $BC_2(V\times L)$ plant (e) and atrophic anthers in a MS $BC_4(V\times T)$ plant (f). Pollen viability in eggplant cultivar 'Tsakoniki' (g), in a MS $BC_4(V\times T)$ plant (h) and in a MF $BC_4(V\times T)$ plant (i). Viable (stained) and non-viable pollen grains are indicated by black and white arrows, respectively.

3.4 Genetic Segregation of the Male Fertility Phenotypes

In the population $BC_1(V\times L)^2$ produced after backcrossing the $F_1(V\times L)^1$ to cv. 'Langada' only PMF and MS plants were obtained (Table 2; cross 2); whereas, all of the three fertility phenotypes were present in $BC_1(VxE)^9$ and $BC_1(V\times T)^{17}$ populations (Table 2; crosses 9 and 17). In general, it was noticed that backcrossing of MS plants constantly produced MS offspring regardless the pollen parent (Figure 1, Table 2). In contrast, the segregation ratios obtained after backcrossing of the PMF and MF plants varied with respect to the recurrent eggplant parent. Backcrossing of the only PMF plant found in population $BC_1(V\times L)^2$ to cv. 'Langada' resulted only in MS offspring in $BC_2(V\times L)^{4b}$ (Table 2; cross 4). The observed frequencies in the population $BC_1(VxE)^9$ derived after backcrossing $F_1(V\times E)^8$ to cv. 'Emi' fitted well the 1MF:3PMF:4MS ratio (Table 2; cross 9; P = 0.824); however, backcrossing of the individual plant $BC_1(V\times E)^{9b}$ that was considered as MF to cv. 'Emi' produced only PMF and MS progenies in the population $BC_2(V\times E)^{11b}$ (Table 2; cross 11). The segregation frequencies fitted better to the 1PMF:1MS ratio (P = 0.491) than the expected 1MF:3PMF:4MS (P = 0.257). In addition, backcrossing of the individual plant $BC_2(V\times E)^{11b}$ that was initially considered as PMF to cv. 'Emi' produced only MS progenies in the population $BC_3(V\times E)^{13b}$, instead of the expected 1PMF:1MS ratio (Table 2; cross 13).

On the other hand, the inheritance of the MF phenotype was more consistent in the backcross populations derived from $F_1(V\times T)^{16}$ and their selfed progenies (Table 2; crosses 17 to 24). Thus, MF plants were successfully obtained in the respective advanced backcrossing and selfing populations allowing a more detailed genetic study. Backcrossing of individual MF plants from populations $F_1(V\times T)^{16}$, $BC_1(V\times T)^{17}$, $BC_2(V\times T)^{19b}$ and $BC_3(V\times T)^{21b}$ to cv. 'Tsakoniki' produced offspring segregating in a 1MF:1PMF:2MS ratio with P-values ranging from 0.472 to 0.936 (Table 2; crosses 17, 19, 21 and 25). In addition, the progeny obtained after backcrossing the individual PMF plant $BC_3(V\times T)^{21a}$ to cv. 'Tsakoniki' segregated in a 1PMF:1MS ratio (P = 0.670, Table 2; cross 23).

Table 2. Genetic analyses of nuclear restorer alleles in the genetic backgrounds of eggplant cultivars 'Langada' (L), 'Emi' (E) and 'Tsakoniki' sharing the cytoplasm of *Solanum violaceum* (V)

Cross				Observed progeny							
No.[z]	Parents[y]	Parental phenotypes[x]	Putative parental genotypes	Populations examined[w]	Fertility phenotype			Expected ratio	df	χ^2	Probability *P*
					MF	PMF	MS				
1	V×L	MF×MF	could not be determined	F_1(V×L)[1]	7	0	0	-	-	-	-
2	F_1(V×L)[1]×L	MF×MF	"	BC_1(V×L)[2]	0	1	15	-	-	-	-
3	BC_1(V×L)[2a] ×L	MS×MF	"	BC_2(V×L)[3a]	0	0	12	-	-	-	-
4	BC_1(V×L)[2b]×L	PMF×MF	"	BC_2(V×L)[4b]	0	0	14	-	-	-	-
5	BC_2(V×L)[3]×L	MS×MF	"	BC_3(V×L)[5]	0	0	25	-	-	-	-
6	BC_3(V×L)[5]×L	MS×MF	"	BC_4(V×L)[6]	0	0	27	-	-	-	-
7	BC_4(V×L)[6]×L	MS×MF	"	BC_5(V×L)[7]	0	0	20	-	-	-	-
8	V×E	MF×MF	($Rf_1Rf_1Rfm_1Rfm_1Rfm_2Rfm_2$) ×A(=$rf_1rf_1rfm_1rfm_1rfm_2rfm_2$)	F_1(V×E)[8]	6	0	0	-	-	-	-
9	F_1(V×E)[8]×E	MF×MF	($Rf_1rf_1Rfm_1rfm_1Rfm_2rfm_2$)×A	BC_1(V×E)[9]	3	8	14	1:3:4	2	0.387	0.824
10	BC_1(V×E)[9a]×E	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1Rfm_1rfm_1rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1rfm_2rfm_2$)×A	BC_2(V×E)[10a]	0	0	11	-	-	-	-
11	BC_1(V×E)[9b]×E	PMF×MF	($Rf_1rf_1Rfm_1rfm_1rfm_2rfm_2$ or $Rf_1rf_1rfm_1rfm_1Rfm_2rfm_2$ or $Rf_1rf_1rfm_1rfm_1rfm_2rfm_2$)×A	BC_2(V×E)[11b]	0	8	11	1:1	1	0.474	0.491
12	BC_2(V×E)[10a]×E	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1Rfm_1rfm_1rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1rfm_2rfm_2$)×A	BC_3(V×E)[12a]	0	0	17	-	-	-	-
13	BC_2(V×E)[11b]×E	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1Rfm_1rfm_1rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1rfm_2rfm_2$)×A	BC_3(V×E)[13b]	0	0	13	-	-	-	-
14	BC_3(V×E)[12]×E	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1Rfm_1rfm_1rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1rfm_2rfm_2$)×A	BC_4(V×E)[14]	0	0	26	-	-	-	-
15	BC_4(V×E)[14]×E	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1Rfm_1rfm_1rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2rfm_2$ or $rf_1rf_1rfm_1rfm_1rfm_2rfm_2$)×A	BC_5(V×E)[15]	0	0	22	-	-	-	-
16	V×T	MF×MF	($Rf_1Rf_1Rfm_1Rfm_1Rfm_2Rfm_2$)× B(=$rf_1rf_1rfm_1rfm_1Rfm_2Rfm_2$)	F_1(V×T)[16]	5	0	0	-	-	-	-
17	F_1(V×T)[16]×T	MF×MF	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$)×B	BC_1(V×T)[17]	8	6	15	1:1:2	2	0.310	0.856
18	BC_1(V×T)[17a]×T	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2Rfm_2$)×B	BC_2(V×T)[18a]	0	0	10	-	-	-	-
19	BC_1(V×T)[17b]×T	MF×MF	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$)×B	BC_2(V×T)[19b]	9	6	21	1:1:2	2	1.50	0.472
20	BC_2(V×T)[18a]×T	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2Rfm_2$)×B	BC_3(V×T)[20a]	0	0	17	-	-	-	-
21	BC_2(V×T)[19b]×T	MF×MF	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$)×B	BC_3(V×T)[21b]	8	8	14	1:1:2	2	0.133	0.936
22	BC_2(V×T)[19b] ⊗	MF ⊗	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$) ⊗	BC_2(V×T)S_1[22]	12	10	11	9:3:4	2	3.909	0.142
23	BC_2(V×T)[19c]×T	PMF×MF	($Rf_1rf_1rfm_1rfm_1Rfm_2Rfm_2$)×B	BC_3(V×T)[23c]	0	10	12	1:1	1	0.182	0.670

24	$BC_3(V\times T)^{20a}\times T$	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2Rfm_2$)×B	$BC_4(V\times T)^{24a}$	0	0	21	-	-	-	-
25	$BC_3(V\times T)^{21b}\times T$	MF×MF	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$)×B	$BC_4(V\times T)^{25b}$	12	9	21	1:1:2	2	0.430	0.807
26	$BC_3(V\times T)^{21b}\otimes$	MF ⊗	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$) ⊗	$BC_3(V\times T)S_1^{26}$	22	10	13	9:3:4	2	1.00	0.608
27	$BC_2(V\times T)S_1^{22a}\otimes$	MF ⊗	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$) ⊗	$BC_2(V\times T)S_2^{27a}$	7	4	5	9:3:4	2	1.028	0.598
28	$BC_2(V\times T)S_1^{22b}\otimes$	MF ⊗	($Rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$) ⊗	$BC_2(V\times T)S_2^{28b}$	9	3	5	9:3:4	2	0.176	0.916
29	$BC_2(V\times T)S_1^{22c}\otimes$	MF ⊗	($Rf_1Rf_1Rfm_1rfm_1Rfm_2Rfm_2$) ⊗	$BC_2(V\times T)S_2^{29c}$	17	4	0	3:1	1	0.150	0.696
30	$BC_4(V\times T)^{24a}\times T$	MS×MF	($rf_1rf_1Rfm_1rfm_1Rfm_2Rfm_2$ or $rf_1rf_1rfm_1rfm_1Rfm_2Rfm_2$)×B	$BC_5(V\times T)^{30}$	0	0	15	-	-	-	-

Note. [z]cross numbers correspond to cross numbers given in Figure 1; [y]Superscripted numbers indicate cross number from which the maternal parent was obtained; letters (a, b and c) indicate different maternal parent of the same population; [x]MF = male fertile, PMF = potentially male fertile, MS = male sterile. Symbol ⊗ indicates self-pollination of individual MF plants; [w]Superscripted numbers represent cross number from which the examined population was obtained; letters (a, b and c) indicate origin from different maternal parent of the former population.

The populations obtained after selfing of MF individuals generally segregated in the expected 9MF:3PMF:4MS and 3MF:1PMF ratios (Table 2; crosses 26 to 29). The only exception was observed in the population $BC_2(V\times T)S_1^{22}$ which segregated in an unexpected ratio (P = 0.142; Table 2; cross 22). This population included some chlorotic seedlings and was obtained after selfing of the individual MF plant $BC_2(V\times T)^{19b}$. However, population $BC_3(V\times T)S_1^{26}$ which was obtained after selfing of the individual MF plant $BC_3(V\times T)^{21b}$ segregated in a 9MF:3PMF:4MS segregation ratio (P = 0.608) and consisted only of green seedlings (Table 2; cross 26). Finally, selfing of three individual MF plants originating from population $BC_2(V\times T)S_1^{22b}$ produced two $BC_2(V\times T)S_2$ populations with 9MF:3PMF:4MS ratios (P = 0.598 and P = 0.916, respectively) and one population with 3MF:1PMF ratio (P = 0.696) (Table 2; crosses 27 to 29).

3.5 Anther Morphology

Normal anther morphology was observed in *S. violaceum*, the eggplant cultivars, their respective interspecific hybrids and the MF plants (Table 3). On the contrary, MS plants formed flowers with either normal or abnormal anthers and the frequency of abnormalities tended to increase in the succeeding backcross generations (Table 3, Figures 2d-2f). PMF plants initially developed some flowers with normal anthers, but the flowers formed in later stages had anthers with either normal or abnormal morphology. It was noticed that the type of abnormalities in anther morphology varied with respect to the eggplant recurrent parent. For example, both the petaloid and atrophic anther types were present in the BC populations with the genetic background of cv. 'Langada' and cv. 'Emi', whereas, only the atrophic type was found in the genetic background of cv. 'Tsakoniki' (Table 3, Figures 2e and 2f). It was also observed that petaloidy was the prevalent morphological abnormality of the anther in the advanced backcross progenies of $F_1(V\times L)$, but diminished in the respective progenies of $F_1(V\times E)$ (Table 3).

Table 3. Number of flowers (in percent) with normal (Nor), petaloid (Pet) or atrophic (Atr) anthers in *Solanum violaceum*, three eggplant cultivars, their interspecific hybrids and the respective backcross and selfed progenies in three fertility phenotypes

Populations examined[z]	Fertility phenotype														
	Male fertile					Potentially male fertile					Male sterile				
	Nor	Pet	Atr	N_1[y]	N_2	Nor	Pet	Atr	N_1	N_2	Nor	Pet	Atr	N_1	N_2
S. violaceum (V)[x]	100.0	0.0	0.0	18	90	-	-	-	-	-	-	-	-	-	-
cv. 'Langada' (L)	100.0	0.0	0.0	18	90	-	-	-	-	-	-	-	-	-	-
cv. 'Emi' (E)	100.0	0.0	0.0	18	90	-	-	-	-	-	-	-	-	-	-
cv. 'Tsakoniki' (T)	100.0	0.0	0.0	18	90	-	-	-	-	-	-	-	-	-	-
F_1(V×L)[1]	100.0	0.0	0.0	7	35	-	-	-	-	-	-	-	-	-	-
BC_1(V×L)[2]	-	-	-	-	-	100.0	0.0	0.0	1	5	68.9	13.3	17.8	15	45
BC_2(V×L)[3a]	-	-	-	-	-	-	-	-	-	-	55.6	8.3	36.1	12	36
BC_3(V×L)[5]	-	-	-	-	-	-	-	-	-	-	26.7	6.7	66.7	14	75
BC_4(V×L)[6]	-	-	-	-	-	-	-	-	-	-	25.6	32.9	41.5	27	82
BC_5(V×L)[7]	-	-	-	-	-	-	-	-	-	-	26.7	43.3	30.0	20	60
F_1(V×E)[8]	100.0	0.0	0.0	6	25	-	-	-	-	-	-	-	-	-	-
BC_1(V×E)[9]	91.7	0.0	8.3	3	12	95.8	0.0	4.2	8	24	76.2	19.1	4.8	14	42
BC_2(V×E)[10a]	-	-	-	-	-	-	-	-	-	-	57.6	18.2	24.2	11	33
BC_2(V×E)[11b]	-	-	-	-	-	87.5	-	12.5	8	24	54.6	27.3	18.2	11	33
BC_3(V×E)[12a]	-	-	-	-	-	-	-	-	-	-	52.9	0.0	47.1	17	17
BC_4(V×E)[14]	-	-	-	-	-	-	-	-	-	-	34.6	0.0	65.4	26	78
BC_5(V×E)[15]	-	-	-	-	-	-	-	-	-	-	33.3	0.0	66.7	22	66
F_1(V×T)[16]	100.0	0.0	0.0	5	30	-	-	-	-	-	-	-	-	-	-
BC_1(V×T)[17]	100.0	0.0	0.0	8	24	100.0	0.0	0.0	6	18	51.1	0.0	48.9	15	45
BC_2(V×T)[18a]	-	-	-	-	-	-	-	-	-	-	56.7	0.0	43.3	10	30
BC_3(V×T)[20a]	-	-	-	-	-	-	-	-	-	-	62.8	0.0	37.3	17	51
BC_2(V×T)S_1[22]	100.0	0.0	0.0	12	36	66.7	0.0	33.3	10	30	30.3	0.0	69.7	11	33
BC_4(V×T)[24a]	-	-	-	-	-	-	-	-	-	-	46.0	0.0	54.0	21	63
BC_4(V×T)[25b]	88.9	0.0	11.1	12	36	33.3	0.0	66.7	9	27	14.3	0.0	85.7	21	63
BC_3(V×T)S_1[26]	87.9	0.0	12.1	22	66	30.0	0.0	70.0	10	30	30.8	0.0	69.2	13	39
BC_2(V×T)S_2[29c]	100.0	0.0	0.0	17	51	84.6	0.0	15.4	4	13	-	-	-	-	-
BC_5(V×T)[30]	-	-	-	-	-	-	-	-	-	-	35.6	0.0	64.4	15	45

Note. [z]Superscripted numbers indicate the cross from which the examined population was obtained and correspond to the cross numbers given in Figure 1; letters (a, b and c) indicate origin from different maternal plants of the former population; [y]N_1 = total number of examined plants per fertility phenotype. N_2 = total number of examined flowers per fertility phenotype; [x]For *S. violaceum* and the eggplant cultivars data for six years are presented.

3.6 Pollen Viability

In the examined plant material the viable pollen grains were round with typical size and were stained after acetocarmine treatment, while non-viable grains appeared shriveled and empty (Figure 2g-2i). It was established that pollen viability was high in the eggplant cultivars and *S. violaceum* and moderate in their corresponding interspecific hybrids, whereas, pollen viability of the MS plants was generally low and tended to decrease further in the subsequent backcross generations (Table 4). On the other hand, it was observed that pollen viability in the PMF and MF plants was moderate and moderate to high, respectively, and remained high and rather stable in the advanced BC populations (Table 4).

3.7 Cytoplasm Inheritance

HRM analysis of the cp-DNA showed that the examined plants in BC_4 and BC_3S_1 populations grouped together with *S. violaceum* regardless of their fertility phenotype; while the eggplant parents were clearly separated

(Figure 3A). Similarly, the *trn*L intron sequencing data indicated that the examined BC_4 and BC_3S_1 plants shared the same sequence with the wild species, but these sequences were different in the eggplant cultivars (Figure 3B). In addition, the RFLP analysis of the mt-DNA demonstrated that the restriction patterns of the examined MS and MF plants were identical to those of *S. violaceum*, but different from those of the eggplant parent (Figure 4).

Table 4. Mean pollen acetocarmine stainability (in percent) in *S. violaceum*, three eggplant cultivars, their interspecific hybrids and their backcrossed and selfed progenies in three fertility phenotypes

Populations examined[z]	Fertility phenotype					
	Male fertile	N[y]	Potentially male fertile	N	Male sterile	N
S. violaceum (V)	84.5 ± 0.4[x]	18	-	-	-	-
cv. 'Langada' (L)	96.1 ± 0.2	18	-	-	-	-
cv. 'Emi' (E)	92.6 ± 0.4	18	-	-	-	-
cv. 'Tsakoniki' (T)	91.3 ± 0.3	18	-	-	-	-
F_1(V×L)[1]	52.0 ± 1.5	7	-	-	-	-
BC_1(V×L)[2]	-	-	27.9	1	11.9 ± 2.4	15
BC_2(V×L)[3a]	-	-	-	-	33.6 ± 4.4	12
BC_2(V×L)[4b]	-	-	-	-	21.4 ± 4.9	14
BC_3(V×L)[5]	-	-	-	-	15.9 ± 3.0	25
BC_4(V×L)[6]	-	-	-	-	22.2 ± 3.6	27
BC_5(V×L)[7]	-	-	-	-	7.9 ± 2.3	20
F_1(V×E)[8]	45.1 ± 1.8	6	-	-	-	-
BC_1(V×E)[9]	50.0 ± 1.5	3	40.3 ± 1.9	8	12.5 ± 2.8	14
BC_2(V×E)[10a]	-	-	-	-	20.1 ± 4.3	11
BC_2(V×E)[11b]	-	-	67.4 ± 3.2	8	20.1 ± 4.6	11
BC_3(V×E)[12a]	-	-	-	-	13.7 ± 2.8	13
BC_4(V×E)[14]	-	-	-	-	8.0 ± 3.3	26
BC_5(V×E)[15]	-	-	-	-	8.3 ± 2.2	22
F_1(V×T)[16]	38.6 ± 1.2	5	-	-	-	-
BC_1(V×T)[17]	59.4 ± 3.0	8	30.3 ± 4.2	6	12.5 ± 2.6	15
BC_2(V×T)[19b]	74.6 ± 4.2	9	39.4 ± 2.5	6	7.7 ± 1.7	21
BC_3(V×T)[20a]	-	-	-	-	23.8 ± 3.8	17
BC_3(V×T)[21b]	62.7 ± 4.6	8	32.9 ± 3.1	8	11.3 ± 2.2	14
BC_2(V×T)S_1[22]	79.8 ± 2.4	12	69.6 ± 4.6	10	23.2 ± 7.1	11
BC_4(V×T)[24a]	-	-	-	-	4.8 ± 1.7	21
BC_4(V×T)[25b]	81.3 ± 3.3	12	51.0 ± 8.3	9	7.6 ± 3.9	21
BC_3(V×T)S_1[26]	77.3 ± 3.0	22	31.3 ± 5.7	10	11.7 ± 5.8	13
BC_2(V×T)S_2[29c]	85.1 ± 1.7	17	73.9 ± 12.2	4	-	-
BC_5(V×T)[30]	-	-	-	-	6.6 ± 1.8	15

Note. [z]Superscripted numbers indicate the cross from which the examined population was obtained and correspond to the cross numbers given in Figure 1; letters (a, b and c) indicate origin from different maternal plants of the former population; [y]N = number of examined plants per fertility phenotype; [x]Means±standard errors. For *S. violaceum* and the eggplant cultivars the means of six years are presented.

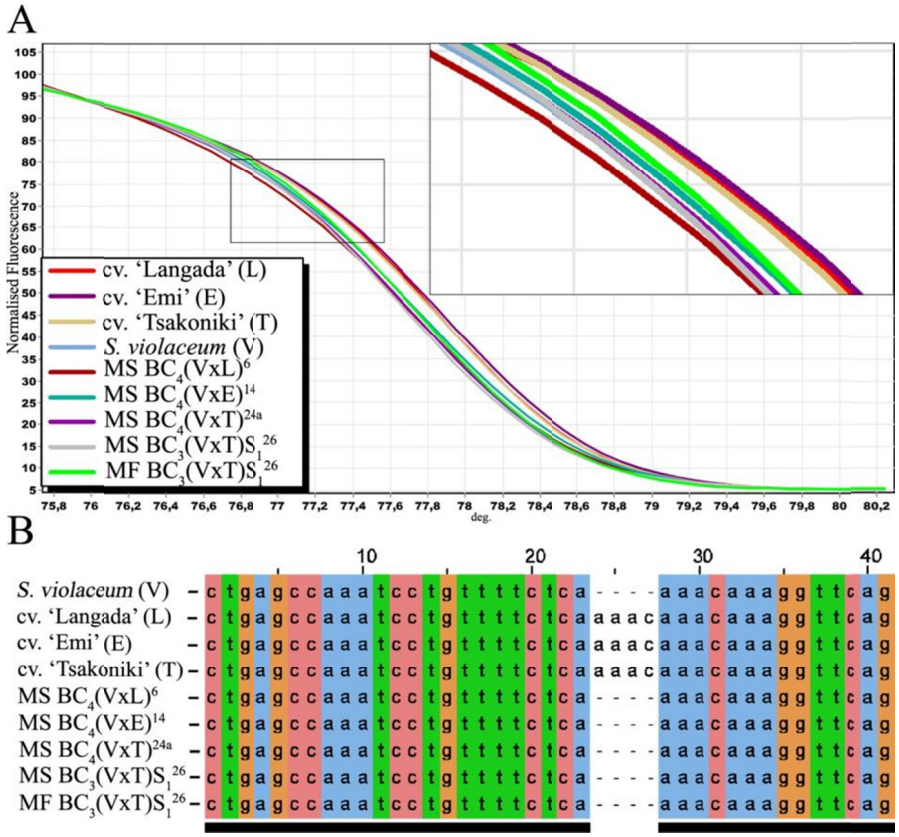

Figure 3. Chloroplast DNA analysis in three eggplant cultivars, *S. violaceum*, male sterile (MS) plants from populations $BC_4(V\times L)^6$, $BC_4(V\times E)^{14}$, $BC_4(V\times T)^{24a}$, $BC_3(V\times T)S_1{}^{22}$ and a male fertile (MF) plant from population $BC_3(V\times T)S_1{}^{22}$. Normalized high resolution melt curves of *trnL* amplicons generated from the examined plant material (A) and partial nucleotide sequences obtained from *trnL* sequencing (B)

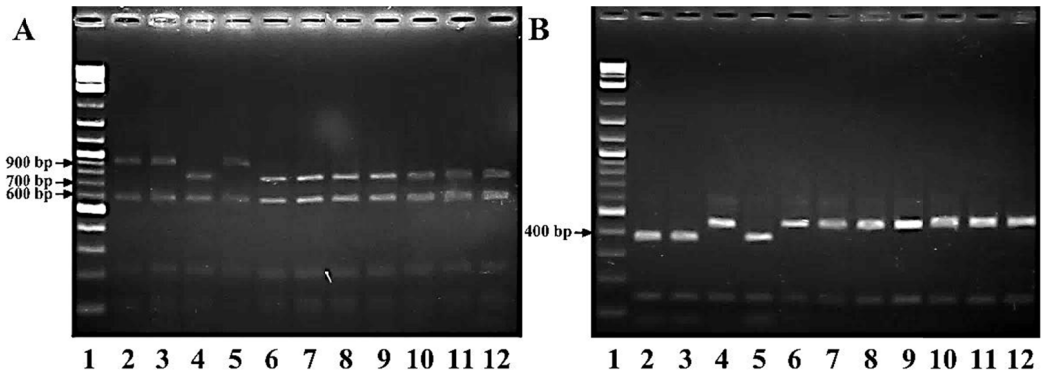

Figure 4. Restriction patterns of the *Alu*I digested nad7/3-4 (A) and *Scr*FI digested V7 (B) regions of small ribosomal subunit RNA gene. Lane 1: Marker, lanes 2-5: cv. 'Langada', cv. 'Emi', *S. violaceum* and cv. 'Tsakoniki', respectively. Lane 6-8: MS plants from populations $BC_4(V\times L)^6$, $BC_4(V\times E)^{14}$, $BC_4(V\times T)^{24a}$, respectively. Lanes 9-10: MS and MF plants from population $BC_4(V\times T)^{25b}$. Lanes 11-12: MS and MF plants from population $BC_3(V\times T)S_1{}^2$

4. Discussion

In the past, a number of studies showed that several species can serve as cytoplasm donors for the development of CMS eggplant lines (Fang et al., 1985; Khan & Isshiki, 2008, 2009, 2010, 2011; Saito et al., 2009). In the present study, the alloplasmic CMS lines of the eggplant cultivars 'Langada', 'Emi' and 'Tsakoniki' were

successfully produced *via* the backcross method using *S. violaceum* as the female parent (Figure 1). The stability of the male sterile phenotype confirms that *S. violaceum* is a suitable cytoplasm donor for the induction of CMS in eggplant, as it was previously reported by Isshiki & Kawajiri (2002). The presence of some dwarf and chlorotic plantlets was restricted in the BC_1 and $BC_2(V\times T)S_1^{22}$ populations and indicated adverse effects of interspecific hybridization on plant vigor. Reduced vigor was observed in the reciprocal interspecific hybrids *S. melongena* × *S. macrocarpon* (Bletsos, Roupakias, Tsaktsira, & Scaltsoyjannes, 2004). However, in the present study these abnormalities were eliminated from the subsequent generations where only healthy plants were recovered.

A common feature of CMS is that the female gametophyte is not affected from the presence of the alien cytoplasm (Nair, 1993). In an eggplant CMS line, high fruit set and seed number are essential because hybrid seed is the final product. Our results demonstrate that the CMS induced by the *S. violaceum* cytoplasm had no negative effect on the female fertility of the examined plant material. That was apparent from the observed gradual increase in the percentage of successful backcrosses, the number of seeds per fruit and the germination percentage in the BC populations (Table 1). The seed number per fruit was lower in the BC_5 populations when compared to the respective eggplant parents (Table 1), perhaps due to incomplete replacement of the eggplant alleles for increased seed number. However, selection of individuals with increased number of seeds is expected to resolve that problem (Isshiki & Yoshida, 2002). From a practical point of view, CMS 'Tsakoniki' is a more suitable seed parent because of the higher backcrossing percentage and seed number per fruit when compared to the other CMS lines (Table 1). The overall female fertility results reported here are in accordance with those previously reported in several other CMS eggplant systems (Isshiki & Kawajiri, 2002; Khan & Isshiki, 2008, 2009, 2010, 2011).

On the other hand, CMS can adversely affect the appearance and functionality of the male organs of the flower, as well as male gametogenesis (Hanson & Bentolila, 2004). The CMS phenotype in alloplasmic eggplant was associated with the transcription patterns of novel open reading frames (ORFs) in the 5' flanking region of *atp*1 mitochondrial gene (Yoshimi et al., 2013). In the present study the negative effect of the *S. violaceum* cytoplasm on male fertility of the MS plants was expressed with reduced pollen release ability, abnormalities in anther morphology and low pollen viability (Figure 2b, Tables 2-4). Since the terminal pore of the anthers in our CMS lines did not dehisce at anthesis (Figure 2b), this type of CMS can be classified as a functional CMS. Anther non dehiscence was a common feature in our three CMS lines and the one developed by Isshiki and Kawajiri (2002), which also carried the cytoplasm of *S. violaceum*. Therefore, it can be concluded that the specific cytoplasm induces the functional CMS type in eggplant, regardless the genetic background of the eggplant nucleus donor. Anther non dehiscence was attributed to the malfunction of the tapetum tissue in GMS eggplant and *S. insanum*, a species closely related to eggplant (Chauhan, 1984; Karihaloo & Malik, 1995).

The replacement of the anthers with petaloid structures in the lineages of CMS 'Langada' and CMS 'Emi' (Figure 2e), indicated that cytoplasmic/nuclear interactions induced homeotic changes in flower morphology. Similar CMS induced alterations were reported in eggplant and several other plant species including carrot, leaf mustard, rapeseed and wheat (Carlsson, Leino, Sohlberg, Sundström, & Glimelius, 2008; Fang et al., 1985; Isshiki & Yoshida, 2002; Linke, Nothnagel, & Börner, 2003; Yang et al., 2005; Zhu et al., 2008). However, the petaloid anther type was absent from the lineage of CMS 'Tsakoniki' (Table 3), suggesting that the type of anther abnormalities depends on the genotype of the eggplant parent. This is further supported by the fact that petaloidy was the prevalent anther abnormality in CMS 'Langada', but the less frequent in CMS 'Emi' (Table 3). On the other hand, the atrophic anther type was common in the three CMS eggplant lines (Table 3, Figure 2f). Perhaps this type of abnormality is induced by the cytoplasm of *S. violaceum* through a different mechanism. Similar abnormalities including underdeveloped, abortive and brown anthers were attributed to pre-meiotic malfunction of the tapetum in GMS eggplant, CMS carrot and CMS *Rosmarinus officinalis* L. (Chauhan, 1984; Hidalgo, Hesse, Ubera, & Frosch-Radivo, 1999; Kozik, Nowak, Nowakowska, & Dyki, 2012).

Pollen viability of the MS plants was very low and did not recover in the advanced BC populations (Table 4). These findings combined with the increased female fertility of the advanced BC populations (Table 1), suggest that low pollen viability is induced by a kind of disharmony between the *S. violaceum* cytoplasm and the eggplant nucleus, rather than meiotic abnormalities. Although meiotic associations were not studied in the present work, available cytogenetic evidence regarding the same species combination strongly supports this hypothesis. For example, near perfect meiosis was reported in the interspecific hybrids between *S. violaceum* × *S. melongena* (Isshiki & Kawajiri, 2002; Rajasekaran, 1970) and in several MS plants in the respective BC_4 progenies (Isshiki & Kawajiri, 2002). Problems during pollen maturation including incomplete starch accumulation and hydrolysis may account for the low pollen fertility observed in our functional CMS lines, as it

was recently demonstrated in the eggplant functional CMS lines carrying the cytoplasm of *S. violaceum* and other wild species (Khan, Hasnunnahar, Iwayoshi, Ogura-Tsujita, & Isshiki, 2015).

In the CMS eggplant systems studied so far, the *Rf*-genes are available only in the pollen non-formation type caused by the cytoplasms of *S. integrifolium*, *S. anguivi* and *S. grandifolium* (Hasnunnahar et al., 2012; Khan & Isshiki, 2010, 2011; Saito et al., 2009). In the abovementioned CMS systems, the fertility restored plants exhibited marked differences in the procession of the co-transcripts of a novel *orf* and *atp1* in relation to the CMS counterparts (Yoshimi et al., 2013). However, the presence of the *Rf*-genes was not confirmed in the pollen non-releasing (functional) CMS eggplant lines carrying the cytoplasms of *S. violaceum*, *S. virgianum* and *S. kurzii* (Isshiki & Kawajiri, 2002; Khan & Isshiki, 2009, 2010). In the present study, some plants appeared to possess the appropriate *Rf*-genes that suppress or compensate for the adverse effects of the *S. violaceum* cytoplasm on male fertility and were characterized by normal anther functionality and morphology, high pollen viability (Figure 2c, Tables 3 and 4). Therefore, our study is the first to report the inheritance of the *Rf*-genes in the advanced BC progenies in eggplant functional CMS lines.

In the pollen non-formation eggplant lines with the cytoplasms of *S. integrifolium*, *S. anguivi* and *S. grandifolium* the fertility restoration was reported to be under the control of two independent duplicated loci (Hasnunnahar et al., 2012; Khan & Isshiki, 2010, 2011); whereas, Saito et al. (2009) reported that the CMS caused by the cytoplasm of *S. grandifolium* was restored by a single dominant allele. However, the segregation ratios observed in our study clearly deviated from those previously reported in the other eggplant CMS/*Rf*-genes systems. These differences may be attributed to the different cytoplasm and nucleus donor combinations used for the development of the previous eggplant CMS/*Rf*-genes systems. The presence of PMF plants in our segregating populations suggested a modifying gene action on the fertility restoration reported for the first time in eggplant functional CMS. Modifying gene action was also reported in some GMS and CMS/*Rf*-genes systems studied in the past. For example, the expression of anther dehiscence in GMS eggplant was suggested to be affected by environmentally sensitive modifiers (Hazra et al., 2008), showing an interesting analogy with our results. In addition, in the fertility restored CMS maize some contrasting segregation ratios observed in diverse environments were also attributed to modifier gene action (Duvick, 1956).

In our interpretation, the observed segregation ratios of the fertility phenotypes in the examined populations with the genetic backgrounds of cultivars 'Emi' and 'Tsakoniki' can be better explained by the action of one major genetic locus plus a duplicated modifier locus (Table 2; crosses 8 to30). In both genetic backgrounds, the presence of a dominant allele in the major locus (designated Rf_1) is essential for fertility restoration; thus, genotypes homozygous for the recessive allele (rf_1rf_1) are male sterile, regardless the allelic constitution of the modifying loci. The presence of a dominant allele in both modifying loci (Rf_{m1} and Rf_{m2}) is also needed for stable expression of male fertility. On the other hand, recessive homozygosity in one of these modifying loci seems to inhibit the expression of Rf_1 under certain conditions, resulting in the PMF phenotype (Table 2). According to the proposed model, *S. violaceum* and cv. 'Tsakoniki' have the same allelic constitution in the second modifying locus ($Rf_{m2}Rf_{m2}$, Table 2; crosses 16 to 30).

The presence of the *Rf*-gene was confirmed in the $BC_1(V\times E)^9$ and $BC_1(V\times T)^{17}$ populations, but not in $BC_1(V\times L)^2$. In addition, MF plants were obtained *via* backcrossing only in the advanced BC populations of $F_1(V\times T)^{16}$ and their selfing progenies. Besides, our results indicated a more complex genetic control of fertility restoration in $BC_1(V\times E)^9$ than in $BC_1(V\times T)^{17}$, probably due to different allelic constitution in the second modifier locus (Table 2). While these findings seem rather inconsistent, it is important to note that MF plants could not be obtained in the CMS line of eggplant cv. 'Uttara' with the cytoplasm of *S. violaceum* (Isshiki & Kawajiri, 2002). Perhaps, when diverse eggplant nuclear genomes are combined with the cytoplasm of *S. violaceum*, a different number of genetic loci and/or gene interactions are involved in fertility restoration, resulting in different segregation ratios. This is supported from studies in CMS rape-seed, rice and pepper where the gene interactions between the fertility restoring loci varied according to the restorer line used in the crosses (Ma et al., 2013; Pahwa, S. K. Banga, Gogna, & S. S. Banga, 2004; Sarkar, Zaman, & Singh, 2002). In addition, Hazra et al. (2008) reported a strong genotypic effect on the expressivity of the genes involved in eggplant GMS and fertility restoration.

The inconsistency observed in the inheritance of the fertility restoration between the three BC_1 populations could also be attributed to the special characteristics of each recurrent eggplant parent. For example, cv. 'Langada' is considered to be a medium late cultivar and its anthesis occurs about 2 weeks later than cvs. 'Emi' and 'Tsakoniki'. It is possible that in $BC_1(V\times L)$ the action of the modifier(s) on the *Rf*-gene took place before the anthesis so all plants except one appeared to be MS. This may also apply to the backcross progenies of plant $BC_2(V\times E)^{11b}$ where only MS plants were obtained, contrary to the expected 1PMF:1MS ratio (Table 2; cross 13).

In the case of $BC_1(V\times E)$ the formation of parthenocarpic fruits may have masked the effect of low pollen viability and led to the miss-selection of PMF plants instead of the MF ones. For example, plant $BC_1(V\times E)^{9b}$ was initially characterized as MF due to pollen release ability throughout the observation period, but the respective backcrossing segregation ratio suggested that it was probably a PMF one (Table 2; cross 11). In addition, it cannot be ruled out that some alleles involved in the fertility restoration were lost during the backcrossing process due to the minor effect on the fertility phenotype, relative small population size, environmental effects on phenotypic expression of the fertility (Duvick, 1956; Yoshimi et al., 2013) or linkage with the dwarf or chlorotic phenotypes mentioned in the results section. Nevertheless, further experiments should be pursued in order to incorporate the *Rf*-genes in the genetic backgrounds of cvs. 'Langada' and 'Emi'. This process may be facilitated by the development of molecular markers linked to the *Rf*-genes. That was demonstrated to be applicable for the *Rf*-genes of the pollen non-formation eggplant CMS lines (Khan, Hasnunnahar, Iwayoshi, & Isshiki, 2014).

From the genealogy of the plant material and the direction of the crosses (Figure 1, Table 2), it can be derived that the dominant alleles responsible for fertility restoration were inherited from *S. violaceum*. Similarly, the recessive alleles responsible for the CMS were inherited from the eggplant parent. The fact that backcrossing of MS plants to the eggplant parent constantly produced MS progenies further supports this hypothesis suggesting the fixation of the eggplant alleles (Figure 1, Table 2). Moreover, the occurrence of MS plants in the selfing progenies of MF plants indicates that the CMS induced by the cytoplasm of *S. violaceum* is of the sporophytic type. Probably, these MF plants were heterozygous for the fertility restoring loci (Rf_1rf_1) and the recessive allele was transmitted through the functional pollen grains (Table 2; crosses 26 to 28). The 3MF:1PMF segregation ratio observed in the population $BC_2(V\times T)S_2^{29c}$ suggests that the essential Rf_1 allele was fixed (Table 2; cross 29). This, along with the high pollen viability of the MF plants (Table 4), denotes the possibility of obtaining lines homozygous for the *Rf*-genes through selfing, which is necessary for the development of self-maintained restorer lines.

The inheritance of cytoplasm organelles in angiosperms is mainly maternal with only some exceptions (Mogensen, 1996). In our study, analyses of cpDNA and mtDNA confirmed the maternal inheritance of the chloroplasts and mitochondria in the BC_4 and BC_3S_1 progenies from *S. violaceum* (Figures 3 and 4). It seems that maternal inheritance of cytoplasm organelles is a common situation in the interspecific hybrids between eggplant and its wild relatives (Khan & Isshiki, 2009, 2010, 2011), although one case of biparental transmission of the chloroplast was documented (Khan & Isshiki, 2008). Moreover, the results obtained by HRM analysis, a fast molecular approach with a wide applications range (Ganopoulos, Madesis, Darzentas, Argiriou, & Tsaftaris, 2012; Madesis et al., 2012; Madesis, Ganopoulos, Bosmali, & Tsaftaris, 2013; Reed, Kent, & Wittwer, 2007) showed that this method could be used as an additional tool in similar experiments in the future. Overall cytoplasmic analysis data indicated that the eggplant cytoplasm was successfully substituted with the cytoplasm of *S. violaceum* by the backcrossing method.

The comparative study of three CMS eggplant lines developed in the present work may have some important implications for the development of CMS lines of the pollen non-release type. First, since the seed number per fruit of the CMS lines corresponded to that of the eggplant parents, it may serve as a criterion for the selection of eggplant cultivars prior to the development of CMS lines. Second, since the expression of male sterility/fertility is affected by the genotype of the nucleus donor (eggplant), it would be desirable to employ as many eggplant cultivars as possible in the interspecific crosses with the cytoplasm donor. Third, the populations segregating for the *Rf*-genes should be studied, if possible, in different climatic conditions or cultivating seasons in order to detect potential effects of modifier loci. Fourth, it would be interesting to ascertain if the *Rf*-genes discovered herein can restore the fertility in the rest of the pollen non-releasing CMS eggplant lines which lack the *Rf*-genes, in a similar way reported in the pollen-non formation CMS lines (Khan et al., 2014). In such a case, these pollen non-releasing CMS lines will be made available for eggplant hybrid seed production broadening the cytoplasmic sources of eggplant CMS, without the burden of re-developing the respective *Rf*-gene carrying lines.

4. Conclusions

The present study confirmed the suitability of *S. violaceum* as a cytoplasm donor for the development of functional CMS eggplant lines. The female fertility of CMS eggplant was not affected by the alien cytoplasm; however, the male fertility was negatively affected. The flowers of CMS plants were characterized by abnormal anther morphology, low pollen viability and prevention of anther dehiscence. Most importantly, the inheritance of the appropriate *Rf*-gene for this type of CMS was confirmed for the first time in eggplant functional CMS. Consistent inheritance of the *Rf*-gene was observed only in the advanced backcross progenies of CMS 'Tsakoniki'. The *Rf*-gene was dominantly inherited by the wild species and compensated for the negative effects

of the alien cytoplasm, thus restoring the male fertility. In addition, a modifying gene action on the *Rf*-gene was observed for the first time in eggplant functional CMS. The development of molecular markers linked to the *Rf*-genes would facilitate the incorporation of these genes in various eggplant genetic backgrounds. It was established that the nuclear genotype of the eggplant parent affected the phenotypic expression of CMS in the cytoplasmic background of *S. violaceum* as well as the mode of fertility restoration. In conclusion, our findings contribute towards the development and understanding of a novel CMS/*Rf*-gene system in eggplant.

References

Bletsos, F., Roupakias, D., Tsaktsira, M., & Scaltsoyjannes, A. (2004). Production and characterization of interspecific hybrids between three eggplant (*Solanum melongena* L.) cultivars and *Solanum macrocarpon* L. *Scientia Horticulturae, 101*(1-2), 11-21. http://dx.doi.org/10.1016/j.scienta.2003.09.011

Budar, F., & Pelletier, G. (2001). Male sterility in plants: occurrence, determinism, significance and use. *Comptes Rendus de l'Académie Des Sciences, Series III, Sciences de La Vie, 324*(6), 543-550. http://dx.doi.org/10.1016/S0764-4469(01)01324-5

Cao, B., Huang, Z., Chen, G., & Lei, J. (2010). Restoring pollen fertility in transgenic male-sterile eggplant by Cre/loxp-mediated site-specific recombination system. *Genetics and Molecular Biology, 33*(2), 298-307. http://dx.doi.org/10.1590/S1415-47572010005000043

Carlsson, J., Leino, M., Sohlberg, J., Sundström, J. F., & Glimelius, K. (2008). Mitochondrial regulation of flower development. *Mitochondrion, 8*(1), 74-86. http://dx.doi.org/10.1016/j.mito.2007.09.006

Chauhan, S. V. S. (1984). Studies in genic male-sterile *Solanum melongena* L. *Indian Journal of Genetics, 44*(3), 367-371.

Costa-Font, M., Gil, J. M., & Traill, W. B. (2008). Consumer acceptance, valuation of and attitudes towards genetically modified food: Review and implications for food policy. *Food Policy, 33*(2), 99-111. http://dx.doi.org/10.1016/j.foodpol.2007.07.002

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin, 19*, 11-15.

Duvick, D. N. (1956). Allelism and comparative genetics of fertility restoration of cytoplasmically pollen sterile maize. *Genetics, 41*(4), 544-565. Retrieved from http://www.genetics.org/content/41/4/544.short

European Commission. (2015). *Plant variety database-European Commission*. Retrieved November, 2015, from http://ec.europa.eu/food/plant/plant_propagation_material/plant_variety_catalogues_databases/search/public/index.cfm

Fang, M., Mao, R., & Xie, W. (1985). Breeding of cytoplasmically inherited male sterile lines of eggplant (*Solanum melongena* L.). *Acta Horticulturae Sinica, 12*, 261-266.

Ganopoulos, I., Madesis, P., Darzentas, N., Argiriou, A., & Tsaftaris, A. (2012). Barcode High Resolution Melting (Bar-HRM) analysis for detection and quantification of PDO "Fava Santorinis" (*Lathyrus clymenum*) adulterants. *Food Chemistry, 133*(2), 505-512. http://dx.doi.org/10.1016/j.foodchem.2012.01.015

Hanson, M., & Bentolila, S. (2004). Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *The Plant Cell Online, 16*(Suppl. 1), S154-S169. http://dx.doi.org/10.1105/tpc.015966

Hasnunnahar, M., Khan, M. R., & Isshiki, S. (2012). Inheritance analysis of fertility restoration genes (Rf) in a male sterile system of eggplant using cytoplasm of *Solanum grandifolium*. *Australian Journal of Crop Science, 6*(3), 475-479.

Hazra, P., Roy, T., & Choudhury, J. (2008). Characterization of genic functional male sterility in brinjal and its expression in different genetic backgrounds. *Indian Journal of Genetics, 68*(1), 47-51.

Hidalgo, P., Hesse, M., Ubera, J., & Frosch-Radivo, A. (1999). Microsporogenesis in male sterile *Rosmarinus officinalis* L. (*Lamiaceae*), an ultrastructural study. *Grana, 38*(6), 343-355. http://dx.doi.org/10.1080/00173130050136136

Isshiki, S., & Kawajiri, N. (2002). Effect of cytoplasm of *Solanum violaceum* Ort. on fertility of eggplant (*S. melongena* L.). *Scientia Horticulturae, 93*(1), 9-18. http://dx.doi.org/10.1016/S0304-4238(01)00314-4

Isshiki, S., & Yoshida, S. (2002). Characteristics of the cytoplasmic male sterility in the eggplant (*Solanum*

melongena L.) carrying the cytoplasm of *S. violaceum* Ort. *Bulletin of the Faculty of Agriculture, Saga University, 87*, 87-93.

Kakizaki, Y. (1931). Hybrid vigor in egg-plants and its practical utilization. *Genetics, 16*(1), 1-25.

Karihaloo, J., & Malik, S. (1995). A case of functional male sterility in *Solanum insanum* L. *Indian Journal of Genetics, 55*(1), 46-49.

Khan, M. M. R., & Isshiki, S. (2008). Development of a male sterile eggplant by utilizing the cytoplasm of *Solanum virginianum* and a biparental transmission of chloroplast DNA in backcrossing. *Scientia Horticulturae, 117*(4), 316-320. http://dx.doi.org/10.1016/j.scienta.2008.05.006

Khan, M. M. R., & Isshiki, S. (2009). Functional male-sterility expressed in eggplant (*Solanum melongena* L.) containing the cytoplasm of S. kurzii Brace & Prain. *The Journal of Horticultural Science & Biotechnology, 84*(1), 92-96.

Khan, M. M. R., & Isshiki, S. (2010). Development of the Male-sterile Line of Eggplant Utilizing the Cytoplasm of *Solanum aethiopicum* L. Aculeatum Group. *Journal of the Japanese Society for Horticultural Science, 79*(4), 348-353. http://dx.doi.org/10.2503/jjshs1.79.348

Khan, M. M. R., & Isshiki, S. (2011). Development of a cytoplasmic male-sterile line of eggplant (*Solanum melongena* L.) with the cytoplasm of *Solanum anguivi*. *Plant Breeding, 130*(2), 256-260. http://dx.doi.org/10.1111/j.1439-0523.2010.01788.x

Khan, M. M. R., Hasnunnahar, M., Iwayoshi, M., & Isshiki, S. (2014). Fertility restoration in three CMS systems of eggplant by the *Rf* genes of each other's systems and their SCAR marker. *Scientia Horticulturae, 172*, 149-154. http://dx.doi.org/10.1016/j.scienta.2014.04.013

Khan, M. M. R., Hasnunnahar, M., Iwayoshi, M., Ogura-Tsujita, Y., & Isshiki, S. (2015). Pollen degeneration in three functional male-sterile lines of eggplant with the wild *Solanum* cytoplasms. *Horticulture, Environment, and Biotechnology, 56*(3), 350-357. http://dx.doi.org/10.1007/s13580-015-0015-3

Kozik, E., Nowak, R., Nowakowska, M., & Dyki, B. (2012). Level of sterility and morphological flowers differentiation of petaloid male-sterile plants of carrot. *Journal of Agricultural Science, 4*(2), 187-194. http://dx.doi.org/10.5539/jas.v4n2p187

Linke, B., Nothnagel, T., & Börner, T. (2003). Flower development in carrot CMS plants: mitochondria affect the expression of MADS box genes homologous to GLOBOSA and DEFICIENS. *The Plant Journal, 34*(1), 27-37. http://dx.doi.org/10.1046/j.1365-313X.2003.01703.x

Ma, Y., Huang, W., Ji, J.-J., Gong, Z.-H., Yin, C.-C., Ahmed, S. S., & Zhao, Z.-L. (2013). Maintaining and restoring cytoplasmic male sterility systems in pepper (*Capsicum annuum* L.). *Genetics and Molecular Research: GMR, 12*(3), 2320-31. http://dx.doi.org/10.4238/2013.January.4.8

Madesis, P., Ganopoulos, I., Argiriou, A., & Tsaftaris, A. (2012). The application of Bar-HRM (Barcode DNA-High Resolution Melting) analysis for authenticity testing and quantitative detection of bean crops (*Leguminosae*) without prior DNA purification. *Food Control, 25*(2), 576-582. http://dx.doi.org/10.1016/j.foodcont.2011.11.034

Madesis, P., Ganopoulos, I., Bosmali, I., & Tsaftaris, A. (2013). Barcode High Resolution Melting analysis for forensic uses in nuts: A case study on allergenic hazelnuts (*Corylus avellana*). *Food Research International, 50*(1), 351-360. http://dx.doi.org/10.1016/j.foodres.2012.10.038

Mogensen, H. L. (1996). The hows and whys of cytoplasmic inheritance in seed plants. *American Journal of Botany, 83*(3), 383-404. http://dx.doi.org/10.2307/2446172

Nair, C. (1993). Mitochondrial genome organization and cytoplasmic male sterility in plants. *Journal of Biosciences, 18*(3), 407-422. http://dx.doi.org/10.1007/BF02702998

Nuttall, V. (1963). The inheritance and possible usefulness of functional male sterility in *Solanum melongena* L. *Canadian Journal of Genetics and Cytology, 5*(2), 197-199. http://dx.doi.org/10.1139/g63-029

Pahwa, R. S., Banga, S. K., Gogna, K. P. S., & Banga, S. S. (2004). Tournefortii male sterility system in *Brassica napus*. Identification, expression and genetic characterization of male fertility restorers. *Plant Breeding, 123*(5), 444-448. http://dx.doi.org/10.1111/j.1439-0523.2004.00960.x

Phatak, S. C., Liu, J., Jaworski, C. A., & Sultanbawa, A. F. (1991). Functional male sterility in eggplant: Inheritance and linkage to the purple fruit color gene. *Journal of Heredity, 82*(1), 81-83.

http://dx.doi.org/10.1093/jhered/82.1.81

Qureshi, S. J., Khan, M. A., Arshad, M., Rashid, A., & Ahmad, M. (2009). Pollen fertility (viability) status in Asteraceae species of Pakistan. *Trakia Journal of Sciences, 7*(1), 12-16. Retrieved from http://www.uni-sz.bg

Rajasekaran, S. (1970). Cytogenetic studies of the F1 hybrid *Solanum indicum* L.× *S. melongena* L. and its amphidiploid. *Euphytica, 19*(2), 217-224. http://dx.doi.org/10.1007/BF01902949

Reed, G. H., Kent, J. O., & Wittwer, C. T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics, 8*(6), 597-608. http://dx.doi.org/10.2217/14622416.8.6.597

Rodríguez, B., Prohens, J., & Nuez, F. (2008). Performance of hybrids between local varieties of eggplant (*Solanum melongena*) and its relation to the mean of parents and to morphological and genetic distances among parents. *European Journal of Horticultural Science, 73*(2), 76-83.

Saito, T., Matsunaga, H., Saito, A., Hamato, N., Koga, T., Suzuki, T., & Yoshida, T. (2009). A novel source of cytoplasmic male sterility and a fertility restoration gene in eggplant (*Solanum melongena* L.) lines. *Journal of the Japanese Society for Horticultural Science, 78*(4), 425-430. http://dx.doi.org/10.2503/jjshs1.78.425

Sambandam, C. N. (1962). Heterosis in eggplant (*Solanum melongena* Linn.). *Economic Botany, 16*(2), 71-76. http://dx.doi.org/10.1007/BF02985293

Sarkar, C. K. G., Zaman, F. U., & Singh, A. K. (2002). Genetics of fertility restoration of "WA" based cytoplasmic male sterility system in rice (*Oryza sativa* L.) using basmati restorer lines. *The Indian Journal of Genetics and Plant Breeding, 62*(4), 305-308.

Toppino, L., & Kooiker, M. (2011). Reversible male sterility in eggplant (*Solanum melongena* L.) by artificial microRNA mediated silencing of general transcription factor genes. *Plant Biotechnology Journal, 9*(6), 684-692. http://dx.doi.org/10.1111/j.1467-7652.2010.00567.x

Yang, J.-H., Zhang, M.-F., Jing-Quan, Y., Shuo, Z., Tao, W., & Zhu-Jun, C. (2005). Identification of alloplasmic cytoplasmic male-sterile line of leaf mustard synthesized by intra-specific hybridization. *Plant Science, 168*(4), 865-871. http://dx.doi.org/10.1016/j.plantsci.2004.10.018

Yoshimi, M., Kitamura, Y., Isshiki, S., Saito, T., Yasumoto, K., Terachi, T., & Yamagishi, H. (2013). Variations in the structure and transcription of the mitochondrial atp and cox genes in wild *Solanum* species that induce male sterility in eggplant (*S. melongena*). *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik, 126*(7), 1851-9. http://dx.doi.org/10.1007/s00122-013-2097-6

Zhu, Y., Saraike, T., Yamamoto, Y., Hagita, H., Takumi, S., & Murai, K. (2008). Orf260Cra, a novel mitochondrial gene, is associated with the homeotic transformation of stamens into pistil-like structures (pistillody) in alloplasmic wheat. *Plant & Cell Physiology, 49*(11), 1723-33. http://dx.doi.org/10.1093/pcp/pcn143