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Wild *Nicotiana* Species as a Source of Cytoplasmic Male Sterility in *Nicotiana tabacum**

by

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SUMMARY

The results of our experiments executed to obtain tobacco male sterile lines through interspecific hybridization are summarized. Ten wild species from the genus *Nicotiana: N. excelsior (exc), N. amplexicaulis (amp), N. rustica (rus), Nicotiana glauca (gla), N. velutina (vel), N. benthamiana (ben), N. maritima (mar), N. paniculata (pan), N. longiflora (lon)* and *N. africana (afr)* were used as cytoplasmic donors and *N. tabacum*, cv. Harmanliiska Basma (HB) as a donor of the nucleus. Genetic effects of cytoplasmic-nuclear interaction of the studied species are discussed. Our results suggested that cytoplasmic male sterility (CMS) was expressed when the cytoplasms of the above mentioned wild *Nicotiana* species were combined with the nucleus of *N. tabacum*. The 10 sources of CMS obtained in tobacco were characterized by altered flower phenotypes. Flowers are classified into types according the stamen, pistil and corolla modification. All these CMS sources were backcrossed to Oriental tobaccos, cvs. Tekne, Nevrokop B-12, Kroumovgrad 90 and Djebel 576, to develop corresponding CMS lines. The investigated cytoplasms produced compete male sterility in all those cultivars. The CMS lines preserved flower types, specific for every "sterile" cytoplasm. The extent of male organ modifications varied from apparently normal (but pollenless) stamens in CMS *(pan), (afr),* some plants of *(vel) (mar)* through different degrees of malformations (shriveled anther on shortened filaments *(lon),* pinnate-like anthers on filaments of normal length *(amp),* petal - *(ben),* pistil- or stigma-like structures *(rus), (gla)*) to lack of male reproductive organs in *(exc)* and in some plants of *(vel), (mar), (rus)* and *(gla).* Most of the above mentioned cytoplasms had normal female gametophyte and good seed productivity. Alterations of the pistils were observed in CMS (*rus), (exc)* and *(ben)* causing reduction of the seed set. Electrophoresis of seed proteins of the tobacco cultivars and their CMS lines also suggested that the nuclei of wild species was entirely displaced by the nucleus of *N. tabacum*. CMS lines with cytoplasms of *N. velutina, N. maritima, N. paniculata, N. longiflora* and *N. amplexicaulis* were selected as suitable for seed production in tobacco. [Beitr. Tabakforsch. Int. 20 (2002) 301–311]

ZUSAMMENFASSUNG

In der vorliegenden Arbeit werden die Ergebnisse unserer Experimente zur Herstellung männlich steriler Tabaklinien durch interspezifische Hybridisierung zusammengefasst. Zehn Wildtypen der Gattung *Nicotiana*: *N. excelsior* (*exc*), *N. amplexicaulis* (*amp*), *N. rustica* (*rus*), *N. glauca* (*gla*), *N. velutina* (*vel*), *N. benthamiana* (*ben*), *N. maritima* (*mar*), *N. paniculata* (*pa*n), *N. longiflora* (*lon*) und *N. africana* (*afr*) wurden als Cytoplasmadonoren und *Nicotiana tabacum*, Sorte Harmanliiska Basma (HB) als Kerndonor verwendet. Genetische Effekte der Cytoplasma-Kern-Wechselwirkung bei den untersuchten Arten werden diskutiert. Unsere Ergebnisse zeigen, dass cytoplasmatische männliche Sterilität (CMS) dann entsteht, wenn das Cytoplasma der oben genannten wilden *Nicotiana*-Arten mit dem Kern von *Nicotiana tabacum* kombiniert werden. Die erhaltenen 10 Quellen für CMS bei Tabak unterscheiden sich charakteristisch in ihrem Blütenphänotypus. Die Blüten können aufgrund veränderter Staubgefäße, Stempel und Blütenkrone klassifiziert werden. Alle diese CMS-Quellen wurden mit den Orienttabak-Sorten Tekne, Nevrokop B-12, Kroumovgrad 90 und Djebel 576 rückgekreuzt, um von ihnen CMS-Analoge zu entwickeln. Das eingekreuzte Zytoplasma verursachte bei all diesen Sorten vollständige männliche Sterilität. In den CMS-Analogen blieben die für jedes "sterile" Cytoplasma charakteristische Blütentypen erhalten. Das Ausmaß der Veränderung der männlichen Organe variierte von offensichtlich normalen (aber pollenlosen) Staubgefäßen bei (*pan*), (*afr*) und einigen Pflanzen von (*vel*) und (*mar*) über verschiedene Grade von Missbildungen (gefaltete Antheren auf verkürzten Filamenten bei (*lon*), gefiederte Antheren auf Filamenten normaler Länge bei (*amp*), blütenkrone-ähnlichen (*ben*), stempel-oder narben-ähnlichen Strukturen (*rus*), (*gla*) bis zum völligen Fehlen männlicher Reproduktionsorgane bei (*exc*) und einigen Pflanzen von (*vel*), (*mar*), (*rus)* und (*gla*). Die meisten der oben beschriebenen Cytoplasmen hatten normale weibliche Gametophyten und eine gute Saatgutproduktivität. CMS-Analoge von (*rus*), (*exc*) und (*ben*) wiesen Veränderungen bei den Stempeln auf, was zu einer verringerten Saatgutausbeute führte. Die Elektrophorese der Samenproteine der

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Tabaksorten und ihrer CMS-Analogen lieferten weitere Beweise dafür, dass der Kern der Wildtypen vollständig durch den Kern von *N. tabacum* ersetzt war. CMS-Analoge mit dem Cytoplasma von *N. velutina, N. maritima, N. paniculata, N. longiflora* und *N. amplexicaulis* wurden als geeignete Sorten für die Produktion von Tabak-Saatgut ausgewählt. [Beitr. Tabakforsch. Int. 20 (2002) 301–311]

RESUME

Les résultats de nos études sur le développement des lignées cytoplasmiques mâle-stériles obtenues par hybridation interspécifique sont présentés. Dix espèces sauvages du genre *Nicotiana* : *N. excelsior (exc), N. amplexicaulis (amp), N. rustica (rus), Nicotiana glauca (gla), N. velutina (vel), N. benthamiana (ben), N. maritima (mar), N. paniculata (pan), N. longiflora (lon)* et *N. africana (afr)* ont été utilisées comme donneurs de cytoplasme et *N. tabacum* cv. Harmanliiska Basma (HB) comme donneur du noyau. Les effets génétiques de l'interaction entre cytoplasme et noyau des espèces étudiées sont présentés. Nos résultats suggèrent que la stérilité mâle cytoplasmique (CMS) est exprimée si les cytoplasmes des espèces sauvages du genre *Nicotiana* mentionnées ci-dessus sont combinées avec le noyau de *N. tabacum*. Les dix sources de CMS obtenues chez le tabac sont caractérisées par des phénotypes altérés de la feuille. Les feuilles sont classées en groupes, selon les caractères morphologiques des étamines, pistils et corolles. Toutes les dix sources de CMS ont été rétro-croisées avec des tabacs orientaux, cvs. Tekne, Nevrokop B-12, Kroumovgrad 90 et Djebel 576, pour obtenir des lignées CMS correspondantes. Les cytoplasmes examinés ont produit une stérilité mâle complète chez tous ces cultivars. Les lignées CMS ont préservé la morphologie florale caractéristique pour chaque cytoplasme "stéril". L'importance des modifications de l'organe mâle allait des étamines apparemment normales (mais sans pollen) chez CMS (*pan*), (*afr*) et certaines plantes de (*vel*), (*mar*), à divers degrés de malformation (anthères ridées et filaments raccourcis (*lon*), anthères plumeuses avec des filaments à longueur normale (*amp*), structures de types pétales (*ben*), pistils ou stigmates (*rus*), (*gla*)) et à l'absence complète des organes mâles de reproduction chez (*exc*) et certaines plantes de (*vel*), (*mar*), (*rus*) et (*gla*). Chez la plupart des cytoplasmes mentionnés ci-dessus le gamétophyte femelle est normale et la productivité de semences est bonne. Les pistils sont altérés chez CMS (*rus*), *(exc*) et (*ben*) causant une réduction du rendement de semences. Les spectres électrophorétiques des protéines de semence des cultivars de tabac et leurs analogues CMS suggèrent également que les noyaux des espèces sauvages sont totalement remplacé par le noyau de *N. tabacum*. Des lignées CMS ayant les cytoplasmes de *N. velutina, N. maritima, N. paniculata, N. longiflora* et *N. amplexicaulis* sont considérées comme appropriées pour la production des semences de tabac. [Beitr. Tabakforsch. Int. 20 (2002) 301–311]

INTRODUCTION

Cytoplasmic male sterility (CMS) is of great importance for the production of hybrid varieties with higher yield, increased resistance to disease and better performance in different environments compared with the parental lines (1–4). It avoids manual emasculation which is labour intensive and impractical for plants with bisexual flowers.

CMS is defined as the inability to produce functional pollen with the preservation of normal female fertility and normal vegetative development (1). This trait is maternally inherited through the egg and the CMS phenotype is in most cases associated with mitochondrial DNA alternations (5–12).

In tobacco CMS is usually observed when the nucleus of *Nicotiana tabacum* is combined with the cytoplasm of some wild species of the same genus (13). This sterility is alloplasmic. The transfer of tobacco nuclear genome into the alien cytopasm is achieved by interspecific hybridization followed by several recurrent pollinations with *N. tabacum*. It has been established that CMS results from the incompatibility of the action of the tobacco nucleus with cytoplasmic genes of the wild species (2,14–19).

Introduction of new sources of cytoplasmic male sterility is highly desirable, since already available CMS lines may become susceptible to certain pathogens, such as the outbreak of Southern corn blight due to *Helminthosporium maydis* on USA maize hybrids carrying T-type cytoplasm (20, 21). To overcome this problem, diversification of CMS sources is essential.

In this paper we summarize our results of hybridization of some wild *Nicotiana* species with *N. tabacum* and describe the resulting CMS flower types. We report the development of CMS lines of cvs. Harmanliiska Basma, Tekne, Nevrokop B-12, Kroumovgrad 90 and Djebel 576 with cytoplasms from *N. excelsior, N. amplexicaulis, N. rustica, N. glauca, N. velutina, N. benthamiana, N. maritima, N. paniculata, N. longiflora,* and *N. africana*. The collection was based on a single *Nicotiana tabacum* genotype and different "sterile" cytoplasms as well as on several *Nicotiana tabacum* genotypes combined with only one "sterile" cytoplasm. So it offered an opportunity to compare the effect of the cytoplasms and nuclei in different combinations.

Since seed proteins are coded mainly by nuclear genes (22), electrophoretic patterns of seed proteins were also studied as additional evidence that the nuclei of wild species were entirely replaced by that of the donor parent cv. Harmanliiska Basma (HB).

MATERIAL AND METHODS

Sexual hybridization was achieved through routine crossing techniques. *N. excelsior (exc), N. amplexicaulis (amp), N. rustica (rus), N. glauca (gla), N. velutina (vel), N. benthamiana (ben), N. maritima (mar), N. paniculata (pan), N. longiflora (lon)* and *N. africana (afr)* were used as female parents and *N. tabacum* cv. Harmanliiska Basma (HB) was used as a pollinator. The combination *N. rustica* × *N. tabacum* was carried out via bridge crossing with *N. alata (N. rustica* × *N. alata)* × *N. tabacum*. Seeds from *N. africana* crosses were grown *in vitro* on Murashige and Skoog (23) agar nutrient medium (MS) for embryos (Table 1) because of their reduced germination capacity. Tissue culture methods have been applied to overcome the sterility of F_1 hybrids from the following combinations: *N. velutina*

Table 1. List of supplements added to basic MS agar nutrient medium for different applications

Supplements (mg L^{-1})	Embryo-genesis	Callus	Organo-genesis	Rooting
Caseine-hydrolysate	500	500	0	0
NAA	0.05	2	0	0
Kinetin	0.05	0.5	2	0
IAA			0.5	
Ferulic acid		0	0	2
Sucrose (g L^{-1})	30	30	30	15
Agar agar (g L^{-1})	7	7	7	7
Yeast extract	500			
Inositol	100			
Gibberellic acid	0.1			

NAA = α-naphtene acetic acid.
IAA = indole-3-acetic acid.

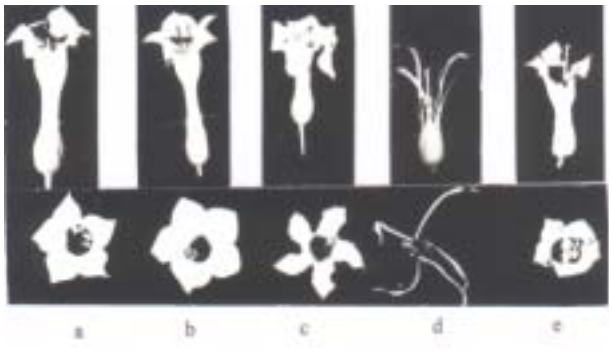

Figure 1 (a–e). Different corolla morphologies of the CMS sources. 1a: Type 1; 1b: Type 2; 1c: Type 3; 1d: Type 4; 1e: Type 5.

× *N. tabacum, N. benthamiana* × *N. tabacum, N. maritima* × *N. tabacum, N. paniculata* × *N. tabacum, N. longiflora* × *N. tabacum and N. africana* × *N. tabacum*. Stem pith parenchyma from their F_1 hybrids was used as explant material for *in vitro* culture. Explants of 2–3 cm were sterilised in 70% ethanol for 1–2 min, followed by 15% potassium hypochlorite solution for 10 min and rinsed 3 times in autoclaved H_2O. They were grown in 12 h photoperiod and 25 ± 2 ˚C. Basic MS agar nutrient medium with supplements (Table 1) was used to induce callus, organogenesesis and rooting. The callus produced was subcultured every 30 d. Organogenesis was induced from the third to the sixth passage depending on the hybrid combination. The F_1 hybrids, the regenerants (R) obtained and BC_1P_2–BC_6P_2 were grown in a green-house and then in the field. Chromosome counts were made on squashed root tips fixed in Clarke's solution and stained with shiff: orceine (*v*/*v* = 1:1) mixture. For meiotic investigations anthers were squashed in 4% acetocarmine. Pollen stainability was determined on temporary carmine-glycerine (*v*/*v* = 1:1) preparations. Flower types of the alloplasmic sources are presented in Table 2 and Figures 1–3. The types of flower malformations are expressed in code consisting of three Arabic numbers separated by points. Figure 1 designates corolla type, Figure 2 the pistil type and Figure 3 stamen type.
Male sterile lines of cvs. Harmanliiska Basma, Tekne, Nevrokop B-12, Kroumovgrad 90 and Djebel 576 with

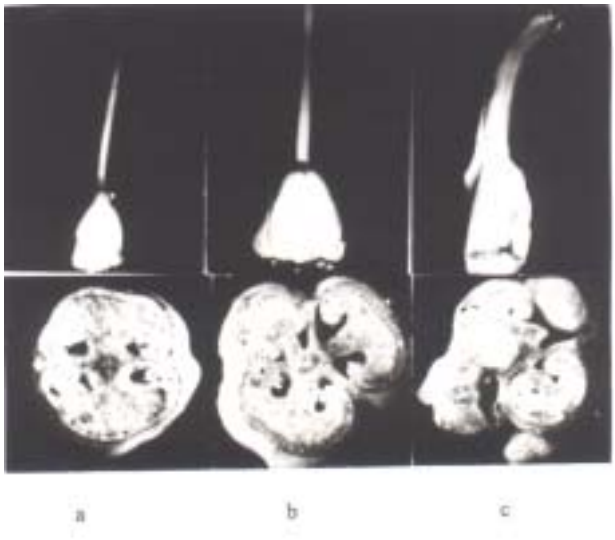

Figure 2 (a–c). Different pistil morphologies of the CMS sources. 2a: Type 1; 2b: Type 2; 2c: Type 3.

cytoplasms from the studied species were obtained through a minimum of six backcrosses (BC_6) of F_1 and R_1 plants. Seed samples (from 5 to 10 individual plants) were ground to a fine powder and 0.01 g of the meal was extracted either with 7 *M* urea dissolved in water:glycerol (2:1, *v/v*), or with 0.05 *M* Tris-HCl buffer, pH 8.0, containing 0.2% sodium dodecyl sulphate (SDS), 5 *M* urea and 2% 2-mercaptoethanol (2-ME). Two gel systems for electrophoretic analysis were used: a) 7.5% acidic polyacrylamide gel, pH 4.3 (24); b) 12.5% SDS-PAGE, pH 8.8, according to LAEMMLI (25). Both gels contained 5 *M* urea (26,27). Dual electrophoretic unit for vertical slab electrophoresis with gel size 90/70/1 mm and 12 wells was applied.

RESULTS AND DISCUSSION

Male sterile plants: CMS obtained through routine hybridization technique

N . excelsior × *N. tabacum*: In earlier experiments (18,28) MS plants were observed in F_1 hybrids of the cross *N. excelsior* × *N. tabacum.* The observed flower malformations in this combination could be classified into the following group: corolla normal (Type 1: Table 2, Figure 1a), partially split with preserved flower tube (Type 3: Figure 1c), completely split (Type 4: Figure 1d); pistil normal, two-loculed (Type 1: Figure 2a); stamens normal anther (without sporogenic tissue in the locules) with shortened filament (Type 2: Figure 3b), modified into secondary stigma (pistil without ovary) (Type 11: Figure 3k), filiform (Type 12: Figure 3l); completely missing (Type 0). Such variation in the flower development as early as in the F_1 generation has not been found in previously existing tobacco CMS sources. The sterility of *N. excelsior* × *N. tabacum*, cv. HB F_1 hybrid was maintained until at least the BC_6 generation confirming its cytoplasm nature. The following flower types were presented in *(exc)* HB BC_6 (Figure 4): corolla – normal, partially split, completely split; pistil – normal, sometimes fasciated (Type 3: Figure 2c); stamens – completely missing.

Table 2. Morphological characterization of modified CMS tobacco flowers[a]

Type	Corolla	Pistil	Anther	Filament		No.
				Length	Additional formations	
0	–	–	absent	absent	–	0
1	normal	normal	normal	normal	–	5
2	normal, excerted stigma	three-loculed	normal	moderate to strongly shortened	petaloidy of variable degree	1–5
3	partially split	fasciated	normal, stigmatoidy on the top	normal	secondary petals, protruding above the corolla	5
4	completely split	–	normal, stigmatoidy on the top	moderate to strongly shortened	petaloidy of variable degree	1–5
5	shortened flower tube		shrivelled	moderate to strongly shortened	–	5
6	–	–	spherical, sometimes with ovules	moderate shortened	–	2–5
7	–	–	consisting of several anthers	moderate shortened	–	1–3
8	–	–	modified into secondary pistil	moderate shortened	–	1–3
9	–	–	modified into secondary petal	strongly shortened	–	5
10	–	–	pinnate like anthers	normal	–	5
11	–	–	modified into secondary stigma	normal to moderate shortened	–	1–3
12	–	–	filiform	strongly shortened	–	1–5
13	–	–	modified into flower buds	strongly shortened	–	1–3

[a]The table comprises all flower types of CMS sources in tobacco developed up to now. It provides the possibility of including every new type of flower malformations.

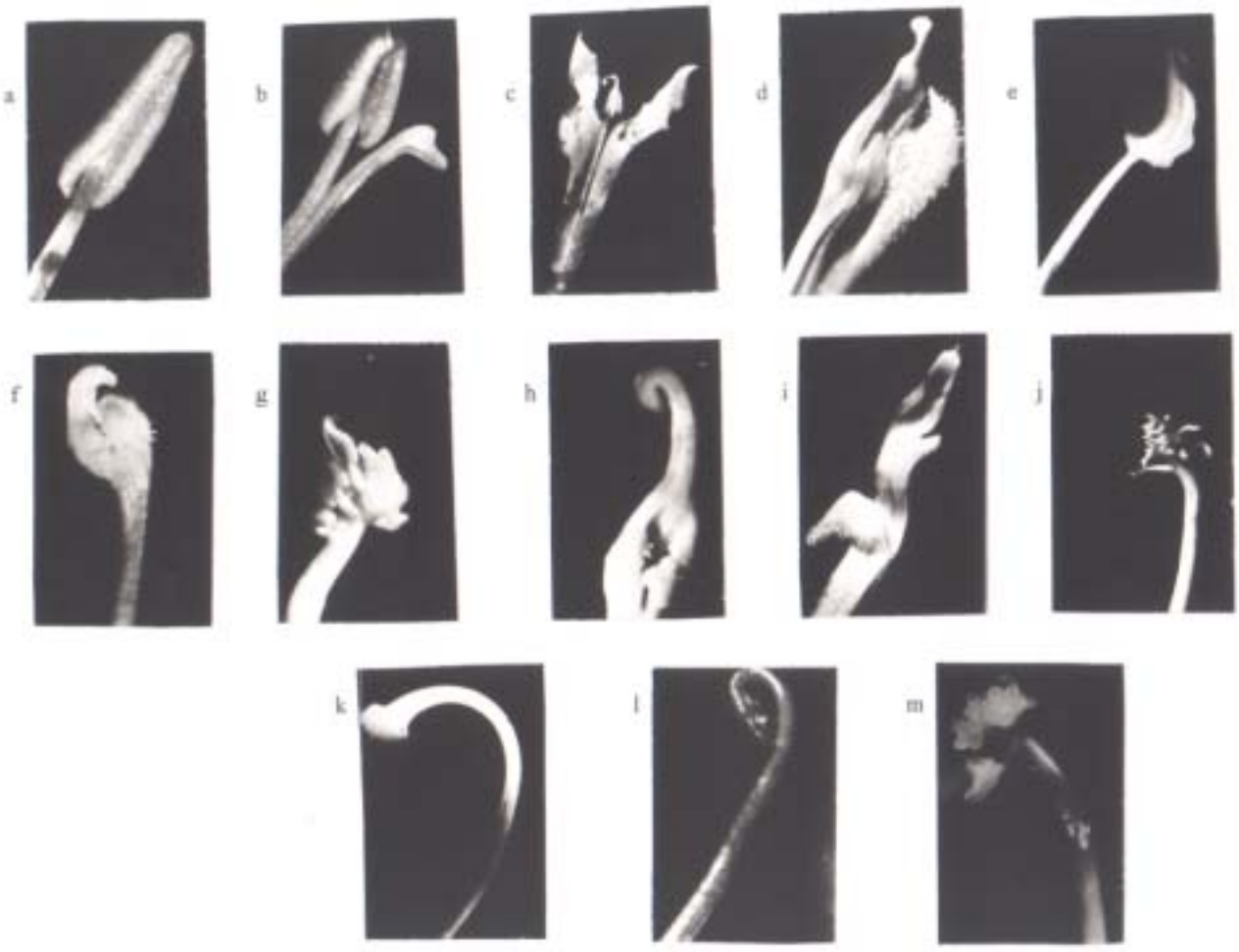

Figure 3 (a–m). Different stamen morphologies of the CMS sources. 3a: Type 1; 3b: Type 2; 3c: Type 3; 3d: Type 4; 3e: Type 5; 3f: Type 6; 3g: Type 7; 3h: Type 8; 3i: Type 9; 3j: Type 10; 3k: Type 11; 3l: Type 12; 3m: Type 13.

Figure 4. Flower morphology of *(exc)* HB BC_6 male sterile plants

The most frequent pistil modification was the occurrence of 3 to 6 locules, most of them without ovules. Polyloculed pistil was often accompanied by considerable shortening and fasciation of the style (flat, hollow with furrowed surface) and a stigma with 3 to 6 lobes.

On the bases of the changes in the flower elements (corolla, pistil and stamens) and their respective combinations in BC_6, four types of flower modifications were observed: 1.1.0; 1.3.0; 3.1.0; 3.3.0; 4.1.0; 4.3.0 according to the nomenclature mentioned above.

N. amplexicaulis × *N. tabacum*: We found male sterile plants in the BC_1P_2 generation of the hybrid combination

Figure 5. Flower morphology of *(amp)* HB BC_6 male sterile plants

(*N. amplexicaulis* × *N. tabacum)* × *N. tabacum* (18,29). They had two corolla types: normal and partially split, a two-loculed pistil, stamens with normal (Type 1: Figure 3a) or pinnate-like anthers of normal filament length (Type 10: Figure 3j). Microsporogenesis did not occur because of

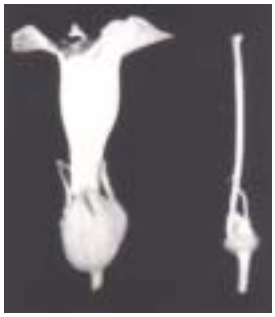

Figure 6. Flower morphology of *(rus)* HB BC_6 male sterile plants

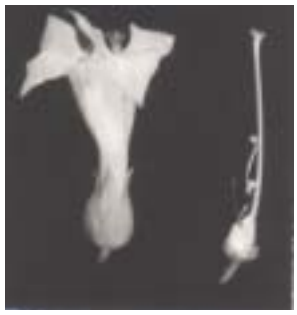

Figure 7. Flower morphology of *(gla)* HB BC_6 male sterile plants

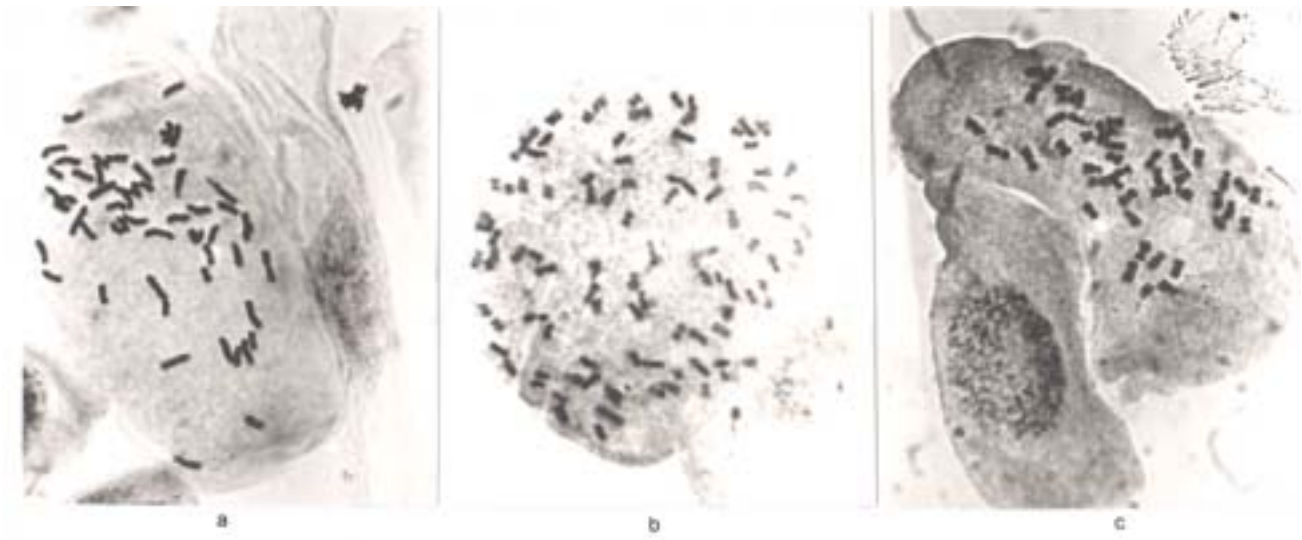

Figure 8. Root meristem metaphase plates of F_1 *N. velutina* × *N. tabacum* regenerants from 6th passage 2n = 54 (a); 9th passage 2n = 83 (b); BC_6 – 2n = 48 (c)

rudimentary archesporial tissue. Male sterility was maternally inherited and maintained in further progeny, which is indicative for its cytoplasmic origin. (*Amp*) HB BC_6 generation had different flower types: corolla normal, or partially split, or completely split; pistil normal; stamens with filaments of normal length, they were pollenless and had pinnate-like anthers (Figure 5). The following flower types were observed: 1.1.10 ; 3.1.10; 4.1.10.

N. rustica × *N. tabacum*: HART (30) obtained CMS plants in BC_1P_2 of a direct cross between *N. rustica* × *N. tabacum*. He has observed anther lobes with or without deformations and nonfunctional pollen degenerating after tetrad stage.
We also obtained male sterile plants in BC_4P_2 from the interspecific hybrid *N. rustica* × *N. tabacum* via bridge crossing with *N. alata* (18,31). These plants displayed a normal corolla and normal or deformed pistil. They were stamenless or with 1 to 2 stamens modified into secondary pistils, sometimes covered with external ovules (Type 8: Figure 3h), or into secondary stigma, or filiform. Male sterility was uniformly inherited up to the BC_5–BC_6 generations, confirming its CMS nature (Figure 6). The flower types of (*rust*) HB BC_6 were 1.1.0, 1.3. 0, 1.1.8, 1.3.8, 1.1.11, 1.3.11, 1.1.12 and 1.3.12.

N. glauca × *N. tabacum*: BERBEC (32) obtained CMS plants by crossing *N. glauca* and *N. tabacum* and backcrossing its F_1hybrids with *N. tabacum*. Normal corolla and feminized stamens were typical for this CMS source.
We also reported male sterility with cytoplasm from *N. glauca* in F_2 of *N. glauca* × *N. tabacum* (33,34). These plants had normal corolla and pistil, were without stamen, or with 1 to 3 stamens with normal anthers of shortened filament, or with 1 to 2 stamens modified into secondary pistils or into secondary stigmas. Histological investigations of the deformed stamens (Type 2) showed decreased anther locules. Meiosis in the pollen mother cells (PMC) were comparable with the fertile plants but the pollen died after the tetrad stage. In some plants PMC perished at the early stages of meiosis, and anther locules degenerated and sometimes instead of sporogenic tissue development of ovules were observed. Male sterility obtained in F_2 was maintained in BC_6P_2 suggesting its cytoplasmic nature (Figure 7). The following flower types were observed in (*gla*) HB BC_6: 1.1.0; 1.1.2; 1.1.8; 1.1.11.

Male sterile plants: CMS obtained using in vitro techniques

N. velutina × *N. tabacum:* Our earlier investigations (17,35) demonstrated that the interaction between *N. tabacum* nucleus and the cytoplasm of *N. velutina* also resulted in CMS. The F_1 hybrids were completely sterile. Tissue culture was used to overcome the sterility of the F_1 *N. velutina* × *N. tabacum* hybrids. Organogenesis was induced in the 3rd to 6th and 9th callus subcultures. The

Figure 9. Flower morphology of *(vel)* HB BC_6 male sterile plants

Figure 10. Flower morphology of *(ben)* HB BC_6 male sterile plants

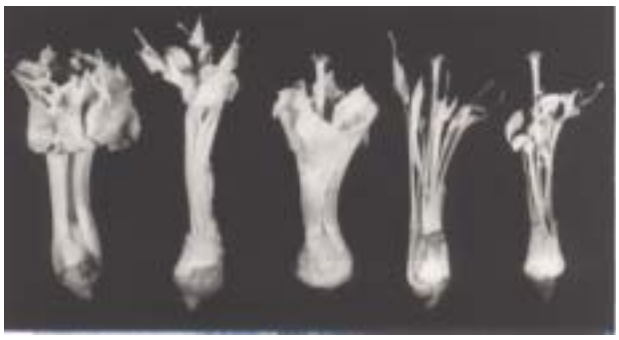

Figure 11. Flower morphology of *(ben)* HB BC_6 male sterile plants – corollas were removed

regenerants of the 3rd to 5th passage that had chromosome numbers close to those of the F_1 hybrid were completely sterile (female and male), while those ones obtained from longer cultured callus (6th passage) were mixoploid with chromosome number 2n = 46–58 (Figure 8a). This phenomenon is probably affected by hormone action, because kinetin and α-naphthalene acetic acid are known to provoke polyploidy (36). The female sterility was overcome and seed capsules formed after pollination with *N. tabacum.* All plants in BC_1P_2 progeny were male sterile. They possessed normally developed corolla, pistils with two locules and stamens with normal anther morphology and shorten stamen filaments. Anthers contained a small number of sterile pollen grains. In the BC_6 generation the following flower types were observed: 1.2.0; 3.2.0; 4.2.0; (Figure 9); corolla normal, partially split, completely split; pistil three-loculed (Type 2: Figure 2b); stamens completely missing. R_1 plants from the 9th passage had chromosome numbers between 2n = 82 and 84 (Figure 8b), close to that of an amphidiploid. The female fertility of these plants was restored but there was no sporogenic tissue in the anther locules. The first backcross of R_1 with *N. tabacum* was male sterile, with normal corolla, two-loculed pistils and stamens with normal anther and filament length, however without sporogenic tissue. This flower type (1.1.1) was maintained after recurrent crosses with *N. tabacum*.

The male sterility of BC_1P_2 plants, that had originated from the 6th to 9th subculturings was maintained in BC_2–BC_6 confirming its cytoplasmic nature. In BC_6 the plants were fully similar in their morphological features (habit, leaf shape and size) with recurrent parent (cv. HB). Chromosome number 2n = 48 (Figure 8c) was counted in root tip meristems. This suggests that in BC_6 progeny the nucleus of the wild *N. velutina* species was fully replaced by that of *N. tabacum*. The following flower types were observed in (*vel*) HB BC_6: 1.1.1, 1.2.0, 3.2.0 and 4.2.0 (Figure 9).

N. benthamina × *N. tabacum:* RAMAVARMA *et al.* (37) obtained CMS in a hybrid with *N. benthamiana* via bridge crossing (*N. benthamiana* × *N. glutinosa*) × *N. tabacum.* In BC_1P_2 single plants with features of male sterility were observed. We also obtained male sterile plants in the same cytoplasm after a direct cross of *N. benthamiana* with *N. tabacum* (17,38). The incompatibility between these two species was overcome using mixed pollen from broadleaf tobacco type burley and from Oriental cultivars.

The F_1 hybrids were completely sterile. The same *in vitro* techniques as described above for *N. velutina* were used to overcome the female sterility of the F_1 *N. benthamiana* × *N. tabacum* hybrid. Organogenesis was induced during the 3rd passage. The regenerants that were obtained, were female fertile and could be successfully pollinated with *N. tabacum.* All plants in BC_1P_2 were male sterile. They exhibited a normal corolla, a deformed pistil with 3 to 6 locules and stamens often modified into secondary petals (Type 3: Figure 3c, Type 4: Figure 3d and Type 9 (Figure 3i) or into stigma-like structures. RAMAVARMA *et al.* (37) observed stamen distortions in BC_1 (*N. bethamiana* × *N. glutinosa*) × *N. tabacum* that had flower type similar to those obtained by us. In our investigation the following types of stamens were observed in BC_6: modified into secondary petals (Type 3, Type 4). Cytological analysis of root meristem cells showed that the plants in BC_1 – BC_5 were mixoploid with chromosome numbers 2n = 32–56, while in BC_6 a stabilization of the chromosome number to 2n = 48 was established. The male sterility noted in BC_1P_2 was maintained in BC_2P_2 – BC_6P_2 progenies and was determined to be CMS. The following flower types were observed in *(ben)* HB BC_6: 1.3.3 and 1.3.4 (Figures 10 and 11).

3. N. maritima × *N. tabacum:* In our experiments (17,39) male sterility was obtained by the interaction of the cytoplasm of *N. maritima* with the nucleus of *N. tabacum*. Tissue culture methods were used again to overcome the

Figure 12. Flower morphology of *(mar)* HB BC_6 male sterile plants

sterility of the F_1 hybrids of *N. maritima* × *N. tabacum*. Cytological analysis showed that some R_1 plants from the 4th passage had higher ratio (70–80%) of stainable pollen. These plants were mixoploid, with chromosome numbers of 2n = 78–82. After self pollination well-seeded capsules were obtained. All R_2 (offsprings of R_1 plants) had male sterile flowers with the following characteristics: normal corolla with shortened flower tube (Type 5: Figure 1e); normal pistil and stamens; a small number of sterile pollen grains. In BC_1 we observed flowers with normal to completely split corolla; with normal (two-loculed) or deformed (three to six-loculed) pistil and stamens with normal anthers without pollen, or with anthers modified into flower buds and shortened filaments (Type 13: Figure 3m) and filiform or completely absent stamens. The following flower types were observed in *(mar)* HB BC_6: 1.1.1; 1.1.0; 3.1.0; 4.1.0 (Figure 12).

4. N. paniculata × *N. tabacum:* We established that the wild species *N. paniculata* is a carrier of cytoplasmic male sterility (17,40). F_1 hybrid of the combination *N. paniculata* × *N. tabacum* was completely sterile. After *in vitro* cultivation we succeeded to restore its fertility (male and female). Organogenesis was induced at 3rd callus passage and average of 40 shoots per callus were formed. The regenerants obtained had chromosome numbers from 42 to 64. In 5 plants 40% viable pollen was found and seed capsules were formed after self-pollination. All R_2 plants were male sterile. They had a normal corolla, well developed two-loculed pistils and stamens with normal anther morphology and filament size, but without pollen. The male sterility observed in R_2 plants was maintained in BC_1P_2–BC_6P_2 progeny which confirmed its cytoplasmic origin. The flower type in (*pan*) HB BC_6 was the same as in the R_2 plants of the same hybrid, 1.1.1 (Figure 13).

5. N. longiflora × *N. tabacum:* We reported the development of male sterile plants resulting from a cross between the species *N. longiflora* and *N. tabacum* (41,42). Hybrids

Figure 13. Flower morphology of *(pan)* HB BC_6 male sterile plants

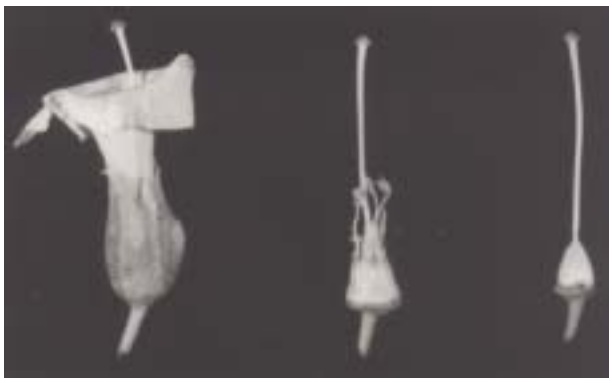

Figure 14. Flower morphology of *(lon)* HB BC_6 male sterile plants

of sexually incongruent species *N. longiflora* and *N. tabacum* were fully male and female sterile. The female sterility was overcome by inducing callus and organ formation from *in vitro* cultured stem pith parenchyma. The regenerants obtained after longer cultivation (6th passages) had chromosome numbers of 2n = 44–93 and restored female fertility. They were successfully pollinated with *N. tabacum*, forming seed-containing capsules. All BC_1P_2 plants obtained were male sterile. The flowers possessed normal corolla with strongly expressed longistily due to the shortened flower tube (Type 5: Figure 1e), normal pistil and stamens with shortened filaments and shrivelled anthers containing only negligible amount of sterile pollen grains. By repeatedly backcrossing of these plants with cv. HB the chromosome number of the somatic cells in BC_6 was gradually stabilised to 2n = 48. In (*lon*) HB BC_6 the plants had normal corolla, shortened tube and slight longistily; normal, two-loculed pistil; stamens with shortened filaments and shrivelled anthers without pollen (Type 5, Figure 3e) of the flower 5.1.5. The male sterile of BC_1P_2 (*N. longiflora* × *N. tabacum*) was preserved after series of backcrosses, which proved its cytoplasmic nature (Figure 14).

6. N. africana × *N. tabacum*: NIKOVA and ZAGORSKA (16,43) demonstrated that the interaction between *N. tabacum* nucleus and *N. africana* cytoplasm resulted in cytoplasmic male sterile plants after sexual hybridization of these species. The incompatibility between them was manifested in the early embryonic stages. Hybrid embryos were rescued and viable F_1 plants were grown by the method of embryocultures (44). The second successful

Figure 15. Flower morphology of *(afr)* HB BC_6 male sterile plants

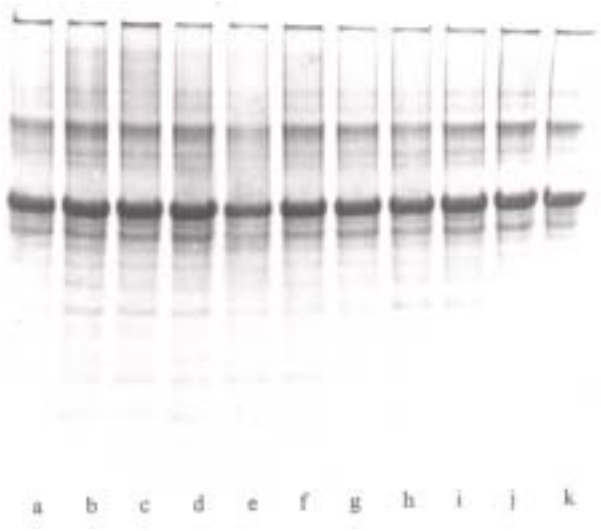

Figure 16. Electrophoretic spectra (7.5% acidic PAGE containing 5 *M* urea) of seed proteins from *N. tabacum,* cv. Harmanliiska Basma and its CMS analogues. 16 a : *N. tabacum,* cv. Harmanliiska Basma; 16 b: *(exc)* HB BC_6; 16 c: *(amp)* HB BC_6; 16d: *(rus)* HB BC_6; 16e: *(gla)* HB BC_6; 16f: *(vel)* HB BC_6; 16 g: *(ben)* HB BC_6; 16 h: *(mar)* HB BC_6; 16i: *(pan)* HB BC_6; 16j: *(lon)* HB BC_6; 16k: *(afr)* HB BC_6.

attempt to achieve this combination by somatic hybridization was that of KUMASHIRO *et al.* (45). The female sterility of the F_1 hybrid from *N. africana* × *N. tabacum* was overcome by in vitro culture. Cells with chromosome numbers close to that of the F_1 hybrid (2n = 44) predominated in the regenerants produced from callus during the first two passages. Most of the plants grown between the 3rd to 7th passage were mixoploid (2n = 46–82). They produced proper seed capsules after pollination with *N. tabacum*. All BC_1P_2 plants (63) were male sterile. They had flowers with well developed corollas, slightly expressed longistily (Type 2: Figure 1b); two-loculed pistils, and stamens with normal anthers and filament morphology (2.1.1) and 100% pollen sterility. Male gametophyte development stopped at the tetrad stage of meiosis.

KUMASHIRO *et al*. (45) obtained only one sterile plant (the remaining 210 regenerants had 100% fertile pollen) from protoplast fusion of *N. tabacum* with *N. africana*. Flowers of the sterile plant had semi-wrinkled and not fully opened corolla and shrunken anthers. According to KUMASHIRO *et al.* (45) CMS plants received by sexual hybridization might have a different flower morphology from the male sterile somatic hybrid. The authors found no sporogenic tissue in

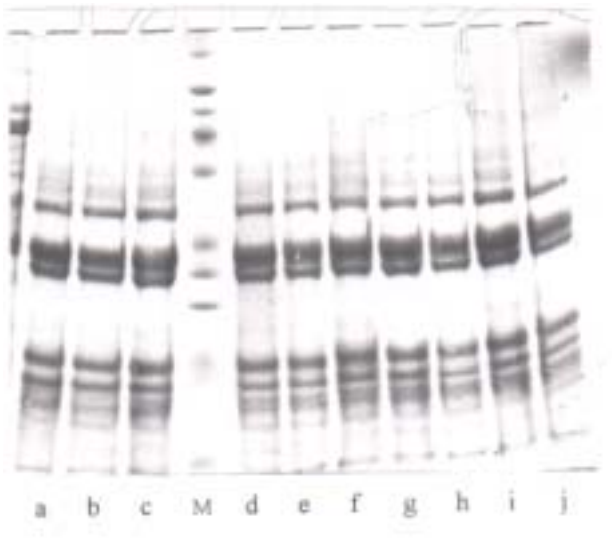

Figure 17. Electrophoretic spectra of seed storage proteins (12.5% SDS-PAGE containing 5 *M* urea) from *N. tabacum,* cv. Harmanliiska Basma and its CMS analogues. 17a: *N. tabacum,* cv. Harmanliiska Basma; 17b: *(exc)* HB BC_6; 17c: *(amp)* HB BC_6; 17d: *(rus)* HB BC_6; 17e: *(vel)* HB BC_6; 17f: *(ben)* HB BC_6; 17g: *(mar)* HB BC_6; 17h: *(pan)* HB BC_6; 17i: *(lon)* HB BC_6; 17j: *(afr)* HB BC_6; M: molecular mass markers (kDa): 205.5, 116.0, 97.0, 84.0+66.0, 55.0, 45.0, 36.0, 29.0, 24.0, 20.0, 14.2.

the sterile plant. In our experiments the flower type observed in BC_1P_2 was stabily inherited in further (BC_2–BC_6) back-crosses with no sporogenic tissue in the BC_6P_2 (Figure 15). The sterility observed in this hybrid combination was cytoplasmic, because it was maternally inherited.

Nuclear background

The obtained CMS sources were taken as the maternal parent and back-crossed six times BC_6 with Oriental tobaccos, cvs. Tekne, Nevrokop B-12, Kroumovgrad 90 and Djebel 576 as the recurrent parents. Examination was made of the BC_6 plants with respect to the morphology of their flower organs and the sexual function of their stamens and pistils. All the backcrossed lines having the cytoplasms from *N. excelsior, N. amplexicaulis, N. rustica, N. glauca, N. velutina, N. bentahamiana, N. maritima, N. paniculata, N. longiflora*, and *N. africana* exhibited complete male sterility. They maintained flower types specific for every sterile cytoplasm, independently of the cultivar. Modifications of male organs varied from apparently normal, but pollenless stamens in CMS (*pan*), (*afr*), some plants of (*vel*), (*mar*) BC6, through different malformations (shriveled anther on shortened filament (*lon*)), petal- (*ben*), pinnate- (*amp*) or pistil-like anthers (*rus*), (*gla*) BC6, to the lack of male reproductive organs in CMS (*exc*) and some plants of CMS (*vel*) and (*mar*) BC6. The CMS (*amp*), (*gla*), (*vel*), (*mar*), (*pan*), (*lon*) and (*afr*) sources had normal female gametophyte and good seed set. Alterations of the female organs three to six-loculed pistils (some of them being without ovules and reduced seed set) were found in CMS (*rus*), (*exe*) and (*ben*). The electrophoretic patterns of CMS lines were indistinguishable from the pattern of initial *N. tabacum* cultivar (Figures 16 and 17). Four of the wild species, donors of corresponding cytoplasms – *N. excelsior, N. rustica, N. paniculata* and *N. longiflora,* were previously

investigated by electrophoresis (26,27,42). Electrophoretic patterns of seed proteins of these particular wild species differed from the pattern of *N. tabacum*, cv. HB. The uniformity of electrophoretic spectra of *N. tabacum*, cv. HB and CMS lines studied therefore provided additional evidence that in the CMS lines the nuclei of the investgated wild species were entirely displaced by the nucleus of *N. tabacum* and that the observed flower malformations were determined by cytoplasmic genes only.

CONCLUSIONS

The study confirmed that CMS can arise as a result of the interspecific exchange of nuclear and cytoplasmic genomes. Our results suggest that successful interspecific hybridization can be achieved between otherwise incompatible species via sexual crosses followed by embryo rescue and tissue culture. The CMS tobacco sources obtained with cytoplasms from the investigated wild species proved the potential possibilities of wide crosses combined with *in vitro* methods for the enrichment of tobacco gene pool which is one of the main requirements for building up of the variety structure of this crop.

The CMS lines with cytoplasms from *N. velutina*, *N. maritima*, *N. paniculata*, *N. longiflora* and *N. amplexicaulis* might be useful for breeding and hybrid seed production in tobacco as they were without flower anomaly that could limit artificial pollination or decrease hybrid yield.

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