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# Application of hybrids with cytoplasmic male-sterility in *Zea mays* L. in China

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**Summary** The abortive behaviour and the cytoplasm quality of cytoplasmic male-sterility (CMS)lines of homocaryon were investigated and the existence of C race of *Bipolaris maydis* were discussed from the point of view of genetic breeding,physiology and pathology in this paper. Then the countermeasures to prevent the danger from disease of *B. maydis* in CMS hybrid production were proposed.

**Key words** corn (*Zea mays* L.),hybrid,cytoplasmic male-sterility,pathology

In the process of hybrid production of corn,it has such advantage as saving artificial emasculation,reducing cost and supplying high quality commercial seeds to make complete sterile hybrids,with the result that the heterosis potential is full exerted.

## Genetic breeding research

Beckett(1971)divided the CMS lines,that were many and diverse in the world,into three groups — T,S and C,accroding to the special reaction of fertile restoration on male flower. Afterwards,Gracen et al. (1974)provided same result. After that,in molecular level,the electrophoresis pattern of mt DNA showed only three kinds of bands of C,S and T groups. According to this result,experts in maize breeding were in agreement on classification of CMS lines. Later on,Pring et al. (1980)divided C group into C Ⅰ,C Ⅱ and C Ⅲ three subgroup further.

The aborted features of male gametophyte sporule in T,C and S groups were deeply studied by predecessor. The process of male gametophyte abortion is as follows.

Stamen primordium→Callose stage→Meiosis stage(T)→Tetrad stage(C)→Young male gametophyte stage→Young pollen stage,Building up [Vegetative nucleus / Generative nucleus](S)→Mature pollen(generative cell produce a pair of gamete)(N)

T and C groups belong in sporophyte abortion which occurres in early period of male development. For this kind of plant the glume is closed and the anther shrivels and includes seriously aborted pollen or produces no pollen.

S cytoplasm belongs to abortion of gametophyte and abortion occurres in late period of male development. In this type of plant,the anther showes different pattern from un-naked type to naked type. The anther fertility usually decreases in cold and moist climate area or short sunshine duration region.

What can we learn from the difference of initial stage in the sporule abortion of T,C and S cytoplasms? The S cytoplasm and normal cytoplasm (N) plants with same nucleus can produce young pollen in development process and the pollen of them can produce vegetative and generative nucleus. When pollens nearly mature,the pollens of S cytoplasm suddenly crumble and the plants become male-sterile. We would suggest that the characteristic of cytoplasm in S and N was close in metabolism,adaptability to environment and disease resistance, but there was great difference on the above characteristic for C, T cytoplasm with N cytoplasm. This problem needs further evidence from production and experiment.

The most early and extensive utilization of male-sterility in the world was T cytoplasm. Research on this field started in 70's in China. Li Jingxiong et al(1961,1963), Yang Yunkui et al(1962), Liu Jilin(1979), Chen Weicheng et al(1979,1986), Wei Jiankun et al (1980), Zheng Yonglian(1982) and Zeng Mengqian et al. (1987) published a series of documents in this field. They established the theoretical basis for utilization of sterile hybrid of maize in China. The CMS38-$11^{T}$ was used as the first line of double hybrid, Nong Da 4 and Nong Da 7 in 50's by Beijing Agricultural University. In the same time, Shuangyue-150 was derived into T double hybrid. Because of endangerment of *B. maydis*, the yield of this hybrid reduced by 46. 3%. Afterwards, the case of yield decreased due to *B. maydis* occurred in succession and even had no output in some place in China. By 1967, the research works utilizing T cytoplasm stopped in China.

In 60's T sterility began to be used in America. The rate of hybrid with T cytoplasm was 85% in 1970. As epidemic of T race of *B. maydis* (Hooker, 1970) the yield lose was 15% of total output of corn, that was about 16. 5 million ton. Also CMS-T cytoplasm was highly specialy infected by *Phylosticaea maydis*.

After 1970, the utilization of CMS-T cytoplasm was stopped and artificial emasculation was used again in America. When C cytoplasm was discovered, which was more resistant to disease, the C cytoplasm hybrid began to be used. In China, the C cytoplasm was going to be used in Henan, Sichuan and Hebei province and others. It can be said that the past few years just were on the eve of the action to use CMS C line greatly in China and all over the world. Meanwhile, there are some questions proposed by breeders and pathologist. The T cytoplasm was very weak in disease resistance, and how was about C cytoplasm? Will the calamity of great reduction of corn yield in America repeat again?

## Physiologic research

To be aimed at above question, the parameters of cell with sterile cytoplasm were tested in four aspects by us(Wei Jiankun et al, 1989).

(1)Cytoplasmic viscosity;

(2)Water permeability;

(3)Solute potential;

(4)Cytoplasmic streaming.

The cytoplasmic viscosity was tested using three methods i. e. plasmolysis, centrifugation and systrophy.

*Cytoplasmic Viscosity*

(1)Centrifugation method In three leaf age, 10 cm long segments of corn stem were cut off from health seedling and were put into certrifuge tube in which there was some spring water; then they were subsequently centrifuged at 2100 rpm. After that, 5 mm long piece of bud sheath was peeled and the subepidermal cell was observed under microscope ($\times$160 or $\times$400)and the chroloplast displacement by centrifugation can be served as relative measure for cytoplasmic viscosity. When 95—100% of chroloplasts moved to one end of cell, the time was noted. The longer time was, the higher cytoplast viscosity was.

The results (Table 1) show that all of five genotypes tend to same result that chroloplast displacement time in four cytoplasms was different. The sequence was N>S>C>T. This result indicated that the viscosity of cytoplasm in four type was N>S>C>T.

**Table 1.** The displacement time of chloroplast in subepidermal coleoptile cell in five inbred lines of homocaryon in maize*

| Genotype | Cytoplasm | Time required for 95-100% chloroplast displacement(min) |
|---|---|---|
| C103 | N | 30.6±1.3c* |
| | S | 16.8±1.5b |
| | C | 15.4±2.0b |
| | T | 12.1±1.3a |
| Mo17 | N | 31.6±2.2d |
| | S | 24.4±3.0e |
| A239 | S(K) | 17.0±1.7g |
| | C | 14.3±0.9f |
| | T | — |
| | N | 26.8±5.2h |
| | S(EK) | 16.3±1.2f,g |
| | S(MY) | 18.2±1.8g |
| | S(SD) | 20.4±1.3h |
| A619 | N | 28.6±2.1i |
| | T | 12.8±2.5j |
| W64A | N | 19.5±1.0k |
| | T | 12.3±0.6l |

* ; Values corresponding to different letters are significantly different at $P=0.01$ level within the same inbred line.

(2) Plasmolysis type There are usually two types of plasmolysis for corn. One is convex form and another is concave form. The cytoplasmic viscosity of the former is lower than that of the latter. The larger rate of convex form is, the lower viscosity is, and in same way, the lagrer rate of concave form is, the higher viscosity is.

When plasmolysed cell is treated by the solution which its concentration is lower than that of plasmolysis solution, the cell deplasmolyse. If concentration of solution is low enough, the cell can renew. The longer time of restoration is, the higher viscosity is.

Seedling of maize in three leaf age was used. Pieces of epidermis were peeled from young bud sheath and were successively put into 0.1, 0.2, 0.3 and 0.4 mol mannitol solution. After that, observation was perfomed under the microscope. The result showed that the rate of concave plasmolysis was 100%, 100% and 60% in N, S and C cytoplasm, respectively, and the rate of convex plasmolysis was 80% in T cytoplasm. It indicated that the quality of S and N cytoplasm was similar and significantly higher than C cytoplasm. The quality of C cytoplasm was better than that of T.

(3) Systrophy There are many particle which are different in size, and a big centre vacuole in plant cell. Under stress condition, the particles tend to gather around the nucleus and even result in cell swallon in serious occurrence. This phenomenon is a physiologic response of living cell to stress of environment condition and named systrophy. The systrophy influents message transfer from a nucleus to another and from cell to cell. Yoshida (1984) reported that the systrophy occurred easily in low viscosity protoplast but happened nothing in high quality cell. In our experiment, the systrophy was not observed in N and S cytoplasm but occurred frequently in C and T cytoplasm.

*Cytoplasmic streaming*

The biological importance of cytoplasmic streaming is described as follows. (1) It indicates that the cell possesses viability; (2) According to Williomson's research, the movement of particles in cell is the result of interaction between myosin and action of ATP. The reduction of circumfluent speed indicates decrease of breath rate and deficiency of metabolic energy; (3) After the cells are infected by pathogens, their protoplastic membrane is injured and the cytoplasmic stream decreases or stopes. The materials of corn with same nucleus and different cytoplasm, N, S, C and T were used in the experiment. The bud sheath in three-leaf age seedling or glume of middle tassel at beginning of flowering stage were used. The cytoplasmic streaming of subepidermal cells was observed under microscope. It could be seen that the varisized microsomes circulated along the cell wall at same direction. The microsomes in N and S cytoplasm were significantly larger than that in C and T cytoplasm, but there were no differences between N and S or between C and T.

The variation of circumfluent speed (unit μm/s) was measured after using methomyl to treat every type of cytoplasm. The results indicated that: (1) In control, sequence of circumfluent rate was N>S>C>T; (2) The treatments of methomyl in 6, 12 and 24 μg/ml had no significant influence to streaming speed of N, S and C cytoplasm but the rate of circumfluence in T cytoplasm decreased significantly. The rate of streaming in T cytoplasm, for example, was 16. 4±0. 3μm/s in control, but it was 13. 1±0. 3μm/s for 24μm/ml methomyl treatment. The variance was in significant level ( $P=0.01$ ). Since Koeppe et al. (1978) indicated that the physiologic effect of methomyl was like T toxin, the methomyl had special toxicity to T cytoplasm.

*Solute potential and water permeability*

There are many particles in cytoplasm. These particles are attached themselves to the netted structure. In cytoplasm, the more particles are, the higher concentration is, and the higher ability of resistance to stress condition is. Though climate is drought and soil salinity is high, the plant with high cytoplasmic concentration can still adapt to environment and maintain regular growth and metabolism.

The relative permeability of protoplast to water can be calculated by following formula:

$$\text{Relative water permeability}=\frac{\text{Time of deplasmolysis of CMS}}{\text{Time of deplasmolysis of normal cytoplasm (N)}}$$

The meaning of relative water permeability was listed in Table 2.
The time of deplasmolysis in N and S is markedly longer than that in C and T(Table 3).

**Table 2.** The solute potential and water permeability of subepidermal paremchyma cells of the coleoptile of different cytoplasm types from two genotypes

| Genotype | Cytoplasm | cell solute potential (MPa) | Relative water permeability | |
|---|---|---|---|---|
| | | | Time of deplasmolysis (min) | CMS time / N time |
| A619 | N | −0. 81±0. 07 | | |
| | T | −0. 59±0. 02 ** | | |
| C103 | N | −0. 76±0. 02 | 5. 6±0. 21 | 1 |
| | S | −0. 69±0. 00 | 5. 0±0. 00 | 0. 89 |
| | C | −0. 56±0. 05 * | 2. 1±0. 11 * | 0. 38 |
| | T | −0. 61±0. 02 ** | 1. 7±0. 14 ** | 0. 30 |

The data are mean of five samples; * Significant at 1% level compared with N and S. ** Significant at 1% level compared with N.

The relative water permeability are 1. 00, 0. 89, 0. 38 and 0. 30 in N, S, C and T cytoplasm, respectively.

The cell membrane is a surface on which the exchanges of substance and message are performed between inside and outside of cell or a cell and other. Because characteristic of bilayer of membrane phosphatide affects the permeability of water, the variation of permeability indicates that the structure and component of cell membrane are different. According to OK Young Lee and Stadelmann's result, the increase of water permeability often shows that the membrane is injured. Under unfavorable environment condition, the permeability in lower quality membrane is easy to increase. The disappear of permeability means the cell to be dead.

*Dehydration*

In three leaf age, one gram of leaf was taken from the young corn seedling in each of four types of cytoplasm in C103 inbred line respectively. A piece was put in test tube in which there was 10 ml 0. 8% mannitol solution accurately. The non-ionic water was used as the control. The conductivity of solution was measured and injured rate was calculated as following formula.

$$\text{Injured rate} = 1 - \left[\left(1 - \frac{T_1}{T_2}\right) \Big/ \left(1 - \frac{C_1}{C_2}\right)\right] \times 100$$

Where $T_1$ and $T_2$ were the conductivity of treatment at the initial time and the terminal time, respectively; $C_1$ and $C_2$ were the conductivity of the control at the same times, respectively.

Injured rate of leaf cells was a measure of membrane stability in stress conditions. The larger injured rate is, the more leakage is. The result(Table 3) indicated that the sequence of injured rate of leaf cells was N<S<C<T. From the results of physiologic research, we can concluded that the relationship of four types of cytoplasm in quality was: N better than S, S better than C, and C better than or similar to T, respectively.

**Table 3.** Injured rate of leaf cells of C 103 inbred line with varied cytoplasm

| Genotype | Cytoplasm | Injured rate(%) |
|---|---|---|
| C 103 | N | 34. 6 |
| | S | 35. 9 |
| | C | 71. 5 |
| | T | 87. 3 |

**Pathologic research**

*The responses of CMS line to Bipolaris maydis*

According to field observation in Guangxi, Sichuan, Henan, Hebei, and other provinces in China, we found that the *B. maydis* had stronger virulence on hybrid in C cytoplasm. Then 116 samples of *B. maydis* coming from 12 provinces or regions in China were screened and studied. Five isolates were found to which the plant with C cytoplasm was highly sensitive but the plants in N, S and T cytoplasm were not sensitive. The virulence experiment of five isolates of *B. maydis* was performed on C103 and VA35 inbred line in greenhouse and data were listed in Table 4.

**Table 4.** The reaction of corn with N and C cytoplasm to five isolates of *B. maydis*.

| Isolate | Genotype of host | Cytoplasm | Reactions | Longest of lesions(mm) |
|---|---|---|---|---|
| 356 | C103 | C | S* | 3. 4 ** |
| | | N | R | 1. 3 |
| 627 | C103 | C | S | 2. 2 ** |
| | | N | R | 0. 5 |
| 156 | VA35 | C | S | 5. 5 ** |
| | | N | R | 2. 0 |
| 353 | VA35 | C | S | 4. 7 ** |
| | | N | R | 1. 7 |
| 523 | VA35 | C | S | 9. 0 ** |
| | | N | R | 1. 4 |
| mean | | C | S | 5. 0 ** |
| | | N | R | 2. 0 |

* S susceptible, R resistant.

** Singificant at $P = 0.01$.

As Table 5 shows, using five isolates of *B. maydis* to inoculate the young seedling of two genotypes of maize indicated that the average length of lesions was 5. 0 mm in CMS-C and 2. 0 mm in N cytoplasm maintainer line. The difference was significant ($P = 0.01$). We difined these five isolates of *B. maydis* as race C(Wei Jiankun et al, 1988).

The reaction of CMS-C line to race C of *B. maydis* was tested by artificial inoculation using the half leaf method (Wu Quan'an et al, 1980). The procedure of the method was:the leaf on plant in CMS-C cytoplasm was divided into two parts at median line. The race C and O were inoculated on each of half leaf respectively. We found that in same leaf one of half leaf treated by race O was infected slightly and the lesions were of necrotic type of typical resistance. Another half leaf treated with race C was infected seriously and the lesions were of wilt type of typical sensitive reaction.

*Stimulation of toxin to phenycalanins ammonia-lyase(PAL) enzyme activity*

T toxin strongly stimulated activity of PAL of CMS-T line but had no significant effect on lines with other cytoplasm (Xue Yinglong et al, 1982). The data in our experiment (Table 5) proved again that T toxin stimulated only the activity of PAL in CMS-T

**Table 5.** The stimulating effect on the activity of PAL by the toxin produced by race T and C

| | Activity of PAL(O. D. 290nm/g fresh wt · h) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | T | | C | | S | | N | |
| | Control | Toxin | Control | Toxin | Control | Toxin | Control | Toxin |
| C toxin | 1. 395 | 1. 480 | 0. 901 | 3. 350 | 0. 895 | 0. 905 | 0. 355 | 0. 530 |
| T toxin | 1. 395 | 3. 565 ** | 0. 901 | 1. 080 | 0. 895 | 0. 940 | 0. 355 | 0. 380 |

** Significance at $P = 0.01$

cytoplasm but had no effect on the CMS cytoplasms. The activity of PAL in T cytoplasm was 3. 56 O. D. 290nm/g fresh wt · h in T toxin treatment but 1. 395 in control. The difference was very significant. Further more, C toxin stimulated the activity of PAL in C cytoplasm only(Table 5) and the activity of PAL was 3. 350 in treatment and it was just 0. 91 in control. The difference was very significant also.

Recently our research in physiology and pathology made some new progress:

1. Besides special effect on poisoning leaf cells with CMS inhibiting pollen tube germenating and elongation, restraining primary root growth, we found that the toxin of *B. maydis* had very sensitive specificity to injure the living cap cells of root with CMS cytoplasm(Guo Lankai, 1991).
2. It was ensured further that the race C specially infected CI subgroup cytoplasm in group C inbred line but had no effect on ES, RS cytoplasm in other subgroups(Liu Keming et al, 1991).
3. C toxin significantly inhibited the activity of SOD of mitochondria in C cytoplasm but had no effect on other cell organs (Cui Yang, 1992).
4. It was found out preliminarily by using chromolography of gel and cillica gel that C toxin was a mixture of several substance with living activity(Cui Yang, 1991).
5. Cui Yang has purified crystal of C toxin by using HPLC technique and got break through in chemical analysis on structure of T, C, O toxin using UNIR and H-NMR technique (1992, unpublished) in the guidance of Prof. Kohmoto.

(1)Toxin I crystal was purified from C toxin. The chemical analysis results showed that there was no toxin I in O and T toxin, and toxin I was a new substance: (2)Toxin I was of high special infection to C cytoplasm in the different genotypes. Inoculation by using toxing I can resolve the problem in which the results of inoculation to use crude extract of C toxin of isolate C can not repeat sometimes.

6. Recently, in cooperation with Laboratory of Research Center of Botanic Science of Cambridge, the techniques of PAPD analysis and DNA finger print were successfully used for identification of O, C and T race of *B. maydis* (Su Hai, 1992, unpublished).

## Discussion

First, We give a review for our results above. The data in Table 6 indicated that some points were as follows: (1)For C and T cytoplasm beginning time of abortion was early. They were characterized by completed abortion, anther degeneration, closed glume, aborted pollen, stable sterility, etc. For S cytoplasm, beginning time of abortion was later. Its fertility was unstable and the restorative mutation to normal cytoplasm occurred sometimes. (2)Among three CMS and N cytoplasm, the physical and chemical properties of cytoplasm were different and there was difference in quality also. All of these differences were termed "cytoplasm distance". The tests of protoplastic parameter indicated that the "distance" was short between C and T or S and N cytoplasm. In brief, for protoplastic quality, i. e. cytoplasmic viscosity, Og value, solute potential, water permeability, membrane stability etc, N cytoplasm was better than S; S was better than C and T. (3) For response of them to *B. maydis*, the lesions on leaf in N and S cytoplasm was of necrotic. N and S were typical type of disease resistance. In early 60's, T cytoplasm was highly infected by *B. maydis* in China, the lesion was of wilt type typical disease sensitivity. It was reported by us in 1988 that the race C existed in nature and was highly sensitive to C cytoplasm. We concluded that the disease-resistant difference in CMS-C, CMS-T and CMS-S was identical with difference of relevant cytoplasm's parameter of protoplast.

**Table 6.** Typical of fertility, protoplastic parameter and results of pathologic test in CMS and N cytoplasm

| Field | Item | Cytoplasms with same nucleus | | | |
|---|---|---|---|---|---|
| | | N | S | C | T |
| Breeding | 1. Initial time of male sterile | Normal | Most late | Earlier | Earliest |
| | 2. Behavior of glume in flower | Open | Close→open | Close | Close |
| | 3. Behavior of anther in flower | Unfold Pollinate | Fold→unfold | Fold | Fold |
| | 4. Anther weight | | $N>S>C>T$ | | |
| | 5. Fertility | Fertile | Sterile to fertile | Sterile | Sterile |
| Physiology | 1. Type of plasmolysis | Concave (100%) | Concave (100%) | Concave (60%) | Convex (80%) |
| | 2. Systrophy | no | no | yes | yes |
| | 3. Time of chloroplast displacement | | $N>S>C>T$ | | |
| | 4. Cytoplasmic streaming | | $N>S>C>T$ | | |
| | 5. Og. value of cell | | $N>S>C>T$ | | |
| | 6. Solute potential | | $N<S<C=T$ | | |
| | 7. Water permeability | | $N<S<C=T$ | | |
| | 8. Membrane stability | | $N>S>C>T$ | | |
| Pathologic test | 1. Reaction to *B. maydis* | R | R | S(C race) | S(T race) |
| | 2. Type of lesion | Necrotic | Necrotic | Wilt | Wilt |

Secondly, on the basis of historical experience in CMS breeding, the first thing is that the utilization of single cytoplasm in long time and wide area must be avoided, and then, another thing is that the research, utilization and development of CMS cytoplasm must be under more and more consideration.

1. The relationship between existed types of CMS cytoplasm and pathogen should be studied further so that more sterile material can be used in production. Liu Keming (1991) et al. found in field and laboratory that C Ⅰ subgroup in CMS-C cytoplasm group was specially sensitive to C race but C Ⅱ and C Ⅲ in same group were not sensitive. This meant that the C Ⅱ and C Ⅲ can be used in production. So far, the hybrids bred from C Ⅱ and C Ⅲ by Chen Weicheng and the breeders of the Xinjiang Academy of Cultivation were widely used in practice and it was characterized by high yield and disease resistance.

Some regenerated plants were established from callus of C Ⅰ subgroup that was treated by C toxin. This fact indicated that gene of sterility was not linked with gene of disease sensitivity to race C. The C Ⅰ subgroup will "be given a new life" (Liang Genqing, 1989; Zhou Hongsheng, 1991).

2. The defect of S cytoplasm group was that the fertility was unstable. This problem could be solved by changing nuclear background, back crossing in pair strictly and strengthening selection pressure. The strong point of S group was that the quality of protoplast closed to normal protoplast. After the hybrids with S cytoplasm, i. e., double type, Tangxu type, $L_1$ type and Ji 1A type, etc., which was used widely in China, the special race of *B. maydis* on S cytoplasm was not found. So far, in China and other countries, the S groups of cytoplasm consisted of more than one hundred types of sterile cytoplasm. On the basis of the synthetical research, Slaco (1985) suggested that S group should be divided into five subgroups. Therefore, extensive research and development on S group will have great potential and a bright future.

3. For selecting new CMS type and establishing new CMS type, it should be under consideration that the cytoplasm of close relative species of Maize is introduced into CMS line.

We introduced CMS-EP with perennial cytoplasm from America in 1984. Laughnan et al. (1983) deduced that it was a new CMS-type. The field observation was performed in Hebei Province of China for several years. The results showed that the CMS-EP line presented stable sterility and, until now, the maintainer line can not be found except A619.

Early in 70's, it was observed in our experiment that the diseases of leaf blight and leaf spot of corn, virosis and bacterial wilt of corn were not found on plants of perennial cytoplasm type. We had established a new CMS line with cytoplasm of perennial Maize, in which the perennial Maize was used as female parent and the different cultivars of corn were used as the male parents, passing through back crossing, screening of sterile plant from mutagenic mass (Wei Jiankun, 1992). Even though this kind of pioneer work was carried out for long time and was very difficult, the utilization of close relative genus of Maize ( *Teosinte* and *Tripsacum* ) should be paid more attention to.

4. Under strategic consideration of avioding the genetic frailty due to genetic homogeneity, the multi-cytoplasm hybrid should be created by using varial types of cytoplasm. Two methods of following can be used simultaneously or separately.

(1) Accoding to the results of observing on growth and decline of physiologic race, the single cytoplasm is taken turn.

(2) To creat multi-cytoplasmic hybrid (Zhang, 1987; Gao Kai, 1992)

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