Relation between Cytoplasmic Male Sterility and Mitochondrial Enzyme Activities in Maize

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In maize, cytoplasmic male sterility (cms) was widely used in breeding and seed production. However, in 1970, plants possessing Texas cytoplasm (T cytoplasm, the most widely used cytoplasm for male sterility in maize) were damaged severely by the southern corn leaf blight. They were affected strongly by the phytotoxin produced by the pathogen of the disease, that is, the race T of *Bipolaris maydis* (Nisikado) Shoemaker.[2,9] Since then, biochemical studies of cms in maize have been stimulated. Phytotoxins produced by *B. maydis* or *Phylosticta maydis* were shown to affect the oxidative phosphorylation and other functions of mitochondria of T cytoplasm plants unlike those of normal (N) cytoplasm plants.[5,6] This fact strongly indicates the presence of defects in the mitochondrial function which is responsible for the cms as well as the sensitivity of maize lines with T cytoplasm to the phytotoxin of the fungus. If this assumption is valid, it is anticipated that male sterility with other types of cytoplasms may also be related to similar defective mitochondrial functions. Humaydan and Scott[3] reported that a methomyl insecticide (Lannate 90) was selectively phytotoxic to several lines of sweet corn with T cytoplasms. Methomyl was also found to inactivate mitochondria of a line with T cytoplasm like phytotoxin of race T of *Bipolaris maydis*.[4]

In the present study, the author showed that low mitochondrial enzyme activity is closely related to cms in maize plants. Furthermore, the author extended Humaydan's work to establish that treatment with methomyl was a useful method to differentiate cytoplasm of "Texas group."

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Alteration of cytochrome oxidase and malate dehydrogenase activities during anther development

In this study the author measured activities of cytochrome oxidase (CO) and malate dehydrogenase (MDH) of anthers of maize lines with male-sterile T, S and C cytoplasms as well as normal (N) cytoplasm. These three male-sterile cytoplasms are typical of the three groups of cytoplasms classified by Beckett.[1] The author followed change of the activities during anther development, checking stages of pollen by microscopic observation using squash method. Large difference between enzyme activities of anthers of normal and male-sterile cytoplasms was found. Minor differences between lines with male-sterile cytoplasms, T, S and C, were also observed.

Maize inbred line WF9 with N cytoplasm and its isogenic lines with T, S and C sources of male-sterile cytoplasms have been maintained by sib-mating. Developing tassels in flag leaves were sampled from plants growing in the field and used in the experiment. Changes in plant height, anther weight, and enzyme activities with the development of microspore and pollen are shown in Table 1.

1) Anther and pollen development

In N cytoplasm plants, increase of anther weight was remarkable after uninucleate stage. In male-sterile plants, however, microspore

Table 1. Changes in plant height, anther weight and enzyme activities[a] with the development of microspore and pollen

Cytoplasm	Plant height (cm)	Anther weight[b] (mg)	Stage of microsporogenesis	Enzyme activity of the anther		Enzyme activity of the flag leaf	
				CO	MDH	CO	MDH
N	141	0.204	Synapsis	(8)[d]	(152)[d]	14	44
	149	0.306	Uninucleate	55	312	25	58
	158	0.721	Uninucleate and binucleate	46	201	17	63
	165	1.305	Binucleate and trinucleate	82	211	21	41
	171	1.437	Trinucleate	70	94	9	41
	176	1.985	Trinucleate	158	293	18	48
	184[c]	2.062	Trinucleate	256	412		
S	144	0.296	From diakinesis to tetrad	(13)[d]	(133)[d]	15	44
	151	0.419	Tetrad and uninucleate	48	271	29	68
	154	0.988	Binucleate	39	143	20	67
	163	0.889	Binucleate and trinucleate	57	132	20	69
	171	0.788	Binucleate and trinucleate	27	124	12	33
	174	0.807	Uninucleate	36	79	20	53
	191[c]	0.851	No nucleus	29	118		
T	139	0.162	Synapsis	(23)[d]	(139)[d]	25	50
	145	0.580	Uninucleate	13	106	19	50
	147	0.634	Uninucleate	22	134	21	60
	161	0.816	Uuinucleate	8	71	16	32
	176	0.606	Uninucleate	8	92	22	115
	171[c]	0.483	Uninucleate or no nucleus	23	149	29	125
C	143	0.373	From pachytene to diakinesis	32(17)[d]	250(90)[d]	13	40
	152	0.404	Tetrad and uninucleate	47	214	21	70
	152	0.591	Uninucleate	35	155	26	69
	158	0.594	Uninucleate	24	116	15	44
	178	0.305	Uninucleate and no nucleus	22	69	19	144
	178[c]	0.258	No nucleus	50	134	30	169
	188[c]	0.153	No nucleus	41	124		

a) Total activity per one gram of fresh samples. Relative values are given. See the text for details.
b) Average weight of one anther.
c) At the time tassels of N cytoplasm plants flowered.
d) Values in parentheses show activities of mitochondrial fractions only.

development was blocked at a certain stage, which is characteristic to cytoplasm type, and the anther growth stunted. In S cytoplasm plants, microspores developed to the binucleate or trinucleate stage and some of them were filled with starch-like grains. Then they degenerated. In T and C cytoplasm plants, microspore development was blocked at the uninucleate stage and degenerated.

2) *Enzyme activity of the anther*

Anthers at various stages of development were sampled, homogenized and fractionated by centrifugation. CO and MDH activities were assayed for each fraction by the method described previously.[7,8] Table 1 shows the total enzyme activity (that is, the compiled value of enzyme activities) for the fractions. Throughout the period after entering into the uninucleate stage, CO and MDH activities of anthers were lower in male-sterile cytoplasm plants than in the N cytoplasm counterpart.

In N cytoplasm plants, enzyme activities greatly increased from the binucleate stage. CO activity which showed a particularly steep increase, attained about five times higher activity at flowering than at the uninucleate stage. MDH activity increased twice in the same period.

In cms plants such marked increase of activities was not observed. Among the three cytoplasmic types, T cytoplasm showed the sharpest contrast to the normal cytoplasm, particularly in CO activity. CO activity of T cytoplasm plants was the lowest of the three throughout the

experiment. Both CO and MDH activities of C cytoplasm plants were highest at the tetrad and uninucleate stages and decreased thereafter. CO activity of S cytoplasm plants was maximum at a later stage, when anthers contained binucleate and trinucleate pollen grains, whereas MDH activity was highest at the tetrad through uninucleate stages.

3) *Enzyme activities of flag leaves*

Flag leaves were sampled together with tassels and their enzyme activities were assayed in the same way as for anthers. Enzyme activities of flag leaves were generally lower than those of anthers. In T cytoplasm plants, CO activity was not as low as the extremely low activity of anthers. Altogether, no significant difference in the enzyme activities of flag leaves was observed between N cytoplasm plants and the three male-sterile plants.

Effect of pollen fertility restoration on enzyme activities and content of starch and other components in anthers with male-sterile cytoplasm

As seen in the preceding section, two mitochondrial enzyme activities, CO and MDH, increased rapidly as the pollen matured in anthers of maize with normal cytoplasm, while those of anthers with male-sterile cytoplasms failed to show such an increase.[7,8] In the present section, the author studied to determine whether anthers of maize lines with male-sterile cytoplasms which were restored for their pollen fertility showed high mitochondrial enzyme activity. The change in starch, soluble sugars, and protein content of the anther was also studied in relation to the change of CO and MDH

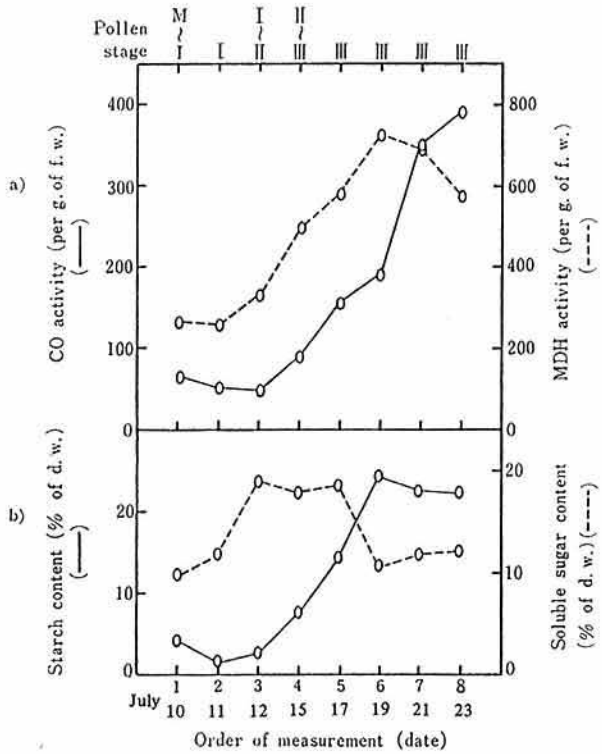

Fig. 1. Change of a) CO and MDH activities and b) starch and soluble sugars content in anthers of WF9N during pollen development. Symbol of pollen stage is as follows; M: meiosis, I: uninucleate stage, II: binucleate stage and III: trinucleate stage.

activities. As a result, CO activity was shown to be usable as an index of pollen fertility.

1) *Change in CO and MDH activities and starch, soluble sugars and protein content of the anther during pollen development*

CO and MDH activities, starch, soluble sugars and protein content of anthers of the three types of lines were measured during pollen development to determine the effect of restoration of pollen fertility, using the line with normal cytoplasm WF9N, the line with male-sterile T cytoplasm WF9T×F5DD1 which was restored for pollen fertility, and the male-sterile lines WF9S×F5DD1 which have the same nuclear background as that of the restored line. Results are shown in Figs. 1–3.

CO activity of anthers of the restored line WF9T×F5DD1, increased rapidly from the binucleate stage like in WF9N (Figs. 1 and 2).

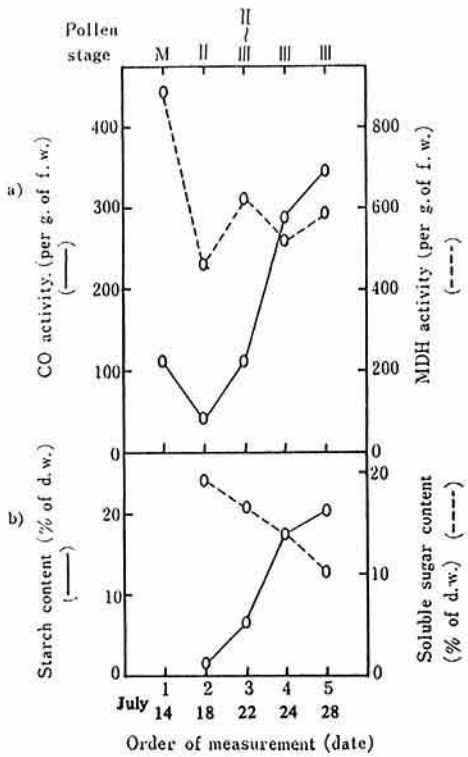

Fig. 2. Change of a) CO and MDH activities and b) starch and soluble sugars content in anthers of WF9T×F5DD1 during pollen development. As for pollen stage, see the legend to Fig. 1.

In the male-sterile line with S cytoplasm WF9S×F5DD1, neither CO nor MDH activity showed a rapid increase after the binucleate stage (Fig. 3). CO activity increased slightly, but remained at a lower level.

In the two male-fertile lines, the starch content of the anther began to increase rapidly after the binucleate stage, when the CO activity of both lines also began to increase. In the male-sterile line with S cytoplasm WF9S×F5DD1, starch content increased slightly after the binucleate stage, but it remained at a lower level until flowering time. This mode of variation was similar to that of the CO activity of the line. These facts indicate that CO activity and starch content are closely related in anthers of maize.

The content of soluble sugars increased at the binucleate stage and early trinucleate stage in anthers of the two male-fertile lines, but

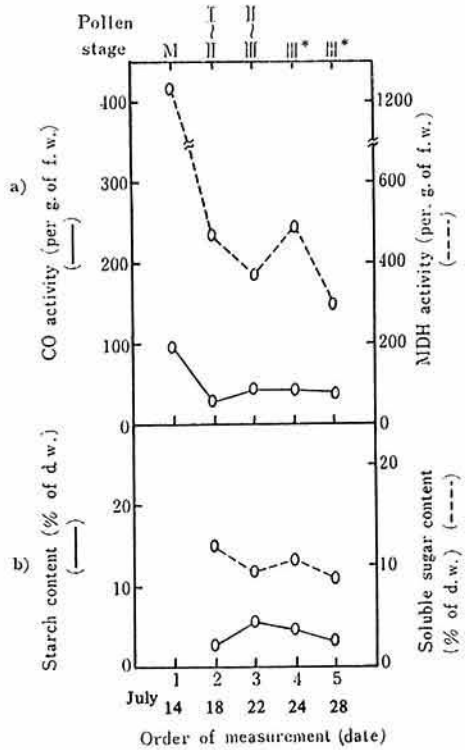

Fig. 3. Change of a) CO and MDH activities and b) starch and soluble sugars content in anthers of WF9S×F5DD1 during pollen development. As for pollen stage, see the legend to Fig. 1. In the stage III*, many pollen did not contain nucleus.

Table 2. Comparison of CO and MDH activities and their ratio between male-sterile and male-fertile lines of maize

Maize line	Pollen fertility	Cytoplasm	CO activity	MDH activity	CO activity / MDH activity
Plants grown in a greenhouse					
W9T × F5DD1	fertile	T	237	398	0.60
R213T × F5DD1	fertile	T	327	398	0.82
R213T × CE1	fertile	T	362	325	1.11
W9T × CE1	sterile	T	45	284	0.16
WF9S × CE1	fertile	S	179	319	0.56
Plants grown in the field					
WF9N	fertile	N	354	603	0.59
W9N	fertile	N	324	315	1.03
R213N	fertile	N	284	67	4.21
WF9N × A158	fertile	N	192	436	0.44
WF9T	sterile	T	23	149	0.16
W9T	sterile	T	10	127	0.08
R213T	sterile	T	5	12	0.38
WF9T × W9	sterile	T	24	228	0.11
R213T × W9	fertile	T	227	346	0.66
WF9T × F5DD1	fertile	T	240	264	0.91
W9T × F5DD1	fertile	T	174	456	0.38
R213T × CE1	fertile	T	233	326	0.72
WF9S	sterile	S	23	60	0.38
WF9C	sterile	C	11	23	0.47
WF9C × W9	fertile	C	342	489	0.70
WF9C × A158	fertile	C	300	369	0.82

decreased to about 10% in the later trinucleate stage. Soluble sugar content was high just before and during the increase of starch content.

Protein content of the anther remained at about 10% of dry weight of the anther throughout the pollen development in the three lines.

2) *Comparison of CO and MDH activities of anthers just before flowering among lines with N,T,S or C cytoplasms*

Table 2 shows CO and MDH activities and their ratio in plants just before flowering in 19 lines with normal or male-sterile cytoplasms, grown in a greenhouse or in the field.

CO activity of all the male-fertile lines was high, ranging between 174 and 362, while that of all the male-sterile lines was below 45, showing a distinctly different range of values from those obtained in the fertile plants. In general, MDH activities and the ratio of CO activity to MDH activity of lines with normal cytoplasms and of fertility restored lines with male-sterile cytoplasms were higher than those of male-sterile lines. These facts, together with the results described earlier, show that high mitochondrial enzyme activities, CO and MDH, are necessary for obtaining mature anthers. CO activity can be used as an index of pollen fertility.

Reaction of various types of cytoplasmic male-sterile lines of maize to methomyl insecticide "Lannate"

As described above, cms is closely related to the function of mitochondria. Thus, reagents that affect mitochondrial function may be used to differentiate cytoplasms in maize. Humaydan and Scott[3] reported that methomyl insecticide "Lannate" was phytotoxic to some lines of sweet corn with T cytoplasm and Koeppe et al.[4] reported that methomyl affected mitochondria of a line with T cytoplasm. Because the compound is easy to obtain on a large scale and the effect can be assayed in plant, the author extended his work to 35 lines with 13 different cytoplasms, devised a root inhibition method, and established that methomyl could be used as the indicator of Texas cytoplasm.

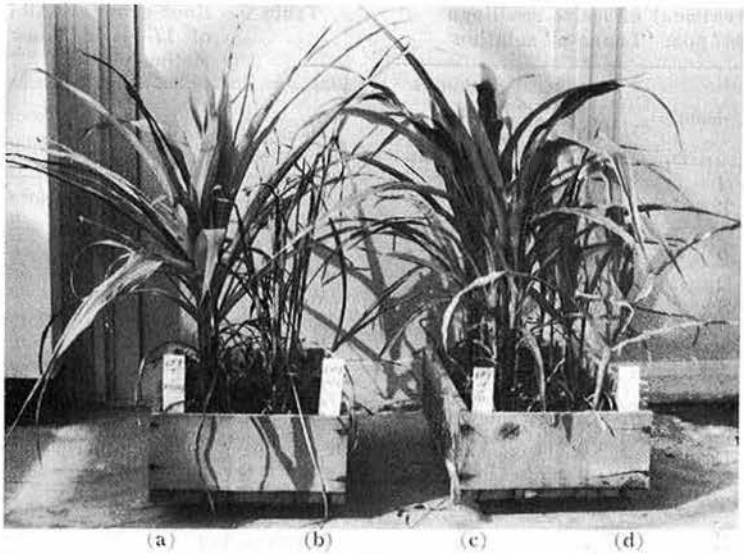

Plate 1. Soil treatment of maize seedlings with 667 ppm "Lannate" solution. About 3 weeks after treatment.
(a) WF9T × F5DDl (b) WF 9HA
(c) WF9N × F5DDl (d) R213T

1) Soil treatment of seedlings in seeding boxes with methomyl

Seedlings were grown in seeding boxes (40 cm long, 25 cm wide and 10 cm deep) in a greenhouse for 17 days, until they reached the 5th or 6th leaf stage. Then 2 *l* of 667 ppm "Lannate" which contained 300 ppm methomyl was applied to each box. Nine days later effects of methomyl were observed. The results are shown in Table 3 and Plate 1. Of the 32 lines with 13 cytoplasms, only plants with T group cytoplasms, T, P, HA and "J" were seriously affected. Plants with normal cytoplasm or male-sterile cytoplasms other than T group were only slightly blighted at the top of the leaf blade. Soil treatment is simpler and more effective than leaf application by spraying or painting.

2) Root growth inhibition experiment

Five seedlings with primary roots shorter than 20 mm were put in a line on two sheets of paper towel at 8 cm from the bottom end. Paper towels were rolled from one end inwrapping the seedlings, fixed by rubber bands and dipped in beakers containing methomyl solution or water (control plot) 2 cm deep. They were incubated in darkness at 30–32°C for three days and the length of the primary roots was measured again. Increment of the length and ratio of the average increment of primary root length of seedlings treated with methomyl to that of control were calculated. By this method results can be obtained in 5 or 6 days.

Table 4 shows concentration dependence. In a 200 ppm solution, root growth of WF9T was inhibited to about 85% whereas WF9N was little affected. Even with a 500 ppm solution, root of WF9N grew to about 50% of the control, As seen in Table 5 root growth of lines with cytoplasm of T group was inhibited by the treatment with 200 ppm of methomyl solution, but there were some differences in the degrees of inhibition among the different lines. This point was confirmed in a separate experiment (data not shown).

Table 6, exhibiting the results of treatment with 500 ppm methomyl solution of seedlings with various cytoplasms, shows that the lines with cytoplasms other than T cytoplasm are significantly resistant than the lines with cytoplasm of T group. Treatment with 500 ppm of methomyl (or 1,100 ppm of "Lannate") is useful to distinguish T group cytoplasms.

Table 3. Soil treatment of maize seedlings with 667 ppm "Lannate" solution

Maize line	Cytoplasm	Group of[a] cytoplasm	Reaction to "Lannate" treatment[b]
WF9N	N	Normal	−
WF9T	T	T	+
WF9HA	HA	T	+
WF9P	P	T	+
WF9Q	Q	T	+
WF9S	S	S	−
WF9G	G	S	−
WF9J	J	S	−
WF9R	R	S	−
WF9C	C	C	−
WF9RB	RB	C	−
WF9D	D		−
R213N	N	Normal	−
R213T	T	T	+
W9N	N	Normal	−
W9T	T	T	+
A111	N	Normal	−
A111"J"	"J"	T	+
KOU 1	N	Normal	−
KOU 1 T	T	T	+
Okuzuru wase	N	Normal	−
Okuzuru wase T	T	T	+
WF9N × F5DD1	N	Normal	−
WF9T × F5DD1	T	T	+
WF9N × CE1	N	Normal	−
WF9T × CE1	T	T	+
WF9N × W9	N	Normal	−
WF9T × W9	T	T	+
WF9N × R213	N	Normal	−
WF9T × R213	T	T	+
W9N × F5DD1	N	Normal	−
W9T × F5DD1	T	T	+

a) Group of cytoplasm of maize defined by Beckett[1]
b) +: Leaf blighted severely. −: Leaf blighted slightly at the tip.

Table 5. Root growth inhibition of seedlings of 17 maize lines in 200 ppm of methomyl

Maize line	Cytoplasm	Group of[a] cytoplasm	Ratio of root growth to control (%)
WF9N	N	Normal	73.1(15.5)[b]
WF9T	T	T	12.2(7.3)
WF9HA	HA	T	8.7(5.3)
WF9P	P	T	17.6(13.6)
WF9Q	Q	T	13.5(8.6)
R213N	N	Normal	66.1(20.3)
R213T	T	T	28.6(11.7)
W9T	T	T	5.7(3.4)
A171"J"	"J"	T	3.5(1.7)
Okuzuru wase	N	Normal	83.9(23.2)
Okuzuru wase T	T	T	7.8(4.5)
WF9T × F5DD1	T	T	24.5(13.9)
WF9T × CE1	T	T	8.5(5.7)
WF9T × R213	T	T	22.6(17.0)
WF9T × W9	T	T	5.4(2.7)
W9T × F5DD1	T	T	9.3(5.1)

a) See the legend to Table 3.
b) See the legend to Table 4.

Table 6. Root growth inhibition of seedlings of 14 maize lines in 500 ppm of methomyl

Maize line	Cytoplasm	Group of[a] cytoplasm	Ratio of root growth to control (%)
WF9N	N	Normal	47.3(13.2)[b]
WF9T	T	T	3.2(3.6)
WF9S	S	S	45.8(14.5)
WF9R	R	S	69.4(19.1)
WF9C	C	C	48.6(31.3)
WF9RB	RB	C	45.1(7.5)
WF9D	D		48.0(7.6)
R213N	N	Normal	34.0(10.3)
R213T	T	T	2.6(2.5)
W9N	N	Normal	33.4(7.6)
A171N	N	Normal	56.1(15.6)
A357N	N	Normal	37.4(12.9)
Okuzuru wase	N	Normal	55.2(14.9)
Okuzuru wase T	T	T	2.9(1.5)

a) See the legend to Table 3.
b) See the legend to Table 4.

Table 4. Dependence of root growth inhibition of maize seedlings on methomyl concentration[a]

Maize line	Methomyl concentration (ppm)					
	0	50	100	200	300	500
WF9N	100	103.4 (6.5)[b]	76.9 (24.5)	72.2 (16.4)	75.4 (7.2)	48.7 (6.2)
WF9T	100	103.2 (9.9)	77.2 (10.4)	14.5 (4.4)	7.7 (2.2)	3.1 (2.7)

a) % of root growth in each concentration of methomyl solution to that in 0 ppm is shown.
b) Values in parentheses are errors estimated from standard deviations of root lengths of both treated seedlings and control seedlings.

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