COMPARATIVE ANALYSES OF SOME OF THE FREE AMINO ACIDS IN ANTHERS OF FERTILE AND GENETIC CYTOPLASMIC MALE STERILE SORGHUM¹

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GENETIC cytoplasmic male sterility in Sorghum is fundamentally an aspect of the problem of gene action and nuclear-cytoplasmic interaction. Our interest in the problem stems not from a concern with the practical use so effectively being made of the failure of certain lines to produce functional pollen but rather from a desire to know more about the basic components of this process as an example of gene-cytoplasm relationships. Moreover, while studies for the better understanding of the biochemical correlates of genetic expression have been conducted extensively in bacteria, viruses, and fungi, which admittedly present better material for such research, need is felt for more information about comparable processes in the higher plants. Such studies are not new, but it was thought that cytoplasmic male sterility offered a somewhat unique situation in which such studies, combined with the possibilities of investigating nuclear-cytoplasmic interaction, might be carried on.

Since the initial work of MAUNDER and PICKETT (1959), it has been considered that genetic cytoplasmic male sterile lines in Sorghum carry the recessive genes (*ms ms*) for male sterility. This, however, is not the only factor involved since other lines with the same gene complement are functional pollen producers. The difference between the two lines resides in the cytoplasm, the first line carrying some factor or factors for sterility, or lacking factors for fertility, the second line possessing such fertility factors or lacking those for sterility. In addition to having such isogenic lines differing only in cytoplasm, fertility restorer lines are available which carry the dominant form (*Ms*) of the male sterile gene or genes (MAUNDER 1960).

Genetic cytoplasmic male sterility could presumably be the result of a relatively simple cause—a deficiency of one or more organic compounds. This deficiency might reflect the nonfunctioning of an enzyme system and result in subsequent accumulation of certain metabolites. Such accumulations might be expected to result if recessive gene action is visualized as inhibiting normal enzyme activity. Both the fertile and sterile lines dealt with in this study carry a recessive gene for male sterility and comparisons are therefore indicated between these lines and the fertility restorer lines. Although critical analyses of

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this comparison will have to await the establishment of the dominant gene or genes in question into backgrounds isogenic with the varieties used, it was hoped that if a clear-cut difference did exist, it might be measurable without the delay involved in the technical problem of establishing isogenic lines. Valid comparisons also are indicated between the fertile and sterile lines; since these lines are isogenic, the comparisons would be between their respective cytoplasms. One might expect therefore to encounter various complicating factors as well as some difficulty in interpretation of results. However, while probably all gene action is to be regarded as expressible only through nuclear-cytoplasmic interaction, this type of sterility represents a specific example of interaction which is accessible for study and amenable to experimental approach.

MATERIALS AND METHODS

The principal observed difference between fertile plants and genetic cytoplasmic male sterile plants occurs after formation of the microspores. In the sterile plants, the microspores enlarge somewhat, form an intine and exine and even in some cases a germinating pore; they fail, however, to undergo normal mitoses or to form starch, and they lead only to aborted pollen. For purposes of discussion the final stage of microgametogenesis in the cytoplasmic male steriles, has been designated "prepollen." Some meiotic abnormalities do occur in these materials, but their frequencies are apparently not consistently sufficient to account for sterility in all cases. Based on this information, it seemed logical to begin work with anthers only, even though it was recognized that the ultimate cause of the differentiation might occur much further back in ontogeny.

Anthers of all varieties used were collected from both greenhouse and field grown materials. The varieties used were Redlan, [(Kafir $\times$ Milo, C. I. 1090) $\times$ Blackhull Kafir C. I. 71], Wheatland (Garden City 38288, Resistant), Combine Kafir-60 and Martin (sel. from Wheatland C. I. 918). Isogenic fertile and sterile lines of each were available. Two fertility restorers, Caprock (Tx 7000) and OK-RY8 (Redlan $\times$ Kaura), were used. All seed was obtained from the Agronomy Department breeding nurseries, Oklahoma State University. Immediately following collection, an acetocarmine smear was made from one anther of each floret and the stage of meiosis determined; it was then assumed that the other anthers of the same floret were in approximately, though not necessarily exactly, the same stage. For each chromatographic run, an average of 30 anthers was used for the prepollen stage, 60 for the microspore and 90 for the pretetrad stage. Each run involved fertiles, steriles and/or restorers, and controls. Each control run consisted of ten microliters of an amino acid control set (L-aspartic acid, L-glutamic acid, L-serine, glycine, L-threonine, and L-alanine) at a concentration of 0.05 mg amino N/ml or of 0.1 mg amino N/ml for each amino acid.

The method used was the descending paper chromatography method of McFARREN (1951) and McFARREN and MILLS (1952). Paper strips were buffered to pH 12.0, and the same pH 12.0 buffer was also added to phenol for the solvent system. The fresh and dry weights of the anthers were determined, and the material was then macerated and extracted in cold 80 percent ethanol,

vacuum dried and taken up in 50 μ l of H_2O , which was then spotted on the buffered strips (Whatman #1). A Fisher stainless steel chromatocab was used; the spray was ninhydrin with four percent acetic acid. The semiquantitative estimates were made with the aid of a Photovolt densitometer using filter #570. A #445 filter was also used in the readings for glycine which gives a yellow-brown spot. The values discussed in this paper are, however, based on the reading with the #570 filter. The estimates for each run were referred to known values of controls run simultaneously. It should be emphasized that the estimates of amino acid content are presented for comparative purposes only and do not represent an attempt to determine actual amounts of each amino acid present. Since only comparative values rather than absolute amounts were of concern, maximum density readings were used.

RESULTS

Comparative lengths, fresh weights, and dry weights: As seen from Table 1, there was a difference in lengths of fertile and genetic cytoplasmic male sterile anthers in every variety at the prepollen stage; the fertile anthers were significantly longer in each case. Two varieties (Redlan and Wheatland) showed a significantly greater length for the fertiles at the microspore stage, while at this stage Martin showed a significantly greater length for the steriles. As seen in Table 3, the average of the length of restorer anthers (1.10 mm) was lower than the average of either fertiles (1.27 mm) or steriles (1.20 mm) at the pretetrad stage and was lower also (1.56 mm) than the average of fertiles (1.79 mm) or steriles (1.73 mm) at the microspore stage. At the prepollen stage, however, the average for the restorers (2.50 mm) was intermediate between that for the fertiles (2.67 mm) and that for the steriles (2.31 mm).

Although in Martin the steriles were significantly higher at the microspore stage, again all fertile varieties showed a significantly higher fresh weight at the prepollen stage than did the steriles. As many factors may contribute to fresh weight measurements, only averages are presented here, as seen in Table 3. As this table shows, the restorers, although lower than the average of either the fertiles or steriles at the two earlier stages, were intermediate in fresh weight between the fertiles and steriles at a prepollen stage.

TABLE 1
Lengths (mm) of anthers at different stages

Variety	Pretetrad		Microspore		Prepollen	
	Fertile	Sterile	Fertile	Sterile	Fertile	Sterile
Redlan	1.26 $\pm$.037	1.24 $\pm$.108	1.71 $\pm$.030	1.57 $\pm$.065	2.53 $\pm$.046	2.30 $\pm$.037
Wheatland	1.54 $\pm$.113	1.37 $\pm$.057	2.16 $\pm$.047	1.90 $\pm$.052	2.98 $\pm$.061	2.37 $\pm$.082
Combine						
Kafir-60	1.15 $\pm$.045	1.17 $\pm$.024	1.74 $\pm$.053	1.75 $\pm$.035	2.53 $\pm$.136	2.33 $\pm$.059
Martin	1.11 $\pm$.050	1.03 $\pm$.009	1.53 $\pm$.047	1.70 $\pm$.021	2.62 $\pm$.067	2.24 $\pm$.044
Caprock	1.30 $\pm$.018		1.69 $\pm$.057		2.47 $\pm$.132	
OK-RY 8	0.89 $\pm$.044		1.42 $\pm$.034		2.53 $\pm$.082	

Concerning dry weight (Table 2), all fertile varieties showed a significantly higher dry weight at the prepollen stage than did the steriles. The fertiles and steriles of Combine Kafir-60 also showed a significant difference at a microspore stage. The average for the restorers (Table 3) became intermediate between fertiles and steriles as development proceeded.

Comparing the percent increase in length and weight gained from one stage to another (Table 4) it is obvious that in the steriles this percentage increase was greater than in the fertiles in both length and fresh weight through a microspore stage, but that following this stage although increase in length and weight occurred, increase, percentage wise, compared unfavorably with that occurring in fertile anthers. One can conclude that although the steriles even at the pretetrad stage were at some comparative disadvantage, the final inferiority in size and weight of the sterile anthers was due to a considerable degree to a failure to maintain whatever metabolic processes were involved in increase in length and fresh weight following microspore formation. The dry weight measurements also suggest that there was failure of normal syntheses in these anthers at this stage. The situation (Table 4) in the restorers is more difficult to interpret; the length

TABLE 2

Dry weight (mg) per anther at different stages

Variety	Pretetrad		Microspore		Prepollen	
	Fertile	Sterile	Fertile	Sterile	Fertile	Sterile
Redlan	.016 ± .001	.013 ± .002	.034 ± .003	.032 ± .002	.086 ± .004	.062 ± .003
Wheatland	.002 ± .003	.020 ± .004	.038 ± .002	.037 ± .003	.080 ± .008	.041 ± .005
Combine						
Kafir-60	.012 ± .000	.014 ± .003	.026 ± .002	.034 ± .002	.089 ± .011	.049 ± .005
Martin	.013 ± .003	.010 ± .001	.022 ± .002	.026 ± .003	.092 ± .006	.046 ± .003
Caprock	.017 ± .001		.033 ± .004		.076 ± .012	
OK-RY 8	.010 ± .001		.022 ± .000		.067 ± .007	

TABLE 3

Comparison of anthers of fertiles, steriles, and restorers

	Average length (mm)		
	Pretetrad	Microspore	Prepollen
Fertiles	1.27	1.79	2.67
Steriles	1.20	1.73	2.31
Restorers	1.10	1.56	2.50
	Average fresh weight (mg)		
	Pretetrad	Microspore	Prepollen
Fertiles	.072	.154	.365
Steriles	.064	.155	.247
Restorers	.050	.131	.315
	Average dry weight (mg)		
	Pretetrad	Microspore	Prepollen
Fertiles	.011	.030	.087
Steriles	.014	.032	.050
Restorers	.014	.028	.072

TABLE 4
Percent increase in length and weight of anthers

	Length	
	Pretetrad-Microspore	Microspore-Prepollen
Fertiles	40.94	49.16
Steriles	44.17	33.53
Restorers	41.82	60.26
	Fresh weight	
	Pretetrad-Microspore	Microspore-Prepollen
Fertiles	113.89	137.01
Steriles	142.19	59.35
Restorers	162.00	140.46
	Dry weight	
	Pretetrad-Microspore	Microspore-Prepollen
Fertiles	172.73	190.00
Steriles	128.57	56.25
Restorers	100.00	157.14

and dry weight data appear patterned after the fertiles, with a greater percent increase occurring after the microspore stage.

Qualitative studies: The phenol pH 12.0 solvent system used in these experiments permits the complete separation of six amino acids. It was early found that five of these were present in all the fertile, sterile, and restorer materials used. Threonine was absent in all these materials at the concentrations used. As no qualitative differences were apparent, it was considered necessary to undertake the semiquantitative studies presented below.

Semiquantitative estimates of comparative amino acid content on a per anther basis: Those differences in amino acid content between fertiles and steriles on a per anther basis which were significant are indicated in Table 5. It will be noted that three of the amino acids, aspartic acid, serine, and alanine were significantly higher in the fertiles than in the male steriles at the prepollen stage in all four varieties. Glutamic acid was also significantly higher in the fertiles at this stage in three varieties with no difference showing up in Combine Kafir-60. These results might be expected in view of the greater length and weight of the anthers at prepollen in fertile materials. Less expected, however, was the significantly higher amount of glycine in the steriles at a prepollen stage on a per anther basis in three varieties.

In Table 6 an attempt has been made to compare amounts of amino acid on a per anther basis in the restorer lines (carrying the dominant form of the male-sterile gene, *Ms*) with that in fertile and sterile lines. Varieties were consequently averaged, a practice which admittedly ignores the amino acid differences stemming from varietal differences and probably having nothing to do with sterility. It will be noticed, however, that at a prepollen stage for every amino acid, except glycine, the restorer lines had a position intermediate between values for fertile and sterile lines. In general, the values for restorers were lower at a pretetrad and at a microspore stage than for either fertiles or steriles, but the

TABLE 5

Significant differences between amounts of amino acids in fertiles and steriles on a per anther basis

Amino acid	Stage	Redlan*	Wheatland	Combine Kafir-60	Martin
Aspartic	Pretetrad	—	—	—	—
	Microspore	—	F	S	F
	Prepollen	F	F	F	F
Glutamic	Pretetrad	F	—	—	—
	Microspore	F	—	S	—
	Prepollen	F	F	—	F
Serine	Pretetrad	—	S	S	—
	Microspore	—	S	S	—
	Prepollen	F	F	F	F
Glycine	Pretetrad	—	—	S	—
	Microspore	—	—	—	—
	Prepollen	—	S	S	S
Alanine	Pretetrad	F	—	S	—
	Microspore	F	F	S	—
	Prepollen	F	F	F	F

* F=fertile line is significantly higher. S=sterile line is significantly higher. —=no significant difference.

TABLE 6

Summary of averages of amino acid content (μ g) on a per anther basis

Amino acid	Fertile	Pretetrad Sterile	Restorer	Fertile	Microspore Sterile	Restorer	Fertile	Prepollen Sterile	Restorer
Aspartic	.0034	.0036	.0016	.0067	.0057	.0040	.0081	.0033	.0075
Glutamic	.0042	.0035	.0041	.0071	.0063	.0048	.0122	.0059	.0097
Serine	.0043	.0045	.0023	.0070	.0082	.0060	.0204	.0101	.0151
Glycine	.0059	.0055	.0016	.0112	.0097	.0058	.0150	.0255	.0152
Alanine	.0022	.0018	.0017	.0059	.0048	.0051	.0261	.0068	.0137

comparative percentage increase in growth (see Table 4) served to bring up the restorers to the intermediate position at prepollen. In the case of glycine, however, which showed a higher value in the steriles at a prepollen stage the restorers were very similar to the fertiles which is suggestive of some correlation between sterility and glycine content.

Semiquantitative estimates of amino acid content on a dry weight basis: Those differences in amino acid content between fertiles and steriles on a dry weight basis which were significant are indicated on Table 7. The only significant difference which would appear to hold up with any consistency across varietal differences was glycine at a prepollen stage. Alanine appeared higher in the fertiles at the prepollen stage in three out of the four varieties and not different in the other. Averages of fertiles, steriles, and restorers on a dry weight basis (Table 8) indicated the restorers to be generally lower in amounts than either

TABLE 7

Significant differences between amounts of amino acids in fertiles and steriles on a dry weight basis

Amino acid	Stage	Redlan*	Wheatland	Combine Kafir-60	Martin
Aspartic	Pretetrad	—	—	—	—
	Microspore	F	F	—	F
	Prepollen	—	F	S	—
Glutamic	Pretetrad	—	—	—	—
	Microspore	F	—	—	—
	Prepollen	F	F	S	—
Serine	Pretetrad	—	—	S	—
	Microspore	—	—	S	—
	Prepollen	F	—	S	—
Glycine	Pretetrad	—	—	S	—
	Microspore	—	—	—	F
	Prepollen	S	S	S	S
Alanine	Pretetrad	—	—	—	—
	Microspore	F	—	—	—
	Prepollen	—	F	F	F

* F=fertile line is significantly higher. S=sterile line is significantly higher. —=no significant difference.

TABLE 8

Summary of averages of amino acid content (μ g) on a dry weight (mg) basis

Amino acid	Fertile	Pretetrad			Microspore			Prepollen		
		Sterile	Restorer		Sterile	Restorer		Sterile	Restorer	
Aspartic	.2340	.2540	.1064	.2331	.1775	.1607	.0953	.1020	.0891	
Glutamic	.2723	.2462	.2168	.2336	.1923	.1455	.1487	.1381	.1243	
Serine	.3133	.3287	.1576	.2524	.2559	.2390	.2811	.2624	.1930	
Glycine	.4432	.4057	.1034	.3907	.2679	.2153	.1826	.5271	.1962	
Alanine	.1543	.1285	.1207	.2034	.1543	.1982	.3663	.1987	.1710	

fertiles or steriles, the principal exception being alanine at the microspore stage and glycine at the prepollen stage.

DISCUSSION AND CONCLUSIONS

Initially one might have expected that any difference between lines isogenic at all loci might be associated with the sterility-fertility differential. Obviously the picture is not this simple. Two of the complicating factors appear to be:

1. The greater length and weight of the fertile anthers.
2. The fact that the isogenic lines are not necessarily isocyttoplasmic.

Due to the first factor, it would seem advisable to not put undue emphasis upon an accumulation of an amino acid in the fertile material on an anther basis. Attention is focused therefore on stages in which significantly higher amounts of an

amino acid occurred in the steriles and particularly on a dry weight basis. As shown in Table 7 this was true for glycine at a prepollen stage. This was the only amino acid of those so far tested for these four varieties which qualified as a fertility-sterility differential, and it may therefore be worth while to further study its effects. It is conceivable that the higher concentration of glycine in the steriles is in itself inhibitory to normal metabolism or causes sufficient imbalance to disturb normal processes; it is planned therefore to test the effect of glycine in different concentrations upon fertile and sterile anthers grown in vitro. If this amino acid is correlated with fertility in some way, it might theoretically be possible to experimentally prevent the formation of functional pollen in fertile anthers by control of glycine concentration itself.

Since the role of glycine in purine synthesis is documented in animal materials, it is equally possible that this accumulation in the steriles represents one of the following: glycine may be tied up by some means and inhibited from entering into purine metabolism, representing then the result of some enzyme reaction failure in the steriles. Inhibition of purine metabolism may in turn be a causal factor involved in failure of microspore mitosis in the steriles. However the inhibition of microspore mitosis by yet unknown factors may be merely reflected in such an accumulation of unmetabolized glycine, the accumulation representing in this case the result of sterility rather than a causal mechanism. It may be speculated that the difference in concentration of glycine between fertiles and steriles is in some way related to failure of microspore mitosis in the latter, and further work should point up which of the alternatives is most probable, if glycine be involved at all. There is also need for further correlated nucleic acid analyses.

What specific role, if any, alanine plays in genetic cytoplasmic male sterility is not clear at this point. The data of KHOO and STINSON (1959) for *Zea mays* indicate a precocious accumulation of alanine in certain sterile lines and not in others. The present data (Table 7), however, indicated that the fertiles of three varieties contained more alanine than their sterile counterparts while the other variety showed no significant difference. However, in this latter, the fertiles were higher at a microspore stage.

The advisability of using a number of varieties in these studies is certainly indicated. For example, on a dry weight basis at the prepollen stage four of the amino acids tested were in higher concentrations in Combine Kafir-60 steriles than in the fertiles of this variety, and there were several stages in other varieties at which one or another amino acid showed up in higher concentrations. These differences do not however, hold up for all varieties and since it would be anticipated that the mechanism controlling genetic cytoplasmic male sterility were the same for all these varieties it is not likely that these differences reflect this type of sterility. What these differences in isogenic lines signify is not yet clear.

Since the picture is not a simple one, it is deemed necessary that isogenic lines of the restorers be established. In the absence of these at present, the restorer data were treated as averages and compared with varietal averages. Differences

among varieties make these somewhat less significant comparisons, but they do point up the tendency towards comparatively lower amounts of each amino acid on a per anther basis in the restorers through the microspore stage (exceptions were glutamic acid at a pretetrad stage and alanine at a microspore stage). At the prepollen stage, however, the restorers were in every case intermediate between fertiles and steriles. In the case of glycine, the amount was very close to that for the fertiles. On a dry weight basis, the restorer materials were quite consistently low compared to both steriles or fertiles except for alanine content at a microspore stage and glycine at a prepollen, again the values being more similar to those for fertiles than to those for steriles.

It is recognized that many other factors were influencing clear-cut results. Although the age of these plants in terms of meiotic stage was a known factor, the data combine material grown in the field and in the greenhouse. Since it is known that the rate of movement of certain free amino acids differs with the season, it would be expected that environmental factors may play a large role. Further experimentation is planned under better environmental control than was feasible when the above experiments were conducted.

It is possible that the reported results were influenced by the fact that at the prepollen stage, metabolic processes within the anthers of the sterile materials are slowing down and beginning to disintegrate. This is the terminal stage for these anthers and breakdown products may be being formed, whereas the fertile materials are continuing to develop and to maintain some active syntheses. Many of the extracts of fertile materials probably included material with pollen grains already containing starch granules and therefore not strictly comparable to the terminal stage in the sterile anthers. It is hoped that several refinements in technique can be applied to this problem now that the experiments to date have pointed up the complexity of the situation and the need for more critical measurements.

The present paper covers the analysis of only six amino acids; the remaining amino acids are currently being investigated. Other components will also be studied since it is possible that carbohydrates and nucleic acids may be involved. Certainly both the failure of microspore mitosis to occur and the increasing evidence for the role of cytoplasmic nucleic acid in nonchromosomal inheritance make the comparative analyses of nucleic acids in these materials of paramount importance. The recent suggestion (EDWARDSON and CORBETT 1961) that at least in certain materials cytoplasmic male sterility transmissible through grafting may behave in a manner suggestive of viral transfer is of interest here also. Nucleic acid studies are underway in this laboratory, but it is hoped that the amino acid studies reported herein will indicate the work in progress, and point out some of the more interesting relationships that are apparent, although as yet incompletely understood. While glycine, and possibly alanine, appear at the present stage of the work to be associated in some way with the expression of sterility, only further analyses will bear out this suggestion.

SUMMARY

In order to elucidate the mode of gene action and of nuclear-cytoplasmic interaction involved in genetic cytoplasmic male sterility, an attempt is being made to conduct comparative studies on fertile and cytoplasmic male sterile lines of selected *Sorghum vulgare* varieties and on certain fertility restorer lines. While work is underway involving nucleic acids, carbohydrates and other amino acids, the present paper reports the chromatographic data on relative amounts of six amino acids in the anthers as well as data on the comparative lengths, fresh and dry weights of anthers at different meiotic stages.

1. Anthers of fertile lines attained a significantly greater length, higher fresh weight and higher dry weight at a prepollen stage than those of their respective isogenic sterile lines. The fertility restorers tested were intermediate in length and weights at this stage.

2. The sterile anthers failed to maintain a growth rate comparable to that of the fertiles following microspore formation.

3. Threonine was absent at the concentration studied in all materials investigated.

4. L-aspartic acid, L-glutamic acid, L-serine, glycine and L-alanine were present in all the materials studied.

5. On a dry weight basis, anthers of sterile lines of the four varieties studied showed a significant accumulation of glycine in the prepollen stage.

6. The average concentration of glycine in the restorer lines at a prepollen stage approximated that for the fertile lines more closely than that for the sterile lines.

7. Certain varieties showed an accumulation of alanine in the fertiles at a prepollen stage.

8. The significance of these data is being tested by *in vitro* experimentation as well as by means of more refined techniques of chromatography.

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