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Male sterility in Indian mustard (*Brassica juncea* (L.) Coss.) – a biochemical characterization

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Summary. Biochemical studies were conducted on some male sterile and their fertile F_1 analogues in Indian mustard. The variation in pH activity during microsporogenesis was normal, except in MS-3. Male sterile anthers had deficient sugar metabolism. Cytochemical analysis of sporogenous and tapetal tissue suggested an effect of sterility elements on the anabolic and catabolic fate of DNA and protein during microsporogenesis. Leaves of male sterile lines had a higher chl a/b ratio. Leaf peroxidase activity was low and different isozymes appeared when separated by starch electrophoresis.

Key words: *Brassica juncea* – Male sterility – Biochemical characterisation – Microsporogenesis – Peroxidase enzyme

Introduction

The final expression of male sterilizing genes arises from a multitude of sporophytic and gametophytic aberrations during microsporogenesis. The sporophytic irregularities are usually accompanied by changes of a cytological or physiological nature, obviously a manifestation of broad pleiotropic spectra. Whether such physiological aberrations are the causes or the effects of male sterility is still in the realm of speculation. However, such studies are important from both an evolutionary and a practical point of view. The studies reported here were conducted on a group of male sterile lines of *Brassica juncea* develop at Punjab Agricultural University, Ludhiana.

Materials and methods

The biochemical studies were conducted on the anthers and leaves of male sterile and fertile F_1 derivatives. Anther samples at the premeiotic (PM), meiotic (M), tetrad (T), mononucleate microspore (NM) and mature pollen stage were analysed for pH range, free sugars, protein and DNA levels. A slight modification of the technique as suggested by Izhar and Frankel (1971) was used to estimate pH range. Approximately 10 anthers in each case were crushed into a 2 ml NaCl solution (pH 6.25). The contents were mixed thoroughly and the pH recorded immediately. The quantitative estimation of free sugars was made by using urine sugar analysis test tape (Eli Lilly and Co, Indianapolis). In each case 10 anthers were crushed in one drop of distilled water on an adhesive tape. The contents were mixed and left for 2 min. The urine test tape was dipped into the sample mixture and allowed to develop colour. The intensity of colour developed within three minutes of dipping was graded according to the manufacturer's specification. DNA and protein estimation was done cytochemically. The floral buds were fixed in acetic alcohol (1:3) for 24 h. Conventional technique of dehydration in tertiary butyl alcohol, and embedding in paraffin (MP 58°–60 °C) was followed. Sample blocks were sectioned to give 10 to 15 slides each of 10 micron thickness. For the localisation of DNA and protein, the deparaffinised sections were stained respectively in Azure B (Flax and Himes 1952) and Bromophenol blue (Chapman 1975). The intensity of colour as observed under light microscope was graded as faint (1), normal (2), strong (3), and intense (4).

The pigment estimation in fresh leaves was carried out by the technique suggested by O'Neill and Cresser (1980). Horizontal starch gel electrophoresis (Smithies 1955) was employed for peroxidase isozyme analysis in fresh leaves. A modification of the technique (Brewer 1970) was used for staining. The starch gel was incubated in a benzedene staining mixture and about 10 ml of 6% H_2O_2 was added. Incubation period was about 90 min at 5–10 °C-until the bands appeared.

Results

pH range

The pH estimates in the anther extracts of male sterile and male fertile lines are presented in Fig. 1. In the fertile anthers, the pH was alkaline (pH: 7.46) during the premeiotic phase of development and became acidic precisely before the release of quartets. Micro-

spore development finally culminating in pollen release was accompanied by an increase in alkalinity, which, however, was lower than that recorded at premeiotic stage. All the male sterile lines with the sole exception of MS-3 followed similar trends. MS-3, on the other hand, recorded minimal value during meiosis, and advent of tetrad stage was associated with a sharp increase.

Free sugars

Low sugar levels at the premeiotic stage was always followed by elevated sugar indices during meiosis and subsequent stages of pollen development in fertile anthers (Fig. 2). Male sterile lines, with the exception of MS-5, on the other hand, showed signs of sugar starvation during all developmental stages, especially at pollen maturation stage. In almost all cases, restored F_1 hybrids had normal sugar metabolism, the exceptions being partially fertile F_1 hybrids involving MS-5 and MS-6, where sugar status at pollen maturation did not deviate significantly from their male sterile parents.

Cytochemical studies

Data regarding the levels of DNA and proteins in sporogenous and tapetal tissue at different stages of microsporogenesis are presented in Tables 1 and 2, respectively. For the sake of clarity these data are being described separately for fertile and sterile anthers.

Fertile anthers

Sporogenous tissue. The sporogenous tissue of normal anthers was characterised by a high level of DNA (3.70) at the premeiotic stage which was followed by a decrease until the tetrad stage (2.65) (Table 1). The decrease was compensated for in mature pollen cells (3.35). Protein level was at a minimum during meiosis (1.17) and started increasing during the tetrad stage (2.56). Mature pollen cells were rich in protein (3.93) compared to all other stages of microsporogenesis.

Tapetal tissue. Both DNA and protein levels (3.55 and 3.70, respectively) were at a maximum at the premeiotic stage (Table 2). A gradual depletion of both took place until the mature pollen stage, when only meagre quantities (0.38 and 0.11, respectively) were left. The decrease in DNA content was gradual but more intense after

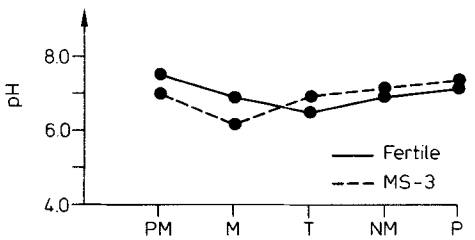

Fig. 1. pH estimation at various stages of anther development

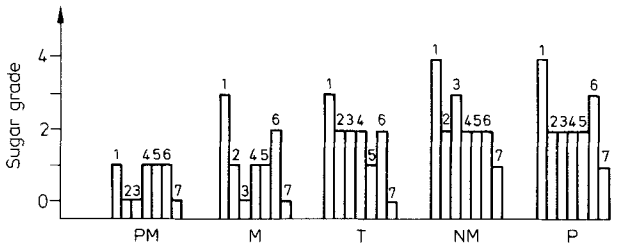

Fig. 2. Qualitative estimation of sugar content in fertile (*1*), MS-1 (*2*), MS-2 (*3*), MS-3 (*4*), MS-4 (*5*), MS-5 (*6*) and MS-6 (*7*)

the nucleate microspore stage whereas protein depletion was slow up to the tetrad stage but more prominent in nucleate microspore stage.

Male sterile anthers

Sporogenous tissue. In contrast to male fertile anthers, the male sterile lines had, in general, low DNA levels at all stages of pollen development, including the premeiotic stage. Decrease in DNA during meiosis was observed in all male sterile lines but this decrease, except in MS-3 line, was not compensated for, as a result of which mature pollens did not have enough DNA.

Similarly, except for MS-2 line, the general trend for protein level was similar to that seen in fertile anthers up to the tetrad stage, after which the decrease in protein level was not compensated for. The pollens were starved of DNA and protein at the mature pollen stage.

Tapetal tissue. It was characterised by a low amount of DNA at all stages of microsporogenesis. Initial DNA levels were also low when compared to fertile plants but the level was invariably high in contrast to normal lines at the mature pollen stage. DNA depletion was not regular in male sterile lines, except for in MS-2.

Protein depletion of tapetal tissue was normal in all the MS lines except for MS-3 and MS-6. So it can be interpreted that in male sterile anthers continuous flow of macromolecules viz., DNA and protein from the tapetum was checked as a result of which there was general starvation of developing microspores.

Leaf pigments

In general, male sterile lines had lower estimates of all three major plant pigments than their corresponding fertile F_1 hybrids. The reverse was, however, true for the chlorophyll a/b ratio. This may be attributed to a comparatively greater increase in chlorophyll b content then chlorophyll a in fertile F_1 hybrids.

Peroxidase isozyme analysis

The leaf peroxidase isozyme patterns of male sterile lines were also compared with their fertile F_1's (Fig. 3).

Table 1. DNA and protein levels (expressed in arbitrary units) in sporogenous tissue of male fertile and male sterile lines

Accession		Developmental stage				
		Premeiotic	Meiotic	Tetrads	Nucleate microspores	Pollens
Fertile	DNA	3.70±0.26	2.95±0.25	2.65±0.18	2.75±0.21	3.35±0.25
	Protein	2.59±0.13	1.17±0.20	2.56±0.32	3.72±0.25	3.93±0.17
MS-1	DNA	2.27±0.13	2.89±0.15	1.50±0.16	1.40±0.09	1.80±0.23
	Protein	2.48±0.03	1.66±0.16	2.45±0.03	2.95±0.17	0.14±0.09
MS-2	DNA	2.18±0.27	1.80±0.24	2.00±0.21	2.10±0.23	2.03±0.17
	Protein	2.05±0.14	0.95±0.23	0.83±0.02	1.71±0.04	1.52±0.09
MS-3	DNA	2.40±0.26	2.25±0.35	2.45±0.19	2.69±0.23	0.00[a]
	Protein	2.15±0.14	1.25±0.06	2.23±0.18	2.05±0.04	0.00[a]
MS-4	DNA	3.05±0.13	2.16±0.17	2.10±0.17	1.25±0.09	2.15±0.01
	Protein	2.44±0.17	1.15±0.32	2.61±0.03	1.49±0.04	1.67±0.16
MS-6	DNA	3.60±0.19	2.17±0.12	1.10±0.09	0.70±0.14	0.60±0.20
	Protein	2.64±0.05	1.30±0.04	2.25±0.05	2.15±0.09	1.63±0.04

[a] Sporogenous tissue was completely reabsorbed

Table 2. DNA and protein levels (expressed in arbitrary units) in tapetal tissue of male fertile and male sterile lines

Accession		Developmental stage				
		Premeiotic	Meiotic	Tetrads	Nucleate microspores	Mature pollens
Fertile	DNA	3.55±0.12	3.16±0.23	2.75±0.21	2.10±0.07	0.38±0.12
	Protein	3.70±0.05	3.60±0.08	3.33±0.12	1.13±0.15	0.11±0.25
MS-1	DNA	2.64±0.15	2.20±0.12	1.90±0.02	2.30±0.14	0.81±0.17
	Protein	3.47±0.10	3.50±0.20	2.98±0.17	1.10±0.10	0.00
MS-2	DNA	2.40±0.26	1.40±0.25	1.30±0.23	1.05±0.05	0.90±0.15
	Protein	3.65±0.08	3.34±0.16	1.99±0.03	0.86±0.05	0.87±0.09
MS-3	DNA	3.15±0.17	3.05±0.23	2.10±0.15	3.25±0.30	3.65±0.03
	Protein	2.67±0.04	1.95±0.25	3.65±0.07	3.45±0.09	4.00±0.00
MS-4	DNA	2.75±0.03	1.93±0.30	1.67±0.17	0.31±0.21	0.00
	Protein	2.95±0.08	2.76±0.16	3.15±0.05	0.75±0.08	0.15±0.10
MS-6	DNA	2.93±0.14	2.70±0.14	1.40±0.15	1.40±0.15	1.36±0.24
	Protein	3.45±0.05	3.10±0.06	3.05±0.60	2.03±0.06	1.95±0.05

Table 3. Quantitative estimation of various leaf pigments in male sterile lines and their fertile F_1 hybrids

Entry	Chlorophyll-*a* (mg/g)	Chlorophyll-*b* (mg/g)	Carotenoids (mg/g)	Chla/Chlb
MS-1	6.76	4.25	0.22	1.59
MS-1 × EJ 20 (F_1)	7.06	4.84	0.39	1.45
MS-2	5.38	2.77	0.18	1.94
MS-2 × RLM 198 (F_1)	6.02	3.54	0.18	1.70
MS-3	6.20	3.49	0.23	1.77
MS-3 × RLM 198 (F_1)	6.37	4.73	0.18	1.35
MS-4	5.33	2.87	0.18	1.85
MS-4 × Varuna (F_1)	6.43	3.64	0.19	1.77
MS-5	6.59	3.67	0.12	1.79
MS-5 × EJ-4 (F_1)[a]	5.60	2.93	0.21	1.19
MS-6	3.15	1.74	0.19	1.81
MS-6 × EJ 33 (F_1)[a]	5.70	3.37	0.26	1.69

[a] Partially fertile

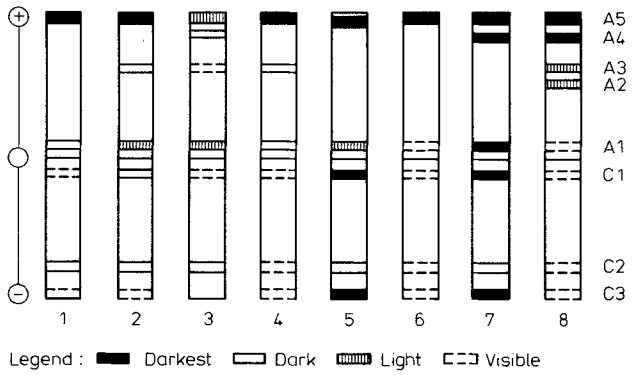

Fig. 3. Peroxidase isozyme analysis in Indian mustard. *1* MS-1 × EI 36; *2* MS-1; *3* MS-2 × EI 20; *4* MS-2; *5* MS-4 × RLM 198; *6* MS-4; *7* MS-3 × 'Burgande'; *8* MS-3

The polypeptide mobilities were apparently identical for fertile F_1's. Polypeptide pattern of the A2 zone was different in male sterile lines, being strong for MS-2 and MS-3, weak for MS-1 and completely absent for MS-4. In MS-3, an additional band was observed at zone A3. Low peroxidase activity in male sterile lines was indicated by diminishing C2 and C3 zones which were restored in fertile F_1 hybrids.

Discussion

Differences in the level of accumulation or depletion of various biochemical products during microsporogenesis indicate a normal vs. mutant metabolism. Sterilizing factors can trigger large scale disturbances of plants in general and anthers in particular. The completion of the tetrad stage is accompanied by a fall in pH, which is correlated with elevated callase activity in *Petunia* (Izhar and Frankel 1971). Subsequently, callose tetrad walls are digested and microspores are released in anther lumen. In MS-3, pH drop was evident even during early meiosis, consequently there is a premature dissolution of callose, and pollens cease to develop and degenerate. Any untimely or abnormal formation and dissolution of callose walls resulted in pollen sterility in peppers (Horner and Rogers 1974). Premature release of microspores without primexine formation on primary microsporocytes is suicidal (Mian et al. 1974). General retardation of sugar metabolism was manifested in anthers of all male sterile lines. This deficiency was corrected in fertile F_1 hybrids, except for in MS-5 and MS-6, where restoration was only partial. Sequestering of normal metabolic events, perhaps simply through deviant nutrient supply, could starve the developing microspores. Prokofeva et al. (1979) have also associated reduced sugar metabolism to male sterility in wheat. The mutant sporophytic behaviour may induce faulty timing and coordination in developmental sequences between the sporocyte and the tapetum.

The initial functional deviations in the form of sporogenous tissue deficits of DNA and proteins appeared only after release of quartets in MS-1. In tapetal tissue, such abnormalities occurred during nucleate microspore stage and were restricted to proteins only. Thus it is apparent that ms gene action resulted in the depletion of DNA and proteins which eventually resulted in the complete destruction of sporogenous cells. The DNA and proteins had similar anabolic patterns in the tapetum but their catabolic fates differed. Differential DNA loss without protein degeneration needs some explanation. It is probable that a protein dependent DNA degrading enzyme may diffuse from the sporogenous cells to the tapetum and thus initiate DNA breakdown (Mian et al. 1974). However, the nature of this genetic control or the biochemical elements which initiate this breakdown needs exploration.

Early onset of sporogenous DNA and protein deficit in MS-2 suggests an effect of sterility elements on DNA and protein synthesis at the premeiotic S phase or the meiotic prophase. DNA level in tapetum was lower than that observed for fertile anthers. Deviant behaviour of the sporogenous and tapetal tissue might be explained by assuming a dislocation in the inflow of macro-molecules including DNA and protein precursors from the tapetum to the sporogenous tissue.

The aberrant behaviour of DNA and protein levels in MS-3 can only be explained on the abnormal tapetal behaviour. The tapetum development in the anthers of male sterile line was normal until the microspore tetrad stage. This was followed by tapetal hypertrophy. The cells became enlarged, acquiring a supranormal shape and size. A plasmodium was then formed by coalescence. Ultimately the loculus was invaded and meiocytes were crushed and even reabsorbed. This explains the steep increases in protein and DNA content in the tapetum during later stages of pollen maturation. Chauhan and Kinoshita (1979) have also reported similar results in *Cucumis milo*. These observations formed the basis of their theory of tapetal function and its role in pollen abortion. The prime aspect of their theory is the presumption that tapetal cells receive little or no food from elsewhere and, as a consequence, take to haustorial activity. This predatory activity is directed towards microspores which collapse as a result.

In MS-4, the initial protein and DNA deficiency observed during meiosis and early mononucleate stage appears to mark the onset of continuous degenerative events leading to sterility. The sterilizing factors in this line appear to have direct effect on tapetal cells where drastic DNA and protein deficits appear as early as meiosis. Several workers have reported cytomixis and transmittance of chromosomal material from the tapetum to developing microsporocytes (Heslop-Harri-

son 1972). Transfer of feulgen positive bodies, originating in the tapetum, passing into anther lumen and ultimately their association with microspores have been reported (Cooper 1952). Thus any disruption in the biochemical link between tapetum and developing microspores should result in abortive pollen grains.

MS-6 had comparatively slow tapetal degeneration with signs of DNA and protein accumulation, particularly during the terminal stages of sporogenesis. The inverse relationship of DNA and protein in tapetal and sporogenous nuclei in fertile anthers has been used by Mian et al. (1974) to postulate that sporogenous cells normally provide a stimulus having two effects: first, depression of tapetal DNA synthesis during meiosis and second, translocation of DNA breakdown products and pollen wall precursors from the tapetum to young microspores. Thus, aberrant tapetal behaviour should be considered as a reflection of deviant metabolism of PMC's.

The male sterile lines had higher chlorophyll a/b ratios than their fertile derivatives, which is contrary to findings in wheat (Svachenko et al. 1974) and barley (Ahokas 1980). Lower ratios in fertile derivatives can be attributed to a comparatively greater increase in chlorophyll b content, and may be linked to a physiological realisation of restoration. Leaf carotenoids have also been linked with anther carotenoid metabolism (Svachenko et al. 1974). Chemical analysis of pollen walls have revealed that while intine is largely cellulose, the exine sporopollenins are oxidative polymers of carotenoids or carotenoid esters (Shaw 1971). Though the real role of carotenoids in anthers remains vague, the level of carotenoids required for the functioning of male cells may provide some clue to metabolic pathways still untraced.

The peroxidase enzyme when separated by starch electrophoresis appeared as different isozymes. The diminishing C2 and C3 zones can be explained as being a result of proteolysis. The frontal culmination of two new bands A2 and A3 in some male sterile lines and their complete absence in restored hybrids can be taken as evidence of some inhibitory activity. Changes in the component patterns of cytoplasmic proteins and isozymes of peroxidase have been ascertained to be associated with male sterility in maize and pepper (Gorya 1979; Markova and Daskalov 1974). The connection between peroxidase activity and male sterility is not yet understood. The observations based on peroxidase analysis do not permit us to ascribe very specific roles, since isozymes can arise as isolation artifacts and thus may not be related to their functional conditions.

Present experiments have shown that many precisely coordinated and interlinked processes are involved during microsporogenesis. Any dislocation in these may result in sterility. This study clearly demonstrated that male sterility is not a uniform phenomenon and that the male sterility genes differ in primary action and in the time and nature of developmental processes influenced. This is confirmed by the fact that corrective or fertility restorer genes are specific for each of the cytoplasmic male sterile lines.

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