Dedicated with humility and

reverence to my

beloved parents

Shri. Kotla Rangaiah

and

Sau. Kotla Vanajatha

-----Chinna

STUDIES ON RESPIRATORY ENZYMES AND OXYGEN UPTAKE BY MITOCHONDRIA OF CMS SYSTEMS OF SORGHUM

By

K. Swathi

Reg No 20129

A thesis submitted to the

**MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI, DIST. AHMEDNAGAR – 413 722,
MAHARASHTRA STATE (INDIA)**

in partial fulfilment of the requirements for the degree

of

MASTER OF SCIENCE [AGRICULTURE]

in

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2002

CANDIDATE'S DECLARATION

I hereby declare that this thesis or part thereof has not been submitted by me or other person to any other University or Institution for a Degree or Diploma

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CERTIFICATE

This is to certify that the thesis entitled, **"STUDIES ON RESPIRATORY ENZYMES AND OXYGEN UPTAKE BY MITOCHONDRIA OF CMS SYSTEMS OF SORGHUM"** submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar, Maharashtra State, for the award of degree of **MASTER OF SCIENCE (AGRICULTURE)** in **BIOCHEMISTRY**, embodies the results of a piece of *bonafide* research work carried out by **Miss. KOTLA SWATHI** under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma

The assistance and help received during the course of this investigation have been duly acknowledged

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Date / /2002

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Date : 29/ 06 /2002

(K. Swathi)

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ABBREVIATIONS

A	Absorbance
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CMS	Cytoplasmic male sterility
cm	Centimetre
CO	Carbon monoxide
Cox	Cytochrome *c* oxidase
^0C	Degree centigrade (celsius)
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetra acetic acid
fr wt	Fresh weight
FAD	Flavine adenine dinucleotide
Fig	Figure
g	Gravitational force
g	Gram
h	Hour
Hepes	(N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid])
kDa	Kilo dalton
Kg	Kilogram
KCN	Potassium cyanide
K_3 Fe $(CN)_6$	Potassium ferricyanide
µl	Microlitre
µm	Micromoles
µM	Micromolar
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mt	Mitochondrial

ABBREVIATIONS (Contd..)

NAD^+	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NRCS	National Research Centre for Sorghum
O D	Optical density
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
s	Seconds
UQ	Ubiquinone
viz ,	Videlicet (namely)

ABSTRACT

"STUDIES ON RESPIRATORY ENZYMES AND OXYGEN UPTAKE BY MITOCHONDRIA OF CMS SYSTEMS OF SORGHUM"

by

Miss. SWATHI K.

A candidate for the degree
of
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Research Guide A A Kale
Department Biochemistry

The main objective of the present investigation was to study some biochemical parameters associated with CMS phenotype in sorghum using isolated mitochondria Mitochondria were isolated from the etiolated seedlings of the two CMS systems of sorghum *viz*, 1049A, 1049B, RSV-33R, SPH-1229 and 104A, 104B, RS-585, CSH-15R The assays of soluble proteins and respiratory enzymes *viz*, cytochrome *c* oxidase and succinate dehydrogenase were carried out from isolated mitochondria Further, the total respiration, cyanide-sensitive, cyanide-insensitive and residual respiration were measured from the isolated mitochondria and etiolated seedlings of these lines The protocol for the isolation and purification of mitochondiral DNA was also standardized.

The isolated mitochondria from both the CMS lines, 1049 A and 104A, contained higher soluble proteins than their respective isonuclear maintainer, restorer and hybrid lines

The cyanide-sensitive respiration was measured by using SHAM, a potential inhibitor of alternative oxidase, while cyanide-insensitive respiration was measured by using KCN, a potential inhibitor of cytochrome *c* oxidase The cyanide-sensitive respiration was higher, whereas cyanide-insensitive respiration was lower in isolated mitochondria and etiolated seedlings of CMS lines than their corresponding maintainer, restorer and hybrid lines When both inhibitors were added simultaneously in the reaction media, residual respiration was observed in isolated mitochondria and etiolated seedlings of both the CMS systems

The cytochrome *c* oxidase and succinate dehydrogenase activities in both the CMS lines were lower than their respective isonuclear maintainer lines and also restorer and hybrid lines The CMS system 104A, 104B, RS-585 and CSH-15R showed comparatively higher cytochrome *c* oxidase and succinate dehydrogenase activities than the other CMS system, 1049A, 1049B, RSV-33R and SPH-1229

The higher protein content, cyanide-sensitive respiration and lower cytochrome *c* oxidase activity in CMS lines than their isonuclear lines indicates that mutation might have occured in the mitochondrial DNA which probably resulted in the synthesis of new protein(s) Specifically the respiratory complex IV might have been mutated because some of the subuntis of this complex are encoded by the mitochondrial genome, reflecting the alteration in the cytochrome *c* oxidase activity and cyanide-sensitive respiration The lower cyanide-insensitive respiration and succinate dehydrogenase activity in CMS lines than their corresponding

Abstr Contd Miss K Swathi

isonuclear maintainer lines suggested that it might be the result of the CMS character and not the cause of CMS, because alternative oxidase and succinate dehydrogenase are encoded by the nuclear DNA The lower alternate oxidase activity in CMS than its isonuclear line may perhaps be correlated to the higher yield or yield contributing characters That's why the hybrid CSH-15R yields more than the hybrid SPH-1229

The protocols for the isolation and purification of mitochondria and mitochondrial DNA were standardized. The alkalilysis method yielded higher mtDNA than freezing and thawing method The freezing and thawing method required less time for the isolation of mtDNA than the alkalilysis method, however the DNA isolated by these two methods was found to be pure By employing these two methods, one can avoid the use of percoll gradient and sucrose density gradient ultra-centrifugation for the isolation of mitochondria and also organic solvents for the purification of mtDNA

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INTRODUCTION

1. INTRODUCTION

India is the second largest producer of sorghum grains only after the United States of America in the world. Among the cereals, sorghum (*Sorghum bicolor*) has the highest dry matter accumulation rate on a daily basis and also has the highest food energy (349 Kcal/100 g edible portion) per unit cultural energy ratio when compared with corn grain and sugarcane (Gopalan *et al* , 1991). Grains of common cereals including sorghum are the major source of carbohydrates and proteins in human nutrition They are also extensively used to produce alcoholic beverages and stockfeed (Fincher, 1989) Sorghum is well known for drought tolerance and can be cultivated in both Sub tropical and tropical environment (Somani, 2000). Almost entire production of grain sorghum goes for the human consumption in one or the other form However, it has remained a food of economically weaker sections, farmers and people of low rainfall areas. This is mainly due to inferior nutritional quality consisting of low levels of proteins and amino acids-lysine, threonine and tryptophan, higher levels of prolamine and leucine, presence of phenolic compounds and lower protein and starch digestibilities. Improvement of sorghum in terms of better nutritional quality and higher yields is possible through plant breeding programmes.

Cytoplasmic male sterility (CMS) is the most important biological tool in crop plants including sorghum for commercial hybrid seed production. CMS, characterized by the inability of plant to shed functional pollen is a maternally-inherited defect common in many higher crop plants It is considered as a prized agricultural commodity since it is less expensive and more reliable than hand and mechanical means of pollination control

Hybrid seed production is facilitated by the development of CMS, maintainer and restorer lines. The male sterile and maintainer (fertile) lines have an identical nuclear background but different cytoplasms. Since the identical nuclear background causes sterility in one line but not in the other (the maintainer), it is clear that a given nuclear background is compatible with one cytoplasm but incompatible with the other. Apparently, the sterility is related to the incompatible interaction of the nuclear genome with the mitochondrial genome (Hakansson and Glimelius, 1991)

CMS in sorghum has been reported in a number of varieties originating in different geographical regions *viz*, India, Africa and America The three male sterile cytoplasms designated as *Maldandi*, Guntur and Vizianagaram are of Indian origin (Sane *et al*, 1996) All the sorghum hybrids developed to date are based on milo cytoplasm. It is important to find out the differences between sterile and fertile cytoplasms of sorghum in a particular nuclear background and also to distinguish the newly evolved CMS lines at both the biochemical and molecular level Conventional method of classification of different sources of CMS cytoplasms to test the reciprocal maintainer-restorer ability among crosses of sterile and maintainer lines is time-consuming and cumbersome

Mitochondrial gene mutations have been associated with CMS phenotype and the involvement of chloroplast genome had been ruled out by Hanson (1991). The mt-genome encodes for a number of polypeptides / proteins including respiratory proteins/enzymes. Earlier, several investigators have reported that mitochondrial genes, *viz*, *atpA*, *atp6*, *atp9*, *coxI*, *coxII*, *coxIII* etc were mutated in different crops resulting in CMS trait (Sane *et al*, 1996; Leaver *et al*, 1988). The restriction enzyme pattern and the restriction fragment length polymorphism (RFLP) was successfully used

for distinguishing isonuclear male sterile and male fertile cytoplasms and also to discriminate the different sources of CMS lines in various crop plants at the molecular level (Ahnert *et al* , 1996)

Although regions associated with male sterility have been identified in several mitochondrial genomes, the biochemical basis of the sterility is not yet clearly understood Attefnpts were made earlier by Munjal *et al* (1988) which discriminated CMS lines from different sources and also distinguished the isonuclear male sterile and fertile lines of sorghum at the biochemical level by examining carbon monoxide sensitivity of cytochrome *c* oxidase

The mitochondrial electron transport is carried out by five major multisubunit complexes consisting of polypeptides which are partly encoded by mitochondrial genome and partly by the nuclear genome Since the sterility is interpreted in terms of nuclear and cytoplasmic incompatibility, the assay of the enzymes involved in electron transport chain may generate basic information which will be helpful for understanding the mechanism of male sterility at biochemical level

It was, therefore, proposed to undertake the present investigation with the following objectives

1 Standardization of the protocol for the isolation of mitochondria and mitochondrial DNA,
2 Oxygen uptake studies in isolated mitochondria from the vegetative cells of CMS and fertile lines, the cyanide-insensitive respiration in vegetative cells, and

4 To study the variations, if any, in succinate dehydrogenase and cytochrome *c* oxidase activities in isolated mitochondria from the CMS system

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Cytoplasmic male sterility (CMS) is the most important biological tool in crop plants including sorghum for commercial hybrid seed production CMS plants are used as female parents in crosses, so that any seed formed on CMS parent is known to be the product of cross pollination In some crops, cytoplasmically male sterile parent are the only way to economically and effectively produce hybrid seeds For example, production of hybrid seed for grain sorghum on commercial scale is dependent on a CMS system because the flowers are small, difficult to emasculate and the plant is self-fertile The alternatives, *viz*, nuclear genetic male sterility and hand emasculation before cross pollination are often not economically viable

The CMS phenotype has been originated from mutation(s) in the mitochondrial DNA (mt-DNA) of male fertile progenators as a result of aberrant recombination events This results in a different restriction endonuclease digestion pattern of mt-DNA from fertile and male sterile cytoplasms (Pring and Levings, 1978, Dewey *et al*, 1986) Mitochondria isolated from CMS lines of maize (Forde *et al*, 1978, Forde and Leaver, 1980) and sorghum (Dixon and Leaver, 1982) synthesized a characteristic variant polypeptide not found in mitochondria from normal (fertile) lines Study of cytoplasmic nuclear interactions that effect male-sterility in plants might reveal much about the functions and variation of mitochondrial genome

Indian Plant Breeders have evolved several CMS lines of sorghum in the past few decades and the molecular and biochemical studies of these

lines is important to findout the differences between the sterile and fertile cytoplasms in a particular identical nuclear background Thus to understand and interpret the biochemical and molecular differences between the sterile and fertile cytoplasms a clear knowledge of structure, function and organization of respiratory chain in plant mitochondria, mitochondrial genome and the respiratory enzymes is very much essential In view of this the relevant references pertaining to the above mentioned topics are reviewed here briefly

2.1 Higher plant mitochondria

Plant mitochondria are small organelles of cytoplasmic compartment of the cell which are generally spherical to ellipsoid in shape and about 1 µm long Mitochondria are involved in respiration and providing energy in the form of adenosine triphosphate (ATP) and metabolic precursors for cells The plant mitochandria consists of a folded inner membrane invovled in different processes, *viz*, electron transport, metabolite transport via selective carriers and ATP synthesis It encloses a matrix space containing all the tricarboxylic acid (TCA) cycle enzymes as well as the distinct genetic system (DNA, RNA and ribosomes) for protein synthesis (Neuburger, 1980) Thus, plant mitochondria are a part of the living cell and influence the developmental, adaptive and genetic properties of the cell

2.2 Similarity of plant mitochondria with animal mitochondria

Many basic features of mitochondrial structure and function, developed at an early stage of evolution, have been highly conserved in animals and plants despite a billion years of divergent evolution Thus, the

similarity between animal and plant mitochondria are briefly reviewed as follows

a The patterns of phospholipids in membranes from plant and animal mitochondria were virtually identical (Harwood, 1985),

b The sequence of electron carriers that mediated the flow of electron from NADH and succinale to oxygen via cytochrome oxidase and ATPase complex appeared very similar to those found in mitochondria from yeast and animals (Moore and Rich, 1985, Palmer, 1976, Storey, 1980),

c TCA cycle functioning in plant mitochondria resembled in many respects to that in animal mitochondria (Wiskich, 1980),

d There were similarities in energy conservation between plant and mammalian mitochondria (Moore and Rich, 1985),

e Plant mitochondria possessed transport systems for numerous anions similar to those of other organisms (Wiskich, 1977, Day and Wiskich, 1984) and

f The outer membrane of plant mitochondria possessed a 31-kDa channel forming protein called mitochondrial porine similar to that of mammalian and yeast mitochondria and responsible for high permeability of the outer membrane of small molecules (Mannella and Tedeschi, 1987)

2.3 Distinctiveness of plant mitochondria

Eventhough there were certain similiarities in plant and animal mitochondria, plant mitochondria possessed certain peculiarities which were not present in animal mitochondria The distinct differences lies between these two in respect of electron transport system, the presence of specific

dehydrogenases, anion carriers and in size and complexity of their DNA (Ikuma, 1972) In addition, the rate of O_2 consumption on protein basis was much higher in plant than in animal mitochondria (Douce *et al*, 1972, Ikuma, 1972), while fatty acid oxidation was lower or not detectable in plant mitochondria (Gerhardt, 1986) Mackezie and McIntosh (1999) reported that tissues demanding high rates of metabolism during reproduction and fruiting, or in the case of nitrogen fixation, requiring low oxygen concentrations, represent processes peculiar to plants Due to an inability to mobilize so as to avoid environmental stresses, plants have evolved unique adaptations to stress, some of which involve the mitochondrion

2.4 The higher plant mitochondrial respiratory complexes

The fundamental structure, function and organization of respiratory chain components and phosphorylation system of higher plant mitochondria were remarkably similar to that found in mammalian system The plant respiratory chain consists of four protein complexes, complex I, complex II, complex III (usually called the cytochrome bc_1 complex) and complex IV or cytochrome *c* oxidase complex (Douce *et al*, 1987) as shown in Fig 1 Except for cytochrome *c*, these complexes were very hydrophobic and were soluble in the "fluid" lipid bilayer medium of mitochondrial inner membrane, more generally known as the coupling membrane

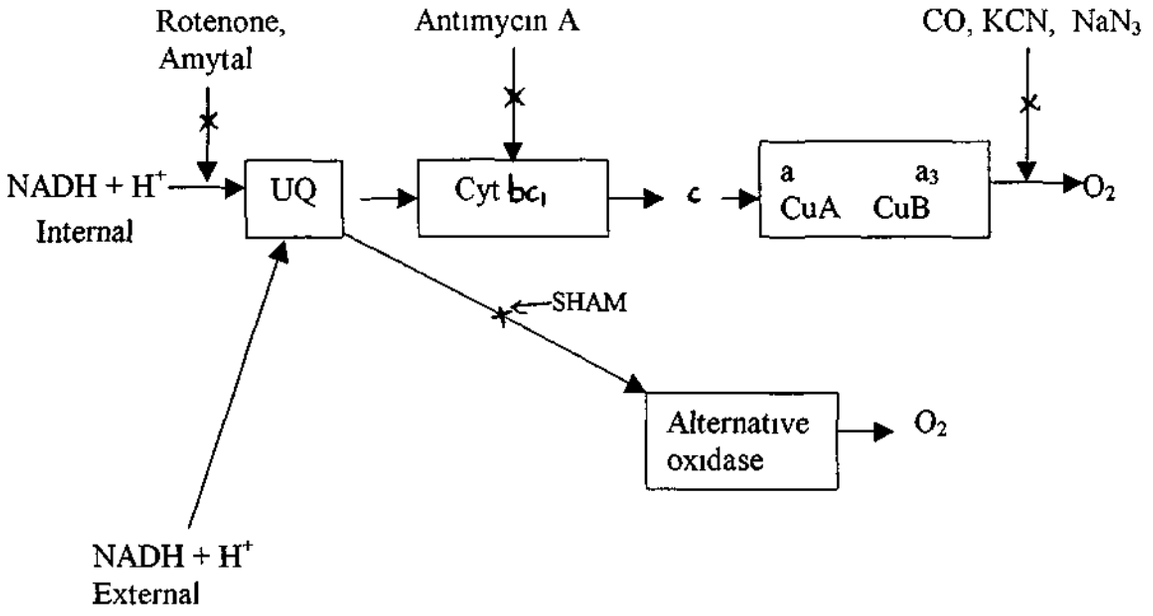
However, distinct differences between plant and animal mitochondria include the cyanide–and antimycin A–insensitive and the respiration linked oxidation of external NAD (P) H and rotenone-insensitive oxidation of internal NADP (Lance *et al*,1975, Laties, 1982)

2.4.1 Mitchondrial respiratory complex I

Complex I is responsible for electron transport from NADH to ubiquinone Fig 1 Consequently, complex I is the entry point for the redox equivalent of NADH produced in the matrix space during the course of substrates (maltae, 2-oxoglutarate, pyruvate, citrate and glycine) Oxidation catalyzed by the various NAD^+ linked NADH dehydrogenases of the TCA cycle, arranged spatially in the mitochondrial matrix into a functioning complex or in case of green leaf mitochondria by the glycine decarboxylase complex Complex I contains a non-covalently bound flavin mononucleotide (FMN), several iron sulphur centres and proably two molecules of ubiquinone However, the number, type and spatial distribution of iron sulphur clusters in the NADH ubiquinone oxidoreductase segment of respiratoary chain is still the subject of considerable uncertainty Rotenone and the powerful inhibitor, piericidin, inhibit the NADH ubiquinone reductase of complex I (Douce *et al* 1987) In the mitochondrial membrane, complex I functions as the 'first coupling site' carrying reversible electron flux from NADH to ubiquinone coupled to generation of energy Thus, it has been shown that passage of a pair of electrons from NAD^+ linked substrates to ubiquinone in mitochondrial respiratory chain result in the translocation of four H^+ ions from matrix into the medium as shown below

$$NADH + H^+ + Q + 4H^+ (in) \longrightarrow NAD^+ + QH_2 + 4H^+ (out)$$

Mitochondrial NADH dehydrogenase complex had more than or equal to 25 subunits, out of these nine subunits were encoded by mitochondrial genome *viz* , NAD1, NAD2, NAD3, NAD4, NAD4L, NAD5, NAD6, NAD7, NAD9 and remaining were encoded by the nuclear genome (Schuster and Brennicke, 1994) The mutations of the mt-encoded subunit lead to the disfunctioning of the respiratory chain causing CMS trait



X = Inhibition of electron transport chain

Fig.1 : Higher plant mitochondrial respiratory chain

2.4.1.1 Oxidation of external NAD (P) H

An important feature of the plant mitochondria has ability to oxidise added NADH at a high rate in the absence of added cytochrome *c* (Palmer, 1976, Moller and Lin, 1986) Douce *et al* (1973) reported that this oxidation did not require NADH translocase, however, a specific NADH dehydrogenase was situated on the outer surface of the inner membrane This dehydrogenase was specific for β-4 hydrogen of NADH and feeds electrons directly to complex III, bypassing complex I and the first site of H^+ translocation (Palmer and Ward, 1985) Consequently, NADH oxidation by this external dehydrogenase was sensitive to antimycin A while insensitive to rotenone or piericidin, and had an ADP/O ratio similar to that of succinate In other words, electrons from the external NADH dehydrogenase had a common pathway with electrons from endogenous NADH at the level of ubiquinone Plant mitochondria oxidize endogenous NADH and NADPH apparently via a Ca^{++-} dependent dehydrogenases located on the outer surface of the inner membrane However, there were two separate dehydrognases on the outer surface of the inner mitochondrial membrane one specific for NADH and the other for NADPH(Arron and Edwards,1970)

2.4.1.2 Oxidation of internal NADH

It has been reported that the two types of internal dehydrogenases were present for the oxidation of endogenous NADH in plant mitochondria One of these internal dehydrogenases, similar to the complex I characterised in mammalian mitochondria, readily oxidizes NADH in a rotenone-sensitive manner (Palmer and Ward, 1985) The second dehydrogenase connected to the respiratory chain via the ubiquinone pool is insensitive to inhibition by

rotenone (Ikuma and Bonner, 1967, Moller and Lin, 1986, Palmer and Ward, 1985) It has been shown that the passage of a pair of electrons from NADH to ubiquinone via this rotenone-insensitive internal dehydrogehase did not result in the translocation of H^+ ions from the matrix into the medium and was therefore not coupled to the generation of $\Delta\mu H^+$ Nonethless, the mechanism of the rotenone-insensitive pathway in plant mitochondria remains obscure

2.4.2 Mitochondrial respiratory complex II

Complex II is responsible for electron transfer from succinate to ubiquinone Isolated preparation of complex II was reported to contain 4 6-5 0 nmole FAD/mg protein and consists of only four protein subunits Complex II can be dissociated by means of chaotropic agents into two sub-complexes The first sub-complex, containing FAD and several nonheme iron centres, was water soluble and consists of two large polypeptides The largest subunit which contains two spin coupled iron-sulphur centres termed as S_1 and S_2 and one molecule of FAD, was located in the matrix space, while the smallest sub unit, which contains a trinuclear (3Fe-4S) centre was termed as S_3 The other subcomplex was very hydrophobic and consists of two small polypeptides These two small polypeptides were needed for binding the enzymes to the membrane and for reduction of ubiquinaone but not required for the enzyme to oxidize succinate in the presence of artificial electron acceptors (Douce *et al* , 1987) The succinate dehydrogenase involved in complex II had four subunits and all were nuclearly encoded (Vary *et al* , 1970)

2.4.3 Mitochondrial respiratory complex III

Complex III (Cytochrome bc_1 complex) is the segment of the respiratory chain responsible for electron transfer from reduced ubiquinone (QH_2) to cytochrome *c* in an antimycin-sensitive fashion as shown in Fig 1 The structure of complex III in higher plant mitochondria is not yet clearly established A cytochrome bc_1 complex has been isolated from various plant tissues (Ducet and Diano, 1978, Degli-Espoti *et al* , 1985)

Complex III isolated from sweet potato mitochondria can be resolved into eight polypeptides of Mw ranging from 51,000 to 1,00,000 Two polypeptides of intermediate molecular weight have been characterized as cytochrome b (MW, 32,000) and cytochromc c_1 (MW 33,000) (Douce *et al.*, 1987) It seems that complex III in plant mitochondria has an absolute preference for electrons generated from complex I This observation suggests that diffusion distance between complex I and complex III is shorter than between complex II and complex III

2.4.4 Mitochondrial respiratory complex IV (Cytochrome *c* oxidase)

Higher plants contain cytochromes not only in mitochondria but also in chloroplasts and other membrane systems In chloroplast, cytochromes are involved in electron transfer, even if the exact details of their functioning is not yet completely known In other membraneous systems, studies are scarce, but suggest the presence of b type cytochromes in microsomes and also in cellular fractions enriched in plasmalemma and perhaps in tonoplast membranes (Ducet, 1985)

Complex IV is the terminal complex of electron transport chain It is located within inner membrane of all eukaryotes The enzyme complex catalyzes the transfer of electrons from reduced cytochrome *c* to molecular

oxygen, thus reducing it to H_2O in four electron transfer process (Wikstrom *et al*, 1981) Cytochrome *c* oxidase offers critical function in cellular respiration in both prokaryotes and eukaryotes

The reaction catalyzed by cytochrome *c* oxidase is the reduction of O_2 by four electrons (Douce *et al*, 1987)

$$4cyt\ C^{2+} + 4H^{+} + O_2 \longrightarrow 4cyt\ C^{3+} + 2H_2O$$

Plesnicar *et al* (1967) used carbon monoxide as an inhibitor of cytochrome *c* oxidase and peroxidase contamination of mitochondria from mung bean Denis and Bonner (1978) used low temperature flash photolysis technique of Chance *et al* (1975) to investigate the cytochrome oxidase-oxygen reaction in mitochondria treated with ferricyanide This treatment included mixed valence state of cytochrome *c* oxidase in which heme A and its associated copper, CuA, were oxidised, while heme A_3 and its associated copper, CuB, were reduced

Reduced cytochrome *c*, the physiological substrate of cytochrome oxidase, transfers electrons to Fe a-CuA site which acts as an electron pool The Fea_3- CuB binuclear centre binds with O_2 and reduces it to H_2O It has been also reported that cytochrome *c* oxidase contains Zn, Mg besides Cu and Fe The Zn and Mg content is one-half to that of Cu and Fe Thus the proposed metal ions ratio is Fe Cu Zn Mg = 2 2 1 1 (Naqui *et al*, 1986) In its purified state (MW, 2,40,000) cytochrome *c* oxidase is composed of atleast seven subunits (I-VII), two heme groups and two copper ions, subunits I, II and III are encoded in the mitochondrial genome, whereas the other subunits are encoded in the nuclear genome and imported into the mitochondria post translationally (Newton, 1988)

2.4.5 Alternate oxidase pathway

The mitochondria of plants possess an alternative respiratory pathway composed of a single terminal oxidase (Vanlerberghe and McIntosh, 1997) Practically all the plant mitochondria showed a residual respiration in presence of CO^-, N^{---} or CN^- (Henry and Nyns, 1975, Lance *et al*, 1975, Laties, 1982, Siedow, 1982) The cyanide resistant electron transport system consists of a branch point from the conventional electron transport system beginning with ubiquinone and terminating with alternative oxidase distinct from cytochrome oxidase (Bendall and Bonner, 1971, Storey, 1976) Substituted benzohydroxamic acid such as salicylhydroxamic acid (SHAM) (Schonbaum *et al*, 1971) the antioxidant n-propylgallate (3,4,5-L-trihydroxybenzoic, acid propyl ester) (Siedow and Girvin, 1980) and disulfiram (tetraethylthirum disulfide) (Grover and Laties, 1981) are potent inhibitors of the alternative pathway Several investigators reported that electrons from TCA-cycle substrates were diverted to the alternative pathway only when the cytochrome pathway approaches saturation, either by inhibition (including state 4) or flooding with electrons (Azcon-Bieto *et al*., 1983, Lambers, 1985) When electron from the quinol pool flow through the alternative pathway, energy is not conserved in the form of an electrochemical gradient and no ATP is formed (Moore and Rich, 1985) Hence, the potential energy of the system is lost as heat

In tissue with high levels of the aternative pathway in the male reproductive structure of cycads and in the flowers or inflorescences of some species belonging to the families, Annonaceae, Araceae, Aristolochiaceae, Cyclanthaceae and Nympheaceae, the functional significance of this pathway is best understood (Siedow, 1982, Lance *et al*, 1985) In Araceae, for example, heat produced by way of electron flow through the alternative

pathway was often used to volatilize insect attractants facilitating insect pollination (Meeuse, 1975)

Expression of gene(s) encoding alternative oxidase is induced by conditions of general stress (Vanlerberghe and McIntosh, 1996, 1997) and demonstrates differential tissue-specific expression of the small gene family in soybean (Finnegan *et al* , 1997) Further more the male-sterile and fertile soyabean tissues showed similar responses to KCN (Obenland, 1988)

The hypothesis that increased alternative pathway activity allows increased carbon flow is difficult to prove but the question is being addressed through the use of transgenic plants with altered levels of the alternative oxidase The alternative oxidase thus appears to work in concert with the TCA cycle to satisfy the plants needs for increased carbon skeletons (Mackenzie and McIntosh, 1999)

Even after great investigation much remains to be done in order to understand the whole cascade of events in relation to alternate oxidase

2.5 Biochemical events associated with CMS trait

Earlier many investigators correlated the enzyme activities, newly synthesized proteins and other metabolic processes occurred in mitochondria and cytoplasm with the cytoplasmic male sterility in crop plants.

2.5.1 Mitochondrial respiratory complex I

Arora *et al* (1993a) reported that rates of electron transport (ET) from NADH to oxygen and partial reactions were almost 1 5 to 2 0 times higher in fertile than sterile line of sorghum Gutierres *et al* (1997) have shown that despite a large difference in size in the mt-DNA deletion, CMS I and CMS II of *Nicotiana sylvestris* display similar alterations Both have an

impaired development from germination to flowering, with partial male sterility that becomes complete under low light In CMS I and CMS II besides NAD 7 two other complex I subunits were also found missing *viz*, NAD 9 and the nuclearly encoded, 38 kDa subunit identified on two PAGE electrophoretic dimensional patterns of mitochondiral proteins They have also isolated mitochondria from CMS leaves which showed altered respiration Although their succinate oxidation through complex II was close to that of the wild type, oxidation of glycine, a priority substrate of plant mitochondria, was significantly reduced The remaining activity was much less sensitive to rotenone, indicating the breakdown of complex I activity Oxidation of exogenous NADH (coupled to proton gradient generation and partly sensitive to rotenone) was strongly increased These results suggest that respiratory compensation mechanisms involving additional NADH dehydrogenases to complex I in CMS lines The capacity of the cyanide-resistant alternative oxidase pathway was enhanced in CMS and higher amounts of enzyme was evidenced by immunodetection

2.5.2 Mitochondrial respiratory complex II

Nakashima (1978) studied the physiological causes of cytoplasmic male sterility in sugarbeet, maize and sorghum and reported that succinic dehydrogenase and glutamic dehydrogenase activities were more intense in fertile than in sterile anthers

Watson *et al* (1977) investigated the mechanism of cytoplasmic male sterility in maize on isolated mitochondria from seedlings and various anther stages and reported that no apparent differences were detected biochemically or electrophoretically between fertile and sterile anthers for succinate dehydrogenase

2.5.3 Mitohondrial respiratory complex III

Arora *et al* (1993a) reported that compositionally the mitochondria of male sterile line of sorghum possessed almost 50% of the cytochrome C_1 of the fertile line The stoichiometry of cytochrome (aa_3 b C_1 C) was 1 2 2 1 for the fertile line and 1 2 1 1 for the CMS line Based on antimycin–A inhibition pattern of mitochondrial ET and stoichiometry of cytochrome it was concluded that the cytochrome b-C_1 complex in the mitochondria of CMS lines is different to that in male fertile lines

2.5.4 Mitochondrial respiratory complex IV

Dixon and Leaver (1982) reported that mitochondria of CMS type of *Sorghum bicolor* synthesized a COX subunit I, 400 dalton larger than that found in fertile plasmatypes

Bino *et al* (1986) analysed the CMS, maintainer and restorer lines of *Petunia* and maize for cytochrome oxidase activity in subsequent stages of microsporogenesis They reported that differences in COX activity between fertile and sterile anthers appeared from meiotic stage in *Petunia* while in maize differences occurred from premeiotic stage onwards In both, *Petunia* and maize, the sterile anthers had lower activity at different stages and decline rapidly as compared to fertile anthers They suggested that the decline in enzyme activity of the CMS in *Petunia* anthers was the result rather than the cause of non formation of functional pollen In case of maize they concluded that CMS correlates with deviations in cox activity In uninucleate stage of anthers cox activity was lower in CMS than fertile line of maize However, at binucleate stage cox activity showed steep increase and attained five times higher activity than found at the uninucleate stages (Ohmasa, 1984) Chen *et al* (1995) studied isozyme patterns of cytochrome

c oxidase in young shoots, of two groups of male sterile lines, two ShuangseA male sterile lines and 3 other male sterile lines The Shuangse A male sterile lines of sorghum showed more bands than the other lines and also observed different intensity of the bands Cytochrome *c* oxidase isoenzymes were distinguished in all 5 lines with different intensity of bands

Watson *et al* (1977) investigated the mechanism of cytoplasmic male sterility in maize by isolating mitochondria from seedlings and various anther stages They had reported that sterile anthers exhibited a lack of biochemical activity and fewer isoenzymatic bands for cytochrome oxidase In their investigation they had also found that sterile seedlings contained significantly more cytochrome oxidase than fertile seedlings

Further it was also reported that cytochrome *c* oxidase activity was significantly lower in male sterile anthers than in male fertile anthers by earlier workers in various crop plants *viz*, wheat (Borisenko and Dmitrieva, 1977), rye (Dmitrieva, 1974), rice (Dai *et al*, 1981) and lucerne (Fursov and Fisenko, 1977)

2.5.5 ATPase and ATP content

Sane *et al* (1997) reported that there was no difference in the rates of uncoupled whole chain electron transport in the 3 lines (cytoplasmic male sterile A_1 (milo), its maintainer and fertility restored line) at any of the temperatures studied (15-45^{0}C) The partitioning of electrons between the cyanide-sensitive and cyanide-insensitive respiratory pathways also did not differ between the 3 lines But differences in the kinetic properties of the isolated mitochondrial F_1 ATPases between the sterile and the fertility restored lines were clearly observed They also proposed that the

differences in kinetic properties of the F_1-ATPase may play a role in the expression of the CMS trait at the time of anther formation

Perl *et al* (1993) examined phosphohydrolase activity on p-nitrophenyl phosphate (pNPpase) and adenosine triphosphate (ATPase) from the soluble and membrane bound fractions of plant leaves, cell suspension cultures and seedlings of *Petunia* of CMS and one fertile (F) lines for each tissue Both pNPpase and ATPase exhibited a broad optimal activity between pH 5, 5-7 and 0 for the membrane bound fractions and between 4, 5-7 and 0 for soluble factions The activity of both were inhibited by divalent ions including Mg^{++} At pH 7 and 2 the activities on various triphosphonucleotides were similar and they were hydrolyzed by a rate of 20-30% of that of ATP They further observed various significant differences between CMS and fertile extracts *viz* , a higher activities in CMS membranes, b lower energy of activation values for activities in CMS membrane functions, c seedling and cell-culture CMS extracts exhibited a higher sensitivity to high temperature denaturation,d the hydrolase activity on mono and triphospho-cytosine compounds was significantly higher in CMS than in F membranes

Arora *et al* (1993b) reported that the rate of ATP translocation was substantially lower in sterile line than in fertile line mitochondria The ability of adenine nucleotides to transolocate proteins of the mitochondrial membrane in sterile was almost one half that of fertile line when the Km for ATP-ADP was determined by two different assays It was proposed that the inadequate supply of ATP in the cytosol resulting from its insufficient translocation may contribute to the male sterility

Wang (1986) found that the ATP content of anthers of maintainer lines was higher than that of male sterile lines, especially in sorghum where

it was 7 times high Pollen from male fertile lines, with high ATP content germinated readily and the pollen tubes grew rapidly, while pollen from male sterile lines with a low ATP content failed to germinate under similar condition Also in experiments designed to test mitochondrial efficiency in ATP export, Liu *et al* (1988) observed organelles from CMS plant performed very poorly when compared with normal lines and in their experiments they indicated a Km for the mitochondrial translocator in CMS times was two lines more i e 161 3-200 μm ADP as compared to male fertile plants 87 43 to 100 μm Nath *et al* (1992) also suggested that fertile line mitochondria were copable of respiring more efficiently than sterile line mitochondria

2.5.6 Alternate oxidase

Musgrave *et al* (1986) compared the cyanide-resistant (alternate) respiration by tissues of 7 male-sterile lines from 4 species (*Glycine max* (L) *Helanium amarum* (Raf) *H. Rock, Plantago lanceolata* and *Zea mays* L) with that of fertile lines Six of male sterile lines lacked alternative respiration in the tissues assayed (leaf or root), while the corresponding fertile tissues displayed a typical alternate pathway equivalent to about 20% of the unihibited respiratory rate The lack of alternative respiration in the male-sterile lines might also serve to explain the commonly observed higher productivity and vigour of male sterile plants compared to fertile ones

Connett and Hanson (1990) found that cells of CMS lines differed from cells of fertile lines in their utilization of the cyanide-insensitive, oxidase pathway Cultured cells from the CMS lines exhibited much lower cyanide-insensitive, salicylhydroxamic acid sensitive respiration than cells from the fertile lines Also immature anthers from CMS plants showed

lower alternative oxidase activity relative to anthers from male fertile plants A cell from fertile plant carrying a nuclear fertility restorer gene and CMS cytoplasm exhibited increased activity of alternative pathway compared with the CMS lines Further Bino *et al* (1986) reported that the importance of alternative oxidase pathway during anther or microspore development is not known

Palmer (1976) reported that the operation of cyanide-insensitive respiration may be important in supplying reducing equivalents for biosynthesis when the cytochrome oxidase pathway is inhibited by high intracellular ATP content Schonbaum *et al* (1971) reported that a small residual oxygen consumption in the presence of both inhibitors (KCN and SHAM), in most of plant cells and tissues was probably due to incomplete inhibition

2.5.7 Glycolysis and TCA Cycle enzymes

In addition to the differences in enzymes involved in respiratory chain (directly and indirectly) differences were also found in the enzymes of glycolysis and TCA cycle Engelhardt and Bezo (2000) reported that when *in vitro* regenerated plants of sugarbeet (*Beta vulgaris* L) with cytoplasmic male sterility (CMS) lines and 0-types were analysed for enzyme polymorphism using starch gel horizontal electrophoresis for malatedehydrogenase (MDH), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), isocitrate dehydrogenase (IDH) and glucose-6-P-dehydrogenase, three PGI isoenzymes and two isoenzymes of MDH and PGM were examined No polymorphism was observed for IDH and PGD isoenzymes

2.5.8 Peroxidase

Nakashima (1978) found that peroxidase activity was higher in sterile than fertile anthers of sorghum Phul *et al* (1987) analysed the peroxidase and acid phosphatase isoenzymes from anthers of 5 CMS and their maintainer lines of sorghum and characterized electrophoretically The band intensity was greater in CMS lines, suggesting that in these lines enzymes were active in breaking down metabolites otherwise important for the formation of fertile anthers Peng *et al* (1988) analysed stamen and pistill peroxidase isoenzymes of *S vulgare* (*S biocolor*) in the isonuclear alloplasmic male sterile lines 3197 A and TX 398A and in the allounclear alloplasmic maintainer line FA Thirteen isoenzymes were detected during development of the organs, with the isoenzyme patterns obtained being very similar in the 2 male-sterile lines Two bands A_8 and A_9 were found in 3197 and TV 398 A but not in FA suggesting that they may be markers for male sterility They also suggested that the cytoplasms regulates nuclear genes during stamen development

Senthil *et al* (1994) measured the peroxidase activity in anthers of CMS, their maintainer, the tester and hybrid lines of sorghum and reported that peroxidase activity was greater in sterile hybrids than in fertile hybrids and parents and recommended this as a criterion for classifying sterile and fertile lines of sorghum Senthil and Manickam (1995) also made an attempt to identify fertility / sterility states of sorghum genotypes on the basis of peroxidase isoenzyme analysis of the anthers and found similar results, that sterile hybrids had a greater enzyme activity than fertile hybrids and parents

Moju and Tigzhao (1998) studied one group of isonuclear alloplasmic (INAP) and two groups of isoplasmic allonuclear (IPAN) male

carrying mitochondria from *N repanda* or *N debneyi* was exclusively correlated with CMS This protein was not present in any of the corresponding male-fertile parental and restored lines

Potz and Tatlioglu (1993) also found that a 18 kDa protein seems to be associated with the cytoplasmic male-sterile phenotype in chives (*Allium schoenoprasum* L)

2.5.10 Other biomolecules

The differences in the metabolism of biomolecules were also observed in male sterile and male fertile lines at different stages In sorghum the starch content decreased during development of sterile but not in fertile anthers Further it was concluded that insufficient nutrient supply at the microspore stage was the main cause of pollen abortion In maize, fertile pollen grains at anthesis contained strach but anther tissue of male sterile plants did not In sugarbeet, sterile anther tissues at the pollen stage contained more starch and lipids than that of fertile anthers In sugarbeet and maize mature fertile anthers contained proline, but sterile anthers did not (Nakashima, 1978)

Tao and Jilin (1993) reported that the activity of amylase and the contents of soluble sugars, starch, soluble proteins, proline in anthers of male-sterile cytoplasmic lines were significantly lower than that of normal cytoplasm at all stages of microsporogenesis and development

2.6 Role of mitochondrial genome in CMS

Plant mitochondrial genome encodes polypeptides which are components of electron transport chain essential for respiration Earlier, some of the mitochondrial genes identified in various crops encoded 9

subunits of NADH dehydrogenase (NAD1, NAD2, NAD3, NAD4, NAD4L, NAD5, NAD6, NAD7 and NAD9), 3 subunits of ATP synthase (*atp*, *atp*6 and *atp*9), 3 subunits of cytochrome oxidase (COXI, COXII and COXIII), apocytochrome b, ribosomal proteins for large and small subunits numbering 10, at least 16 RNA's, 26S, 18S and 5S RNA's (Schuster and Brennicke, 1994)

Kempken *et al*, (1998) suggested that in higher plants mutations and rearrangements in the mitochondrial DNA were believed to cause CMS The recombination events lead to mutations and mutant polypeptides were formed Mutant forms of these polypeptides can cause respiration deficiencies and may cause the sterility in corresponding plant The mitochondrial mutations associated with CMS in several plant species have been identified and characterized In each case the alteration is distinct, CMS associated alteration in mitochondrial genome may involve

1 DNA deletion, e g *Nicotiana* (Chetrit *et al*, 1992)
2 Insertion of sequences of unknown origin, e g Sunflower (Leaver *et al*, 1991)
3 Multiple intragenic recombination events generating chimeric gene arrangements, e g T-maize (Levings, 1993)
4 Production of polycistronic messages e g *Vicia faba* (Grill and Garger, 1981)

Presumable, some defect in mitochondrial function results in the arrest of normal pollen development observed in sterile genotypes Pring *et al*, (1982) also suggested a greater degree of variability in the mitochondrial genome than the chloroplast genome in relation to CMS Differences in mt-DNA restriction patterns and protein synthesis between CMS and male fertile lines have been reported for several plant species (Forde and Leaver,

1980, Boutry *et al*, 1984, Hanson and Conde 1985) Lee *et al* (1989) reported that CMS lines differed from their male fertile counterparts consistently when their mitochondrial DNA was fragmented by endonucleases and their eletrophoretic patterns were examined Xu *et al*, (1995) have shown that sorghum DNA sequences of mitochondrial origin can be used to distinguish different male sterility inducing cytoplasms Nath and Arora (1992) have digested the mt-DNA from male sterile line (2219A) of *Sorghum bicolor* and its restorer line (2219 B) and showed diversities in their total number of fragments as well as their location Further they concluded that a high degree of polymorphism and or deletion might be taking place while achieving CMS in sorghum

Pring *et al*, (1988) have shown that numerous recombination events have resulted in the appearance of an unusual mitochondrial open reading frame, urf 13 T, which encodes 13 kDa polypeptide in the male-sterile T cytoplasm of maize and they have also shown that repeated mitochondrial DNA sequences that participate in recombination in sorghum appear to be randomly distributed among male-fertile or male-sterile cytoplasms

Harada *et al* (1989) investigated the molecular basis of CMS character in sugarbeet They have isolated and sequenced the mitochondrial DNA fragment of fertile (N) cytoplasms which contains the gene for subunit I of cytochrome *c* oxidase (*coxI*) Computer translation of DNA sequences upstream from the coxI gene revealed reading frame of 3' exon of *cox*II Using this cloned sugarbeet *cox*II gene as a hybridization probe, they indicated that sugarbeet *cox*II has an 1 2 kbp intron corresponding to the intron found in rice and carrot The hybridization pattern indicated that N mitochondrial genome contains single copies of the *cox*II gene, whereas

sterile (S) mt-DNA has two copies of the gene However, regarding the 3' flanking region of *cox*II, one of the genes has same restriction enzyme maps as N cytoplasm,but another *cox*II gene has not By further hybridization experiments, it appeared that a recombination event produces a unique extra truncated *cox*II gene in cytoplasm and indicated that *cox*II gene may be the cause of CMS trait in sugarbeet

Pring *et al* (1993) observed two rearrangements of mt- DNA specific to the male sterile cytoplasm of sorghum The first resulted from recombination of *orf* 25 with sequences-similar to those 5' to maize *atp*6 and T-*urf* 13, which produced a 795 bp *orf* (ORF 265) The second chimaeric structure included amino terminus and sequences 5' to sorghum *atp*9, generating a 32 bp *orf* (ORF 107) Transcripts of ORF 107 were changed by the male fertility state, while transcription of ORF 265 were unaffected

'Sane *et al*, (1996) have attempted to characterize three male sterile cytoplasms of sorghum of Indian origin designated as Maldandi, Guntur and Vizianagaram by studying the restriction fragment length polymorphism (RFLP) and expression patterns of 14 mitochondrial genes Their results indicated that these lines were identical to each other with respect to the location of 10 of mitochondrial genes selected, but these were distinguished from each other on the basis of RFLP pattern using *atp*6, *atp*9 and are *rrn* 18 probes Further these three cytoplasms differed from their maintainers in the location of *nad*3, *rps*13 and *atp*A Differences were observed in the pattern of expression of *atp*A between the sterile and respective maintainers

Yan *et al* (1997) found that the sorghum mitochondrial *atp*9 was polymorphic among the male-sterile cytoplasms but each cytoplasm was

characterized by a major 650nt transcript Sane *et al* (1994) proposed that incompatibility in nuclear- cytoplasmic interactions may be explained in terms of incompatible subnits being synthesized by the mitochondria and nucleus for a multisubunit compelx of mitochondrial membrane such as ATPase In sorghum, formation of CMS is strongly correlated with anther specific loss of mitochondrial atp6 RNA editing (Kempken *et al*, 1998) The frequency of atp6 RNA editing was specifically reduced in anthers of male-sterile *Sorghum bicolor*, which increased in frequency in partially restored progeny (Howard *et al*, 1999) Howard and Kempken (1997) also showed that *atp6* transcripts of wheat and selected plasti d transcripts in *S bicolor* showed normal RNA editing, indicating that loss of *atp6* RNA editing is specific for CMS in *S bicolor* mitochondria. Restoration of fertility in F_1 and F_2 lines correlated with an increase in RNA editing of *atp6* transcripts

Dmitrieva (1979) studied the molecular aspects in male sterile maintainer and fertility restorer lines of maize, wheat, rye, sunflower and sorghum and suggested the following sequence of events DNA mutation leads to disturbance in the synthesis of RNA and functionally active proteins, which leads inturn to a reduction in metabolic rate in the anthers and resulted to male sterility Differences were found in metabolic rate between sterility maintainers and fertility restorers

Akagi *et al* (1994) also found that southern blot analysis of the mitochondrial DNA (mt-DNA) of *Oryza sativa* indicates recombination events around a number of genes The appearance of the CMS character is tightly correlated to reorganization around the *atp6* gene The nucleotide sequence downstream from *atp6* contains a pseudogene which was proably

created by recombination of the mitochondrial genome Sense and antisense transcripts of the downstream region of *atp6* were found in CMS and restored CMS (fertile) lines, but not in the normal (fertile) line In the CMS line, several antisense transcripts of the *atp6* gene were also found

Like CMS system in T-maize, sorghum also possess plasmid like, linear mitochondrial DNA (mt-DNA) in cytopasmically male sterile lines in addition to their principal chromosome and were designated as N_1 and N_2 with molecular sizes 5700 and 5300 base pairs, respectively (Pring et al , 1982) Chase and Pring (1985) reported that plasmid like DNA's additional to the linear N_1 and N_2 molecules were also present The recombination events occurred in between existing mitochondrial genome with these plasmid like DNA molecules leading to synthesis of modified polypeptide e g the *cox*I gene in the 9E sorghum cytoplasm or the generation of novel open reading frames (Leaver *et al* 1988)

Mignouna *et al* (1987) showed specific modification in the male sterile cytoplasm of rice by restriction analysis In addition to the major mt-DNA three small plasmid like DNA molecules were detected by agarose gel electrophoresis in both cytoplasms An additional molecule was specifically found in the sterile cytoplasm These mitochondrial DNA modifications support the hypothesis of the mitochondrial inheritance of the cytoplasmic male sterility in rice

Tang *et al* (1996) from their observations indicated that mitochondrial open reading frames associated with CMS in different species can include highly similar motifs, and that fertility restoration could involve a mechanism by which synthesis of a CMS-associated gene product may be precluded through internal transcript cleavage Bailey-Serres *et al* (1986) in their studies of numerous male fertile and male sterile sorghum lines found

that when cytoplasms were transferred to foreign nuclear backgrounds some changes in the products of organello mitochondrial protein synthesis occurred Variant mitochondrial polypeptides were synthesized at lower levels in cytoplasmically male sterile than in male fertile nuclear-cytoplasmic combinations for 4 of the cytoplasms

Jaiswal *et al* (1988) suggested that the analysis of RAPD profiles of total DNA could distinguish the seven sorghum restorer lines that differentially restore (or maintain) the A_1 and A_2 cytoplasmic male sterile cytoplasms on the basis of their ability to restore completely or partially the fertility in the A_1/A_2 CMS cytoplasms Interestingly, RAPD profiles of mt-DNA's of these lines also followed the same pattern as that of the total DNA, and indicated that the different restorer lines possess specific nuclear-cytoplasmic combinations

Johns *et al* (1992) observed that CMS in common bean is associated with the presence of a 3kb unique mitochondrial sequence designated *pvs* The *pvs* sequence encodes atleast two open reading frames (297 and 720 bp in length) with portions derived from the chloroplast genome Fertility restoration by the nuclear restorer gene Fr results in the loss of this transcriptionally active unique region and also their observations support a model of fertility restoration by the loss of a mitochondrial DNA sequence prior to or during microsporogenesis/ gametogenesis

Thus the above brief review depicts that CMS system in crop plants is mainly governed by mutation in mitochondrial genome, differences in the activities of respiratory enzymes and oxygen uptake Therefore the present investigation was undertaken to study respiratory enzymes associated with CMS trait in sorghum

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Seeds

Seed materials of a total of eight lines *viz* , 1049A, 1049B, RSV-33R, SPH-1229, 104A, 104B, RS- 585 and CSH- 15R were obtained from Senior Sorghum Breeder, All India Co-ordinated Sorghum Improvement Project, MPKV, Rahuri (Table 1)

3.1.2 Chemicals

The chemicals were procured from Sisco Research Laboratories Pvt Ltd ,Mumbai and Aldrich Chemical Co ,Wisconsin U S A

3.2 Methods

3.2.1 Growing of etiolated seedlings

The protrays were used for growing the sorghum seedlings Each tray was filled with approximately 0 75 kg of vermiculite and irrigated till saturation The seeds were sown and the trays were kept in dark chamber at $30^0C \pm 2$ for growing etiolated seedlings at Biotechnology Centre, MPKV, Rahuri (Fig 2)

3.2.2 Isolation of mitochondria

The mitochondria were isolated according to the method described by Chase and Pring (1986) with some modifications

Table 1 : Pedigree details of CMS, maintainer, restorer and hybrid lines of sorghum

Sr No	Parents / hybrids	Pedigree
1	1049 A (CMS)	104 B x ICSB 36209
2	1049 B (Maintainer)	104 B x ICSB 36209
3	RSV 33R (Restorer)	SPV 504 x SPV 570
4	SPH 1229 (Hybrid)	1049A x RSV 33R
5	104A (CMS)	296B x Swati
6	104B (Maintainer)	296 B x Swati
7	RS 585 (Restorer)	NRCS
8	CSH 15R (Hybrid)	104A x RS 585

Source Sorghum project, MPKV, Rahuri, Maharashtra

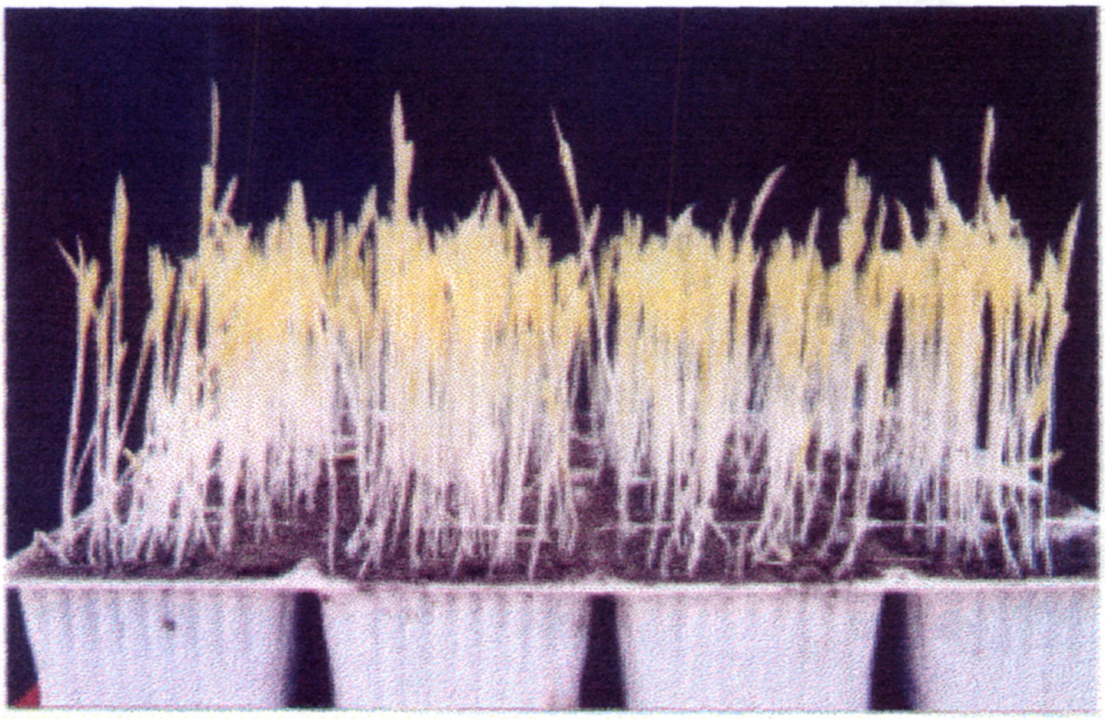


Fig. 2 : Etiolated seedlings

All the glassware and reagents were autoclaved and the manipulations were done at 0-4^{0}C unless otherwise stated

Materials

1. **Isolation buffer :** Hepes-KOH buffer 25mM, pH 7 4 containing 1 mM EDTA, 0 1% BSA, 0 1% cysteine, 5mM mercaptoethanol, 0 4M mannitol and 0 6 % PVP

For preparing 1000 ml of isolation buffer 72 86 g of mannitol, 5 95g of Hepes, 0 3722 g of EDTA, 1 g of BSA, 1 g of cysteine, 6 g of PVP and 0 391 ml of mercaptoethanol were dissolved in about 800 ml of sterile distilled water, the pH was adjusted to 7 4 with KOH and then the final volume was made to 1000 ml with sterile distilled water

2. **Sucrose (0.4 M)**

Sucrose, 68 46 g was dissolved in 450 ml sterile distilled water and the volume was made to 500 ml with sterile distilled water

3. **Sucrose (0.6M)**

Sucrose, 102 69 g was dissolved in 450 ml of sterile distilled water and volume was made to 500 ml with sterile distilled water

4. **Assay buffer :** Tris-HCl 0 05M, pH 7 5 containing 0 3 M sucrose

Tris, 0 6057 g and 10 269 g of sucrose was dissolved in 80 ml of sterile distilled water, pH was adjusted to 7 5 with HCl and volume was made to 100 ml with sterile distilled water

Method

About 100 g of (7-day old) etiolated tissues were ground in a mortar and pestle in 3 volumes of isolation buffer The homogenate was filtered through 4 layers of muslin cloth and centrifuged at 1000 xg at 4^0C for 10 min to remove cell debris, nuclei and plastids The supernatant was then centrifuged at 12,000 xg at 4^0C for 15 min to obtain the mitochondrial pellet The pellet was resupended in 0 4M sucrose and again centrifuged at 1000 xg at 4^0C for 10 min The resultant supernatant was underlaid with 20 ml of 0 6M sucrose and centrifuged at 12,000 xg at 4^0C for 20 min Then the mitochondrial pellet was gently suspended with the aid of small artist's soft paint brush in 10 ml of prechilled assay buffer The resuspended mitochandria were centrifuged at 1000 xg for 10 min at 4^0C, the supernatant was collected with the micropipette and used for estimation of soluble proteins, enzyme assays, oxygen uptake, cyanide-sensitive, cyanide-insensitive respiration etc

3.2.3 Soluble proteins

Soluble proteins of the mitochondria were determined by the colorimetric method described by Lowery *et al* (1951) using bovine serum albumin as standard protein

Materials

1. Reagent 'A' (2 % sodium carbonate in 0.1M sodium hydroxide)

Two g of sodium carbonate was dissolved in 0 1M sodium hyodroxide and volume made to 100 ml with 0 1 M sodium hydroxide

2 **Reagent 'B' (0.5 % copper sulphate in 1 % sodium potassium tartarate)**

Five hundred mg of copper sulphate ($CuSo_4$ $5H_2O$) was dissolved in 1% (w/v) sodium potassium tartarate solution and volume made to 100 ml with 1% sodium potassium tartarate solution

3. Alkaline copper reagent

Fresh alkaline copper reagent was prepared just before use by mixing 50 ml of reagent 'A' with 1 ml of reagent 'B'

4. Folin-ciocalteau reagent

It was prepared by diluting commercial grade reagent of one part with one part of distilled water on the day of use

5. Stock solution of standard protein (Bovine serum albumin)

The BSA (100 mg) was weighed accurately and dissolved in distilled water and volume made to 100 ml with distilled water

6. Working standard solution

The stock solution of 10 ml was pipetted in the 100 ml volumetric flask and diluted to 100 ml with distilled water The concentration of BSA in this solution was 100 µg per ml

Colour development

The fresh mitochondrial suspension, 0 1 ml was pipetted into a test tube and diluted to 1 ml with sterile distilled water Alkaline copper reagent, 5 ml was added in it and mixed well and kept for 10 min at room

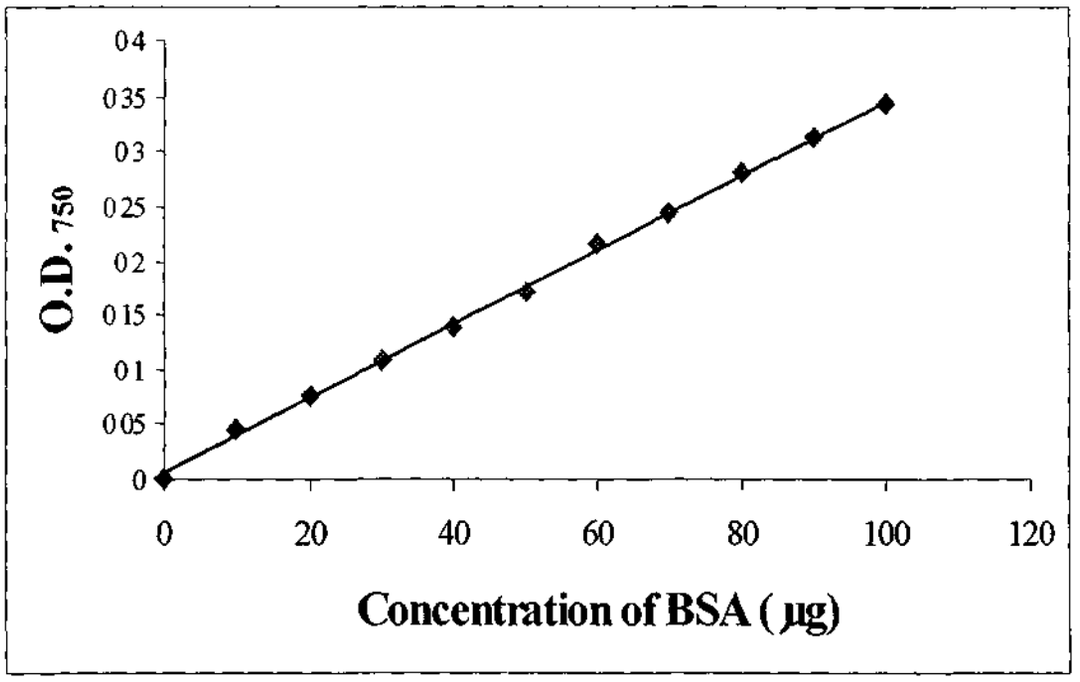


Fig. 3 : Calibration of a standard curve for the estimation of soluble protein

temperature Folin ciocalteau reagent 0 5 ml was added rapidly in it and vortexed on vortex mixer and kept for 30 min at room temperature for colour development The intensity of blue colour was measured after 30 minutes at 750 nm onaSpectronic -20 Spectrophotometer

Calibration of standard curve

The 0, 0 1, 0 2, 0 3 , 1 0 ml of the working standard solution of BSA was taken in a series of test tubes Colour was developed as above and read at 750 nm The standard graph was plotted, absorbance vs concentration of the BSA (Fig 3) The soluble protein was calculated from standard curve and results were expressed as mg of mt-protein per g of fresh weight

3.2.4 Assay of cytochrome *c* oxidase

Cytochrome *c* oxidase activity was recorded by the rate of oxidation of ferrocytochrome *c* which was measured by following the decrease in the absorbancy at 550 nm (Wharton and Tzagoloff, 1967)

Ferrocytochrome *c* + $2H^+$ + ½ O_2 ⟶ Ferricytochrome *c* + H_2O

Materials

1. Potassium phosphate buffer(0.1 M , pH 7.0)

Potassium dihydrogen phosphate 0 1 M, 39 ml and Di potassium hydrogen phosphate 0 1 M, 61 ml were mixed, pH adjusted to 7 0 on pH meter and volume was made to 200 ml with sterile distilled water

2. Ferrocytochrome *c* (1 %)

Cytochrome *c*, 50 mg was dissolved in 5 ml of 0 01M potassium phosphate buffer, pH 7 0 The solution was reduced with a few milligrams of sodium dithionate Then the excess sodium dithionate was removed by dialysis in size 8 visking dialysis tubing against 0 01M phosphate buffer pH 7 0 for 18-24 h with three changes of buffer This cytochrome remains 96-99% reduced upto several months or more

3. Potassium ferricyanide (0.1M)

Potassium ferricyanide, 1 646 g was dissolved in phosphate buffer pH 7 0 and volume was adjusted to 50 ml with phosphate buffer

4. Enzyme

Mitochondrial suspension was diluted to a protein concentration of 0 3 mg/ml

Method

To each of two 3 ml cuvettes with a 10 mm light path, the following were added, 0 3 ml of potassium phosphate buffer, 0 21 ml ferrocytochrome *c* and 2 49 ml of water The blank cuvette was oxidized with 0 03 ml potassium ferricyanide After temperature equilibration at 38^0C, the reaction was initiated by the addition of 30 µl of mitochondrial suspension The decrease in absorbancy was measured at 550 nm every 10 seconds

The activity of cytochrome *c* oxidase was calculated as follows

$$k = 2\ 3 \log \frac{A\ (time_0)}{A\ (time_0 + 1\ min)}\ min^{-1}$$

3.2.5 Assay of succinate dehydrogenase

Succinate dehydrogenase was assayed by spectrophotometric method described by Bonner (1954) by measuring the reduction rate of potassium ferricyanide in the presence of sufficient KCN to inhibit cytochrome oxidase It is a reliable assay for succinic dehydrogense from plants

Materials

1. Potassium cyanide neutralized (0.05 M)

Potassium cyanide, 0 33 g was dissolved in 80 ml of 1 mM KOH solution and pH adjusted to 7 0 with 0 1M KOH, volume made to 50 ml

2. Potassium ferricyanide (0.01M)

Potassium ferricyanide, 0 1646 g was dissolved in 0 1M phosphate buffer, pH 7 2 and volume was adjusted to 50 ml with phosphate buffer

3. Sodium succinate (0.2 M)

Sodium succinate, 2 7014 g was dissolved in 40 ml of sterile distilled water and volume adjusted to 50 ml with sterile distilled water

4. Phosphate buffer (150 mM, pH 7.2)

Potassium dihydrogen phosphate 0 5 M, 4 24 ml and dipotassium hydrogen phosphate 0 5 M, 16 64 ml were mixed and 60 ml of sterile distilled water was added in it, pH was adjusted to 7 2 and the volume was made to 100 ml with sterile distilled water

Method

To a 1 cm cell, 0 6 ml of KCN, 0 3 ml of K_3 Fe $(CN)_6$ and 0 2 ml of sodium succinate were added Phosphate buffer, 2 ml was added to this mixture to give a final concentration of 0 1 M in a total of 3 0 ml To the blank cuvette all these were added except K_3 Fe $(CN)_6$ At zero time 0 2 ml of the suitably diluted mitochondrial preparation was added to both cells and the optical density was measured at 400 nm and is followed as a function of time every 15 sec

The enzyme activity was expressed in units i e 1 unit corresponds to change in O D by 0 001 mg^{-} mt-protein min^{-1}

3.2.6 Measurement of respiration

The respiration studies were carried out in relation to cyanide-sensitive and cyanide-insensitive pathways of electron transport chain by the method described by Connett and Hanson (1990) using oxygen monitoring system YSI model

Studies were carried out both on the tissues of etiolated seedlings and isolated mitochondria

3.2.6.1 Tissues of etiolated seedlings

Materials

1 **Calcium chloride (0.05 M)**

Calcium chloride, 0 5549 g was dissolved in 80 ml of sterile distilled water and volume adjusted to 100 ml with sterile distilled water

2 **Calcium chloride (0.3 M)**

Calcium chloride, 3 3297 g was dissolved in 80 ml of sterile distilled water and volume was adjusted to 100 ml with sterile distilled water

3. Salicylhydroxamic acid(0.1 mM)

Salicylhydroxamic acid, 0 383 g was dissolved in small quantity of ethanol and volume was made to 25 ml with sterile distilled water

4 **Potassium cyanide stock solution (0.05 mM)**

Potassium cyanide, 0 33 g was dissolved in 80 ml 1 mM KOH and pH adjusted to 7 0 with 1 M KOH, volume made to 100 ml with sterile distilled water

5. Hepes (0.1 M , pH 7.2)

Hepes, 1 1915 g was dissolved in 40 ml of sterile distilled water, pH adjusted to 7 2 with KOH and volume was made to 50 ml with sterile distilled water

Methods

The etiolated seedlings (7-day old) were harvested To allow for better oxygen diffusion and penetration of inhibitors seedlings were cut into small (5 mm) segments with a sharp razor blade and suspended in 2 ml of 0 3M $CaCl_2$ (0 2 M), 1 ml of 0 3M Hepes (0 1 M) and allowed to equilibrate for 5 to 30 minutes For respiratory assay, tissue segments were suspended in buffered 12 µl of 0 05M $CaCl_2$ (0 2 mM), 1 ml 0 3 M Hepes (0 1 M) and 1 626 ml of sterile distilled water was added and equilibrated at room

temperature in the stirred oxygen electrode cuvette For measurement of cyanide-insensitive respiration, 60 µl of 0 05 M KCN (1 mM), a potent inhibitor of cytochrome oxidase pathway was added in the reaction media The cyanide-insensitive respiration was measured by adding 300 µl of 0 1 M SHAM (10 mM) in the reaction mixture

3.2.6.2 Isolated mitochondria

Mitochondria were isolated according the method described under 3 2 2

Material

1. **Assay buffer :** Phosphate buffer 10 mM, pH 7 2, containing 0 3 M sucrose , 5 mM $MgCl_2$, 10 mM KCl

Sucrose, 2 56 g 2 5 ml of 0 1M phosphate buffer pH 7 2, 0 1869 of potassium chloride and 0 026 of magnesium chloride were dissolved in sterile distilled water and volume was made 25 ml with sterile distilled water

2 **Potassium cyanide stock solution (0.05M , pH 7.0)**

Potassium cyanide, 0 33 g was dissolved in 80 ml of 1 mM KOH and pH adjusted to 7 0 with 0 1 MKOH and volume made 100 ml with sterile distilled water

3. **Salicylhydroxamic acid stock solution (0.1M)**

Salicylhydroxamic acid, 0 383 g was dissolved in small quantity of ethanol and volume was made to 25 ml with sterile distilled water

4. Adenosine triphosphate stock solution (0.1 M)

Adenosine triphosphate, 60 mg was dissolved in 1 ml of 0 1 M KOH

5. Adenosine diphosphate stock solution (0.1 M)

Adenosine diphosphate (disodium salt), 49 3 mg was dissolved in 0 1 ml of 0 1 M KOH

6 **Succinate (0.4 M)**

Succinic acid, 1 18 g was dissolved in 20 ml of sterile distilled water and volume was made to 25 ml with sterile distilled water

Cyanide-insensitive respiration

In a 3 ml cuvette, 0 2 ml of mitochondrial suspension (0 5 to 1 mg of protein) was diluted by adding 2 7 ml of assay buffer which had been equilibrated at 25^0C for atleast 2 hours Mitochondria were supplied with 75 µl of succinate (10 mM) and 3µl of ATP (0 1 mM) 6 µl of ADP (0 2 mM) was added to initiate state III respiration and 60 µl of KCN (1 mM) was added in the mixture The oxygen uptake was measured by YSI model, oxygen monitoring system

Cyanide-sensitive respiration

The cyanide-sensitive respiration was measured same as described above instead of KCN, 30 µl of SHAM (1mM) was added into the reaction mixture The oxygen uptake was measured by YSI model, oxygen monitoring system

Residual respiration

The residual respiration was recorded by adding both KCN and SHAM into the reaction mixture

The total respiratory activity (V_T) as defined by Musgrave and Siedow (1985) as

$$V_1 = V_{cyt} + V_{alt} + V_{res}$$

Where,

V_{cyt} is the cyanide-sensitive component of respiration mediated by cytochrome oxidase pathway, V_{alt} is the respiration mediated by the alternative oxidase pathway sensitive to SHAM, V_{res} is the respiration insensitive to both SHAM and cyanide

3.2.7 Isolation of mitochinodrial DNA

Mitochondrial DNA was isolated by two methods, alkali lysis method and freezing and thawing method All reagents and glassware used in the following experiments were autoclaved and all manipulations were carried out at 0-4^{0}C unless otherwise stated

3.2.7.1 Mitochondiral DNA isolation by alkali lysis method

Mitochondrial DNA from the etiolated seedlings of sorghum was isolated by alkali lysis method as described by Chase and Pring (1986) with some modifications

Materials

1. **Isolation buffer :** Hepes-KOH buffer 25mM, pH 7 4 containing 1 mM EDTA, 0 1% BSA, 0 1% Cysteine, 5mM mercaptoethanol, 0 4M mannitol and 0 6 % PVP

For preparing 1000 ml of isolation buffer 72 86 g of mannitol, 5 95g of Hepes, 0 3722 g of EDTA, 1 g of BSA, 1 g of cysteine, 6 g of PVP and 0 391 ml of mercaptoethanol were dissolved in about 800 ml of sterile distilled water, the pH was adjusted to 7 4 with KOH and then the final volume was made to 1000 ml with sterile distilled water

2 **DNase buffer :** Tris-HCl, 0 05 M, pH 7 5 containing 0 3 M sucrose

Tris, 152 g and 26 67 g of sucrose were dissolved in 200 ml of sterile distilled water, pH adjusted to 7 5 and volume was made to 250 ml with sterile distilled water

3. DNase

DNase, 2 mg was taken and 1 ml of $MgCl_2$ frozen solution was added (Not autoclaved)

4. Magnesium chloride (1 M)

Magnesium chloride hexahydrate 10 165 g was dissolved in little quantity of sterile distilled water and volume made to 50 ml with sterile distilled water

5 **Shelf buffer :** Tris-HCl 0 01 M, pH 7 2 containing 0 6 M sucrose

Tris, 0 61 g, 102 6 g of sucrose and 3 722 g of EDTA were dissolved in 450 ml of sterile distilled water, pH adjusted to 7 2 and volume was made to 500 ml with sterile distilled water

6. NN buffer : Tris-HCl, 50mM, EDTA, 20mM, pH 8.0.

Tris, 0 60 g and 0 87 g of Na_2EDTA were dissolved in 80 ml of sterile distilled water, pH adjusted to 8 0 and volume was made to 100 ml with sterile distilled water

7. n-Lauryl sarcosine (0.5%w/v)

n-Lauryl sarcosine 0 5 g was dissolved in 100 ml of sterile distilled water

8. Proteinase K

Sterile distilled water, 1 ml was added to 2 mg of proteinase K (Not autoclaved)

9. Extraction buffer

Tris-HCl 0 15 M, pH 8 0 containing 80 mM EDTA, 0 1 NaCl and 1 5% SDS

Tris, 4 54 g, 7 44 g of EDTA, 1 46 g of NaCl and 3 75 g of SDS were dissolved in 200 ml of distilled water, pH adjusted to 8 0 and volume was made to 250 ml with sterile distilled water

10. Potassium acetate (5 M)

To 60 ml of 5 M potassium acetate, 11 5 ml of glacial acetic acid and 28 5 ml of distilled water were added The resulting solution was 3M with respect to acetate As a result we get 100 ml of 5 M potassium acetate

11. Isopropanol

Commercial grade isoproponol was used

12. Ethanol (70%)

Distilled water 150 ml was added to 350 ml of ethanol to prepare 500 ml of 70% ethanol

13 **TE buffer :** Tris-HCl, 50mM, EDTA, 1mM, pH 8.0.-

Tris, 0 61 g and 0 38 g of Na_2EDTA was dissolved in 80 ml of sterile distilled water, pH adjusted to 8 0 and volume was made to 100 ml sterile distilled water

14. Sodium acetate (3 M)

Sodium acetate, 408 24 g was dissolved in 800 ml of sterile distilled water, pH was adjusted to 5 2 with glacial acetic acid and final volume was made to 1000 ml with sterile distilled water

15 **TE buffer :** Tris-HCl, 10mM, EDTA, 1mM, pH 8.0.

Tris, 0 12 g and 0 38 g of Na_2EDTA was dissolved in 80 ml of sterile distilled water, pH adjusted to 8 0 and volume was made to 100 ml sterile distilled water

Method

The seven days old etiolated seedlings was ground in a in a mortar and pestle in 3 volumes of isolation buffer, mercaptoethnaol and sodium diethyl dithiocarbamate were added to yield a final concentration of 5 mM just before isolation buffer was added The homogenate was filtered

through 4 layers of muslin cloth and centrifuged at 1000 xg at 4^0C for 10 min to remove cell debris, nuclei and plastids The supernatant was then centrifuged at 12,000 xg at 4^0C for 10 min to obtain mitochondrial pellet The mitochondrial pellet was resuspended in 20 ml of DNase buffer with a small paint brush and preparation was centrifuged at 1000 xg at 4^0C for 10 min The supernatant was collected and made 0 01M $MgCl_2$ by addition of 100 µl of 1M $MgCl_2$ followed by the addition of 100 µl of DNase The suspension was incubated at 0^0C for 1 hr DNase was removed by carefully underlaying 20 ml of shelf buffer The mitochondria were pelleted through the shelf buffer by centrifuging at 12000 xg for 20 min at 4^0C The mitochondrial pellet was resupended in 10 ml shelf buffer and repelleted at 14,000 xg for 10 min at 4^0C The mitochondrial pellet was suspended in 2 0 ml of lysis buffer, 0 5 ml of n-lauryl sarcosine, 5 µl of RNase and lysed at 37^0C for 1 hr with occasional shaking Then 150 µl proteinase K was added into the lysate After 1 hr 2 4 ml of extraction buffer was added into the mitochondrial lysate suspension This preparation was divided into eight microfuge tubes (1 5 ml) and incubated at 65^0C for 10 min Then 200 µl of 5 M potassium acetate was added to each tube and incubated at 0^0C for 20 min Tubes were centrifuged at 12000 xg for 5 min The supernatant was then pipetted into microfuge tubes containing a prechilled mixture of 400 µl isopropanol and 40 µl of 5 M ammonium acetate Tubes were vortexed and incubated at -20^0C for 20 min The mt DNA was pelleted by spinning in a microfuge at 10,000 xg for 5 min The pellet was suspended in cold 70%(v/v) ethanol and air dried in laminar air flow cabinet The DNA pellet was suspended in TE buffer (50 mM Tris-HCl, 10 mM Na_2EDTA,pH 8 0) All the DNA samples were pooled into one tube containing a final volume of 700 µl DNA was reprecipitated by the addition of 75 µl of the 3M sodium

acetate and 500 µl of isopropanol DNA was pelleted immediately by spinning for 5 min in a microfuge

The pellet was washed with 70% ethanol, air dried in laminar air flow cabinet and rehydrated in 200 µl of TE buffer (Tris-HCl; 10mM, EDTA, 1mM; pH 8.0.) and stored at 4^0C The 5 µl of this sample was taken and mixed with TE buffer (Tris-HCl, 10mM, EDTA, 1mM, pH 8.0) and the volume was made to 2 ml The absorbance was read on UV spectrophotometer at 260 nm and 280 nm and the yield was calculated

3.2.7.2 Mitochondrial DNA isolation by freezing and thawing method

Mitochondrial DNA from the etiolated seedlings of sorghum was isolated by freezing and thawing method as described by Munjal and Narayan (1995)

Materials

All the reagents in the ealier method except DNase buffer, DNase and magnesium chloride were used in this method

Method

The seven days old seedlings were ground into fine powder with liquid nitrogen by freezing and thawing and isolation buffer was added in 1 3 ratio, mercaptoethanol and sodium diethyl dithiocarbamate were added to yield a final concentration of 5mM just before isolation buffer was added The mixture was filtered through a 4 layers muslin cloth and centrifuged at 1000 xg for 10 minutes The supernatant was separated and again centrifuged at 12000 xg for 10 min, now the pellet is retained and supernatant was discarded The pellet was dissolved in 10 ml of isolation

buffer with soft paint brush, centrifuted at 1000 xg for 15 min, the supernatant was underlaid in a 20 ml of prerefrigerated shelf buffer and centrifuged at 12000 xg for 30 min The supernatant was discarded and the pellet was dissolved in 10 ml of shelf buffer with the paint brush and centrifuged at 12000 xg for 15 min, this step was repeated one more time The remaining steps were carried out in the same way as in the previous method

3.3 Statistical analysis

The statistical analysis was carried out by Completely Randomised Design

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

The role of the mitochondrial genome in cells of higher plants is limited to the synthesis of 10-12 polypeptides The majority of these mitochondrial encoded polypeptides are components of three enzyme complexes of respiratory electron transport chain, involved in oxidative phosphorylation and the generation of ATP At biochemical level the CMS trait is correlated with the energy stress in the developing anthers in various crop plants Therefore in the present investigation, attempt was made to establish and to distinguish the male sterile and fertile lines of sorghum by the mitochondrial enzyme assays and measuring the cyanide-sensitive and insensitive respiration The results obtained in this regard are tabulated and briefly summarized below

4.1 The soluble protein content in mitochondria isolated from CMS, maintainer, restorer and hybrid lines

The two CMS systems having CMS, their maintainer, restorer and hybrid lines were subjected for the analysis of mitochondrial protein content and tabulated in Table 2 and 3 It was observed that in the male sterile lines, the content of soluble proteins were higher than its respective maintainer, restorer and hybrid lines The CMS line 1049A contained 41mg g^{-1} fr wt while CMS line 104A contained 54mg g^{-1} fr wt soluble protein Their respective maintainer lines having isonuclear background contained lower amounts of soluble protein *viz* , 1049 B had 32 mg g^{-1} fr wt while 104 B had 26 mg g^{-1} fr wt The restorer and hybrid lines also had lower amount of

Table 2 : Soluble protein content in mitochondria isolated from CMS, maintainer and restorer parental lines of hybrid SPH-1229 of sorghum

Parents/hybrids	Soluble protein (mg g^{-1} fr wt)
1049 A	41
1049B	32
RSV 33R	18
SPJ 1229	24
S E ±	1 08
C D	3 33

Table 3 : Soluble protein content in mitochondria isolated from CMS, maintainer and restorer parental lines of hybrid CSH-15R of sorghum

Parents/hybrids	Soluble protein (mg g^{-1} fr wt)
104 A	54
104B	26
RS 585	20
CSH 15R	28
S E ±	0 95
C D	2 95

soluble protein than the corresponding CMS lines These results indicates that in the mitochondria of the cytoplasmic male sterile lines some new chimeric protein might be synthesized in relation to CMS trait indicating their higher concentration Earlier, Perl *et al* , (1993) analysed triton treated membrane bound protein from seedlings, leaves and cultured cells of male sterile and fertile lines of *Petunia* They reported that these membrane bound proteins were higher in the CMS lines than the fertile lines The results obtained in the present investigation are in agreement with this earlier findings

4.2 Cyanide-sensitive and cyanide-insensitive respiration in mitochondria isolated from CMS, maintainer, restorer and hybrid lines

The cyanide sensitive respiration was measured using salicylhydroxamic acid, a potent inhibitor of alternative oxidase, secondary pathway while cyanide insensitive respiration was measured by using potassiumcyanide, a potent inhibitor of cytochrome *c* oxidase of normal respiratory pathway The results obtained in this respect were tabulated in Table 4 and 5 It was observed that both cyanide and SHAM were found to decrease the respiratory rate of mitochondria from sterile, fertile, restorer and hybrid lines of the two CMS systems when the inhibitors were added individually in the reaction media The cyanide sensitive respiration was found to be significantly higher in CMS lines *viz* , 1049 A and 104A-54 31 nmoles of O_2 $min^{-1}mg^{-1}$mt-protein (73 3 %) and 46 59 nmoles of O_2 $min^{-1}mg^{-1}$mt-protein (74 96%) respectively, than their respective maintainer, restorer and hybrid lines The total respiration was found to be higher in

Table 4 : Cyanide-sensitive and insensitive respiration in mitochondria isolated from CMS, maintainer and restorer parental lines of hybrid SPH-1229 of sorghum

Parents/ hybrids	nmoles of O_2 $min^{-1}mg^{-1}$ mt-protein			
	Total	Cyanide sensitive	Cyanide insensitive	Residual
1049 A	73 65	54 31 (73 74)	10 23 (13 89)	9 11 (12 36)
1049B	91 35	33 43 (36 49)	46 64 (51 05)	11 28 (12 34)
RSV -33R	120 09	35 73 (29 75)	63 51 (52 88)	20 85 (17 36)
SPH-1229	197 5	78 39 (39 69)	97 39 (49 31)	21 72 (10 99)
S E ±	6 97	2 97	4 38	1 37
C D 5%	21 51	9 18	13 53	4 24

Figures in parenthesis indicates the per cent respiration compared with total respiration

Table 5 : Cyanide-sensitive and insensitive respiration in mitochondria isolated from CMS, maintainer and restorer parental lines of hybrid CSH-15R of sorghum

Parents/ hybrids	nmoles of O_2 $min^{-1}mg^{-1}$ mt-protein			
	Total	Cyanide sensitive	Cyanide insensitive	Residual
104 A	62 17	46 59 (74 93)	7 98 (12 83)	7 60 (12 22)
104B	77 65	24 74 (31 86)	41 51 (53 45)	11 40 (14 68)
RS-585	100 60	30 37 (30 18)	56 67 (56 33)	13 56 (13 47)
CSH-15R	133 72	48 98 (36 62)	68 20 (51 00)	16 54 (12 36)
S E ±	3 866	1 84	3 24	1 35
C D 5%	11 920	5 69	9 99	4 16

Figures in parenthesis indicates the per cent respiration compared with total respiration

1049A, 1049B, RSV-33R and SPH-1229 than the 104A, 104B, RS-585 and CSH-15 R lines However, the per cent cyanide-insensitive respiration was more or less similar between the two CMS lines, maintainer, restorer, hybrid lines of the two CMS systems

On the contrary to the cyanide-sensitive respiration of the mitochondria, the cyanide-insensitive respiration was found to be more in fertile lines than CMS lines in both the CMS systems The male sterile lines had about 4 times lower percentage of cyanide-insensitive respiration than their respective maintainer, restorer and hybrid lines The CMS lines had significantly lower cyanide-insensitive respiration than their respective maintainer, restorer and hybrid lines The CMS line 1049A had 10 23, 1049B, 46 64, RSV-33R, 63 51 and hybrid SPH-1229, 97 39 nmoles of O_2 $min^{-1}mg^{-1}$mt-protein cyanide-insensitive respiration whereas 104 A had 7 94, 104-B, 41 51, RS-585, 56 67 and CSH-15R, 68 20 nmoles of O_2 min^{-1} mg^{-1}mt-protein cyanide-insensitive respiration

In addition to the cyanide sensitive and insensitive respiration, residual respiration was also observed in all the eight lines when the inhibitors *viz*, SHAM and KCN were added into the reaction media simultaneously However, the rate of respiration was found to be lower than the cyanide-sensitive and insensitive respiration and it was almost more or less similar in all the lines irrespective of sterile or fertile lines Schonbaun *et al* (1971) observed that residual respiration was found when both the inhibitors were added in the reaction media The results obtained in the present investigation were agreed with this earlier report

Musgrave *et al*, (1986) reported 37% alternative (cyanide-insensitive) respiration in CMS and 63 % in fertile lines of the isolated

mitochondria In the present investigation the percent values of cyanide-insensitive mitochondrial respiration in CMS plants obtained were 3 times lower than the reported values and for fertile liens the reported values were about 1 2 times more than the values obtained for fertile, fertile restored lines in the present investigation. Connett and Hansen (1990) also reported that the percent alternate oxidase (cyanid-insensitive respiration) was more in mitochondria isolated from cell suspension culture of *Petunia* The similar trend was obtained in the present investigation in accordance with the earlier reported trend

4.3 Cyanide-sensitive and insensitive respiration in the tissues of etiolated seedlings from CMS, maintainer, restorer and hybrid lines

The cyanide-sensitive and insensitive respiration measured from the etiolated seedlings of two CMS systems *viz* , 104A, 104B, RSV 33 R and SPH-1229 and 104A, 104B, RS-585 and CSH-15 R are tabulated in Table 6 and 7 It was observed that total respiration of the CMS lines, 1049A (1 59 n moles of O_2 min^{-1} mg^{-1} fr wt) and 104A (1 63 n moles of O_2 min^{-1} mg^{-1} fr wt) were found to be lower than the respective maintainer and hybrid lines The total respiration of these maintainer and restorer lines were in the range of 2 14 to 2 36 n moles of O_2 min^{-1} mg^{-1} fr. wt The total respiration of hybrid lines SPH-1229 CSH-15R ranges between 2 43-2 52 nmoles of O_2 $min^{-1}mg^{-1}$ fr wt It was also observed that total respiration measured from the etiolated seedlings and isolated mitochondria in 104A, 104B, RS585 and CSH 15R lines were higher than the another CMS system 1049A, 1049B, RSV 33 R and SPH 1229 lines The cyanide sensitive respiration in etiolated

seedlings was found more in CMS lines than their corresponding maintainer, restorer and hybrid lines The two different CMS lines had almost same cyanide sensitive respiration However, differences were observed between the two maintainer, restorer and hybrid lines The hybrid line CSH-15R had 0 88 nmoles of O_2 $min^{-1}mg^{-1}$ fr wt (35 6 per cent) more cyanide-sensitive respiration than the hybrid SPH-1229 0 73 nmoles of O_2 min^{-1} mg^{-1} fr wt (30 4 per cent) The CMS line 1049A having 1049B as maintainer and RSV-33R restorer line had more or less similar cyanide-sensitive respiration while in second CMS system variation was observed between the maintainer 104B and restorer RS-585 lines for CMS 104A The cyanide-insensitive respiration from the etiolated seedlings of CMS lines was lower than its respective maintainer, restorer and hybrid lines In CMS lines about 4 to 5 times the per cent the cyanide insensitive respiration was lower than their maintainer, restorer and hybrid lines Connett and Hanson (1990) reported that in cells from CMS plants of *Petunia*, cyanide- sensitive respiration was accounted for most of the total respiratory rate In contrast in cells from male fertile and fertility-restored plants, the activity of the SHAM-sensitive pathway continued to account for large fraction of total oxygen consumption The results obtained in the present investigation were agreed with these earlier reports Musgrave *et al*, (1986) reported that seven male sterile lines lacked alternative respiration in the tissues assayed from leaf or root of *Petunia* However, in the present investigation, alternative respiration (cyanide-insensitive respiration) was observed in etiolated seedlings

Musgrave *et al*, (1986) suggested two hypotheses for coincidence of male sterility and lack of alternative pathway The first involves the

Table 6 : Cyanide-sensitive and insensitive respiration in the tissues of etiolated seedlings from CMS, maintainer and restorer parental lines of hybrid SPH-1229 of sorghum

Parents/ hybrids	nmoles of O_2 min^{-1} mg^{-1} fr. wt.			
	Total	Cyanide sensitive	Cyanide insensitive	Residual
1049 A	1 59	1 0 (62 8)	0 20 (12 57	0 39 (24 52)
1049B	2 14	0 68 (31 77)	0 91 (42 52)	0 55 (25 70)
RSV-33R	2 18	0 68 (31 19)	0 98 (44 95)	0 52 (23 85)
SPH-1229	2 43	0 73 (30 04)	1 12 (46 09)	0 58 (23 86)
S E ±	0 10	0 04	0 04	0 03
C D 5%	0 35	0 16	0 144	0 10

Figures in parenthesis indicates the per cent respiration compared with total respiration

Table 7 : Cyanide-sensitive and insensitive respiration in the tissues of etiolated seedlings from CMS, maintainer and restorer parental lines of hybrid CSH-15 R of sorghum

Parents/ hybrids	nmoles of O_2 min^{-1} mg^{-1} fr. wt.			
	Total	Cyanide sensitive	Cyanide insensitive	Residual
104 A	1 63	0 99 (60 73)	0 18 (11 04)	0 46 (28 22)
104 B	2 22	0 62 (27 79)	0 98 (44 14)	0 62 (27 92)
RS-585	2 36	0 57 (24 15)	1 18 (50 00)	0 61 (25 84)
CSH-15R	2 52	0 88 (34 92)	1 06 (42 06)	0 58 (23 01)
S E ±	0 07	0 03	0 05	0 02
C D 5%	0 24	0 11	0 166	0 08

Figures in parenthesis indicates the per cent respiration compared with total respiration

possibility that lack of alternative respiration is responsible for male sterility However, the occurrence of alternative respiration in CMS- T maize tissue makes the relationship between lack of the pathway and male sterility in other lines appear more coincidental than causative Further, mitochondria isolated from CMS-C tissue which lacked alternative respiration display the pathway *in vitro* suggesting a possible inhibition of the alternative pathway mediated by some component of the cytoplasm A second hypothesis for the coincidence between male-sterility and lack of alternative pathway in tissues would postulate that some regulatory substance influencing both the alternative pathway and the sex of the flowers

In the present investigation the appearance of alternative pathway at lower level in sterile than fertile lines would be result of CMS rather than the cause of CMS trait The lack of alternative respiration in the male-sterile lines might also serve to explain the commonly observed higher productivity and vigor of male sterile plants compared to fertile ones (Musagrave *et al*, 1986) In the present investigation CMS line 104 A had lower cyanide-insensitive respiration (alternative respiration) than the 1049A line, indicating that 104 A line would have higher productivity and vigour than 1049A line The operation of cyanide-insensitive respiration may be important in supplying reducing equivalents for biosynthesis when the cytochrome oxidase pathway is inhibited by high intracullular ATP content (Palmer, 1976) This may be the case during microspore development (Bino *et al*, 1986) however whether the alternative oxidase pathway is of special importance during anther or microspore development is not known

In the present investigation the consistent difference in cyanide-insensitive respiration (alternative oxidase) in isonuclear CMS (104 A and

1049 A) and their respective fertile (104 B and 1049 b) lines did not conclude that the same mitochondrial DNA locus which specifies CMS also specifies altered cyanide-insensitive respiration

In the present study it was found that in case of isolated mitochondria the percent residual oxygen consumption was lower when compared to that of whole plant tissues, presumably because of enhanced accessibility of sites to the inhibitors Further, it was also observed that the total oxygen consumption in isolated mitochondria was considerably higher than the whole plant tissues, because of the ready availability of sites and there by more oxygen diffusion in this organelle

4.4 Cytochrome *c* oxidase activity in mitochondria isolated from CMS, maintainer, restorer and hybrid lines

The cytochrome *c* oxidase activity in mitochondria isolated from 1049A, 1049B, RSV-33 R, SPH-1229 and 104A, 104B, RS-585, CSH-15R were presented in Table 8 and 9 It was observed that CMS 1049 A had 5 06 k min^{-1} cytochrome *c* oxidase activity while 1049B, RSV-33 R and SPH-1229 had 12 09, 12 85 and 7 99 k min^{-1} cytochrome *c* oxidase activity respectively It was observed that CMS line had lower activity than maintainer, restorer and hybrid line The maintainer and restorer line had more or less similar activities In another CMS system CMS line 104 A had 11 99 $kmin^{-1}$ while its maintainer restorer and hybrid had 17 26, 18 23 and 13 33 $kmin^{-1}$ cytochrome *c* oxidase activity respectively In this system also CMS had lower activity than the corresponding maintainer, restorer and hybrid lines and further in this system it was found that the maintainer and restorer lines had more or less similar activity It was observed that CMS system having 1049 A, 1049 B, RSV 33 R parents and SPH 1229 hybrid had

Table 8 : Cytochrome *c* oxidase activity in the mitochondria isolated from CMS, maintainer and restorer parental lines of hybrid SPH- 1229 of sorghum

Parents /hybrids	Activity (k min^{-1})
1049 A	5 06
1049B	12 09
RSV-33R	12 85
SPH-1229	7 99
S E ±	0 394
C D 5%	1 216

Table 9 : Cytochrome *c* oxidase activity in the mitochondria isolated from CMS, maintainer, restorer parental lines of hybrid CSH- 15R of sorghum

Parents /hybrids	Activity (k min^{-1})
104 A	11 99
104B	17 26
RS-585	18 23
CSH-15R	13 34
S E ±	0 676
C D 5%	2 08

lower cytochrome *c* oxidase activity than the CMS system having 104 A, 104 B, RS585 parent and CSH 15 R hybrid

Earlier it was reported that cytochrome *c* oxidase was significantly lower in anthers of CMS than anthers of fertile lines of maize (Watson *et al* 1972; Ohmasa 1984, Bino *et al*, 1986), wheat (Borisenko and Dmitrieva, 1977), rye (Dmitrieva, 1971), rice (Dai *et al* 1978) and Lucerne (Fursov and Fisenko, 1977) In maize, differences were also occurred in the number of cox isoenzymes of male fertile and sterile plants (Watson *et al* 1977) The trend obtained in the present investigation similar to the earlier reports However, Ohmasa (1984) observed that no significant differences in the enzyme activities of flag leaves between fertile cytoplasm plants and three male-sterile plants of maize Watson *et al*, (1977) also reported that the sterile seedlings of maize contained significantly more cytochrome *c* oxidase activity than the fertile seedlings Thus the results obtained in the present investigation were contradictory with the results reported by Ohmasa (1984) Out of 7 subunits of cytochrome *c* oxidase three subunits (COX I, COX II and COX III) are encoded by the mitochondrial genome and aberrant synthesis of one of the mitochondrial encoded proteins initiates the abnormalities i e results in CMS phenotype The mitochondria of CMS type of *Sorghum bicolor* synthesize a *cox* subunit I, 400 dalton larger than the form found in fertile plasmatypes (Dixon and Leaver, 1982) In the present investigation the low activity of the cytochrome *c* oxidase may be due to the mutation in cox complex and specifically in COX I subunit resulting in detrimental protein synthesis causing CMS However, hybrid lines SPH 1229 and CSH 15 R evolved from these 1049 A and 104 A CMS lines had higher activity This might be due to the restorer nuclear gene

which may interact with the cytoplasmic genome and may supress the synthesis of the new protein which prevents the expression of sterility

4.5 Succinate dehydrogenase activity in mitochondria isolated from the CMS, maintainer, restorer and hybrid lines.

The succinate dehydrogenase activity in mitochondria isolated from the two CMS systems *viz*, 1049 A, 1049B, RSV-33R and SPH-1229 and 104 A, 104 B, RS-585 and CSH-15R were tabulated in Table 10 and 11 It was observed that the CMS line 1049 A had 1 9 units of succinate dehydrogenase activity which was lower than the maintainer 1049B (2 8 units), restorer RSV-33R (4 8 units) and hybrid SPH-1229 (3 8 units) line In other CMS system, CMS line 104 A had 3 6 units succinate dehydrogenase activity which was found lower than the 104 B maintainer (4 8 units) RS 585 restorer (6 1 units) and CSH-15R, hybrid (4 3 units) lines In both systems the restorer line had highest succinate dehydrogenase activity corresponding to their CMS, maintainer and hybrid lines Watson *et al*, (1977) reported lower succinate dehydrogenase activity in the CMS-C, CMS-S and CMS-T lines than the fertile line of maize In sorghum, anthers from CMS line had lower succinate dehydrogenase activity than anthers from fertile line (Nakashima, 1978) In the present investigation also, same trend was observed as that of earlier reports However, Watson *et al* (1977) did not find apparent differences in succinate dehydrogenase activity when detected biochemically and eletrophoretically Succinate dehydrogenase complex consists of four subunits, entirely encoded by nuclear genome The reffered differences appeared in the present investigation in isonulear lines of A and B in both systems might be due to the result of CMS and not the cause of CMS trait Thus succinate dehydrogenase activity did not account

Table 10 : Succinate dehydrogenase activity in the mitochondria isolated from CMS, maintainer, restorer parental lines of hybrid SPH-1229 of sorghum

Parents /hybrids	Specific activity (units)
1049 A	1 9
1049B	2 8
RSV-33R	4 8
SPH-1229	3 8
S E ±	0 081
C D 5%	1 251

Table 11 : Succinate dehydrogenase activity in the mitochondria isolated from CMS, maintainer, restorer parental lines of hybrid CSH-15R of sorghum

Parents /hybrids	Specific activity (units)
104 A	3 6
104B	4 8
RS-585	6 1
CSH-15R	4 3
S E ±	0 098
C D 5%	0 303

for cytoplasmic male sterility however it would implicate for inducing male sterility

4.6 Standardization of protocol for isolation of mitochondrial DNA.

In the earlier method described by Chase and Pring (1986) and Munjal and Narayan (1995) used Tris-HCl and TES buffer respectively in the isolation buffer, however in the present study Hepes-KOH buffer was used because it causes higher extractability of the mitochondria and thereby result in higher yield of mitochondrial DNA The Chase and Pring (1986) added the sucrose as osmoticum in the isolation buffer however in the present study mannitol was used as osmoticum because it is not a metabolizable sugar by plant and therefore the concentration of mannitol will remain unchanged and thereby reduce the probability of swelling of mitochondria The EDTA in the isolation buffer acts as chelating agent, mercaptoethanol and cysteine act as sulphydryl group protectant, PVP removes the polyphenols by binding with it Incorporation of sodium-diethyldithio carbonate (DIECA) in isolation buffer during grinding was found essential for isolation of undegraded mt-DNA Without DIECA the mt-DNA was almost totally degraded (Wilson and Chourey, 1984) In alkali lysis method, DNase treatment was given to the isolated mitochondria to get rid of nuclear genome if present possibly attached to the mitochondrial membrane For the DNase activity magnesium chloride is required as co-factor therefore added in reaction medium for freezing and thawing method DNase treatment was omitted because of possible membrane damage caused by liquid nitrogen (Munjal and Narayan, 1995)

The n-lauryl sarcosine treatment was given to the isolated mitochondria to rupture the mitochondrial membrane to release the mt-DNA

into the surrounding media DNase treatment was given to digest the mitochondrial RNA followed by proteinase K treatment to digest mitochondrial proteins alongwith RNase added exogenously in the reaction medi of mitochondrial lysate The SDS in extraction buffer will facilitate protein-SDS complex and potassium acetate facilitate to form insoluble potassium dodecyl sulfate and also aids to remove the polysaccharides and proteins The isopropanol and ammonium acetate treatment was given to facilitate the DNA precipitation The traces of salts was removed by treating the DNA pellet with 70 % ethanol The reprecipitation of DNA was done one more time with isopropanol and sodium acetate in order to produce pure form of DNA The TE buffer was used to facilitate DNA solubilization

The yield of mitochandrial DNA by two methods was measured on UV-Sphechrophotometre and was presented in Table 12 It was observed that by DNase treatment method yield of mitochondrial DNA was more than by the freezing and thawing method This may be due to possible damage to the mitochordrial membrane due to freezing and thawing with liquid nitrogen

The A 260/280 ratio was tabulated in Table 13 It was observed that the ratios obtained by these two methods was around 1 8 which shows that mt-DNA preparation obtained from both the methods was pure

The two protocols followed in the present investigation had their own advantages and disadvantages when compared with each other The advantage of freezing and thawing method is that it avoids the possibility of degradation of mt-DNA by nasty DNase enzyme if remained traces in the preparation and also saves 1 h which was required for DNase treatment and otherwise makes the protocol lengthy However, in alkali lysis method

Table 12 : Mitochondrial DNA yield from two different methods

Method	Yield (µg 100 g^{-1} fr wt)
Alkali lysis	15
Freezing and thawing	11 5
S E ±	0 43
C D at 5%	1 693

Table 13 : Mitochondrial DNA yield from sterile, maintainer and restorer parental lines of SPH-1229 and CSH 15R hybrids of sorghum

Parents / hybrids	Yield (µg 100 g^{-1} fr wt)	Purity (A_{260} /A_{280})
1049 A	12 5	1 796
1049B	12 3	1 801
RSV 33R	14 0	1 798
SPH 1229	10 9	1 8
104A	11 1	1 805
104B	14 0	1 786
RS-585	13 0	1 806
CSH – 15 R	12 0	1 70

DNase treatment is advantages because it digests the nuclear genome if present around the mitochondrial membrane and avoids the doubt about the possible contamination of nuclear DNA with mt-DNA

Further, both these protocols have the marked advantage over other protocols in that, no organic solvent extractions of the mt-DNA with phenol, chloroform or ether is necessary Another advantage of these methods is that ultracentrifugation, for either the isolation of mitochondrial organelles on sucrose or percoll gradients or separation of mt-DNA from plastid and nuclear DNA's on cesium-chloride density gradient is not needed

The quantity of mitochondrial DNA obtained in the present investigation is sufficient for the further RAPD or RFLP studies

SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

Sorghum is an important food grain crop in Maharashtra and India It is grown after rainy season on available soil moisture as a *rabi* crop Most of the people prefer sorghum as food in their diet in the form of *bhakari* It is therefore necessary to improve the nutritional quality of this grain As the population is increasing in geometric proportion, it is necessary to increase the yield of food grains like sorghum to meet the demand The hybrid seed production is an important breeding programme to improve the yield and quality of sorghum grain The cytoplasmic male sterility is the prized agricultural commodity used in the hybrid seed production as it obviates the hand emasculation and pollination The continuous use of a single source of CMS leads to susceptibility to insect pests and diseases, the transfer of CMS trait to the desirable line by conventional backcrossing method is time consuming If the causes of cytoplasmic male sterility are investigated, it will be possible to transfer this trait to the desirable line by the application of genetic engineering methods which will be a time saving process Cytoplasmic male sterility is caused by mitochondrial gene mutation(s) In this context, studies on biochemical aspects of mitochondria seem to be the most important one Studies on mitochondrial respiration and mitochondrial respiratory enzymes may generate some clue regarding the cause(s) of male sterility

The present experiment was conducted using two CMS system *viz* , 1049A, 1049B, RSV-33R, SPH-1229 and 104A,104B, RS-585, CSH-15R The etiolated seedlings were grown to isolate mitochondria The etiolated seedlings and isolated mitochondria from these eight lines were used for

measuring the cyanide-sensitive and cyanide-insensitive respirations The isolated mitochondria were also used for succinate dehydrogenase and cytochrome *c* oxidase assays The protocols for the isolation of mitochondrial DNA, were also standardized. The results obtained in this investiagation are briefly summarized as follows

1 The soluble protein content in mitochondria from the two CMS lines, 104A and 1049A, were higher than their alloplasmic isonulear 104B and 1049B lines of sorghum It was also observed that the respective restorer and hybrid lines also had the lower contents of soluble proteins The higher concentration of soluble proteins in the CMS lines indicates that these lines might have synthesized new protein(s)/ polypeptide(s) which are not synthesized in their respective isonuclear lines The additional proteins synthesized may be associated with the CMS phenotype

2 The cyanide-sensitve respiration in the isolated mitchondria of 104A and 1049A lines was higher than their respective isonuclear maintainer lines, 104B and 1049B

3 The cyanide-insensitive respiration in the isolated mitochondria was found lower in the CMS lines, 104A and 1049A lines than their respective maintainer, restorer and hybrid lines Amongst the CMS lines, the CMS 104A had comparatively lower cyanide-insensitve respiration than the 1049A

4 In all the eight sorghum lines, the residual respiration was noticed when both the inhibitors, SHAM and KCN were added simultaneously in the reaction media The total respiration was found more in the hybrid SPH-1229 and its respective parental lines than the hybrid CSH-15R and its respective parental lines

5 When the cyanide-sensitive and insensitive respiration was measured from the etiolated seedlings, the similar trend of respiration as that of mitochondrial isolates was observed However, the higher respiration observed in isolated mitochondria may be because of the more availability of accessible sites

6 Activities of cytochrome *c* oxidase and succinate dehydrogenase were found lower in the mitochondrial isolates of CMS, 104A and 1049A lines than their respective isonuclear maintainer, restorer and hybird lines

7 The yield of mt-DNA from sorghum lines was comparatively higher in the alkali lysis method than the freezing and thawing method The preparation of mt-DNA appeared pure when A260/280 ratio was measured

In conclusion, the higher protein content observed in the mitochondria isolated from the etiolated seedlings of CMS lines suggested that some additional protein(s) were synthesized by these mitochondria The lower level of partially mitochondrially encoded cytochrome *c* oxidase suggested that genetic determinants of CMS character resided in the mitochondria Amongst the two methods of mt-DNA isolation tried, the yield of mt-DNA was higher by the alkali lysis method than the freezing and thawing method

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6. LITERATURE CITED

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