GENETIC AND BIOCHEMICAL CHARACTERIZATION OF THE *ETCHED (et)* MUTANT OF *Zea mays*

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This is to certify that I, H.G. Sangeetha, have carried out the research embodied in the present thesis tor the full period prescribed under Ph.D. Ordinances of the University.

I declare to the best of my knowledge that no part of this thesis was earlier submitted for the award of research degree of any University.

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TO MY PARENTS

AND RAMESH

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ABBREVIATIONS

Cat-	Catalase
CF	Coupling factor
Chl.a	Chlorophyll.a
Chl.b	Chlorophyll.b
CPI	Chlorophyll protein I
Cyt	Cytochrome
DAG	Days after germination
DAP	Days after pollination
DCMU	Dichlorophenyl dimethyl urea
DCP1P	2,6 dichlorophenol indophenol
et	etched allele
et et	mutant genotype
	heterozygote genotype
et et	wild type genotype
GA	Gibberellic acid
IAA	Indole acetic acid
kD	Kilodaltons
LHCP	Light harvesting chlorophyll protein
MV	Methyl viologen
NAD	Nicotinamide adenine dinucleotide
nm	Nanometres
UD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PEP	Phospho enol pyruvate
PS	Photosystem
RUBP	Ribulose bis phosphate
SEM	Scanning electron microscope
TMBZ	Tetra methyl benzidine
TMPD	N,N,N,N - tetra methyl - p-phenylenediamine

CONTENTS

1. INTRODUCTION

Higher plants offer unique advantages as experimental material to probe the chain of events connecting the gene locus and its phenotypic trait. With an alternation of distinctly different gametophytic and sporophytic generations, higher plants constitute highly suitable systems to study the mechanism by which a segment of the hereditary material is ultimately expressed as a phenotype (Nelson, 1967). However, a major problem encountered in the biochemical genetic analysis of higher plants is that, in relatively very few cases there is a clue in the phenotype of the mutant as to the metabolic lesion that is responsible tor it. Where such a correspondence is obvious, like the flower colour mutants of maize, or mutants affecting carotenoid synthesis, considerable progress has been made in understanding gene expression and its regulation.

Among higher plants, maize lends itself best to studies on genetics and biochemistry of development. Extremely high genetic variability, availability of a wealth of information on its genetics in terms of well mapped chromosomes and easily detectable phenotypes are attributed to the choice of maize, for studies in basic biological research in general and genetics in particular.

One of the most interesting and important problems in eukaryotic genetics today is the regulation and control of gene action during differentiation and development. However, a rigorous analysis of the genetic basis of differentiation

and development is often hampered by the lack of suitable mutants. Further, developmental mutants are often lethal.

In maize, a number of mutations are known which affect either seed or seedlings during specific stages of development and growth. However, not many mutants are available which individually affect the phenotype of seed as well as seedlings. One such mutant is the *etched* mutant of maize which affects the seed as well as seedlings. The *et* allele in homozygous recessive condition causes a pitted and scarred endosperm and also gives rise to virescent seedlings. Etching phenotype of the kernel and virescence of seedlings are genetically inseparable. It is interesting that in the seed the effect of the mutation is in endosperm which is dead-end tissue and the effect is irreversible. On the contrary, the virescence phenomenon is on a growing tissue of seedlings and the effect of the mutation is reversible under light.

The present work aims at an indepth analysis of *et* mutation in terms of seed and seedling phenotypes. Attempts have been made to study the relationship between the *et*, mediated seed aberration and seedling virescence. Studies were performed to look into the nature of possible changes in chloroplast biochemistry including relevant enzymes.

Further, biochemical changes in seeds during development, maturation and germination has been investigated. Our observations suggest a close correlation between aberrations of endosperm phenotype and delayed chloroplastogenesis and associated changes in the enzyme levels in the early seedlings.

2. LITERATURE REVIEW

VIRESCENCE

The virescent mutants of higher plants are an interesting group of mutants, which at early seedling stage are near-white to yellowish, and gradually turn green as they grow. Virescent mutations are found to occur in a wide range of flowering plants including tomato (Rick, 1982), maize (Cholet and Paolillo, 1972), cotton (Benedict et al, 1972) and groundnut (Alberte et al, 197b).

The effect of temperature upon a mutant phenotype is one of the easiest of the environmental variables to study and hence examples of temperature-induced modifications of mutant phenotypes are numerous (Robertson and Anderson, 1961). In alfalfa, a chlorophyll-deficient mutant has been described, whose expression is influenced by temperature (Stanford, 1959). In barley also, several temperature sensitive mutants have been described. (Collins, 1927; Nybom, 1955). In corn, temperature-sensitive mutants have been recognized even in the early days of maize genetics (Carver, 1927; Emerson, 1912; Lindstrom, 1918; Miles, 1915).

The virescent mutant pale-yellow-1, when grown in continuous light at 25 ^{o}c takes about a week, before the onset of greening. Normal plants however, require only one day for greening. At elevated temperatures (35 ^{o}C), however, the onset of greening is observed in less than two days which nearly approaches the rate of greening for normal plants

grown at this temperature (Phinney and Kay, 1954). The seedling of pale-yellow-1, shows a deficiency in carotenes and carotenois (flavoxanthin c, violaxanthin b and lutein) in addition to chlorophyll a and chlorophyll b. When grown in dark, the mutant accumulates 10% of the amount of protochlorophyll-a found in dark-grown normal seedlings (Kay and Phinney, 1956). The dark-grown mutant seedlings contained less carotenois than normal seedlings, but the same amount of carotene as in normal seedlings. It was suggested that the gene may be operating on a common pathway for the synthesis of chlorophyll and carotenoids, but at a position after the synthesis of carotenes and before the synthesis of carotenois and the phytyl portion of the chlorophyll molecule.

The viridis mutant of Gateway barley has been studied extensively. The mutant is deficient in chlorophyll and carotenoids when young, but developed nearly normal levels of pigment with age (Machlachlan and Zalik, 1963). Protochlorophyll amounts were found to be lower in etiolated mutant seedlings. In the young mutant, chloroplasts are observed to be small and irregular with large vesicles and no grana. The level of free amino acids, particularly of serine, was observed to be extremely high. The Viridis mutation is believed to alter the lipoprotein of lamellar structure in the chloroplast, and the other effects are consequential

(Machlachlan and Zalik, 1963).

Quality and intensity of light, and temperature are found to influence the Gateway barley and its virescent mutant (Miller and Zalik, 1965). However, chl.a to chl.b ratio did not differ in either barley lines under treatment except in the mutant at low temperature. The optimum conditions of temperature and light intensity for both lines were found to be 24.5 ^{o}C and 1020 ft C. The mutant was found to be very sensitive to low temperature. Interestingly, this mutant at high temperature is insensitive to light intensity. it is hypothesized that the mutation has resulted either in a reduced rate of synthesis of a moiety of the holochrome other than chlorophyll, or a reduced rate of assembly of the moieties into grana.

Reciprocal crosses of normal and mutant Gateway barley gave F_1 populations with normal green appearance. Since the mutant character was not inherited maternally, the possibility of it being due to a plastome mutation was excluded. The segregation ratios of F_2 progeny obtained from selfing the F_1 plants showed that the chlorophyll-deficient character behaved as a recessive single nuclear-gene mutation. The slight deviation from the expected 3:1 ratio observed in the segregating F_2 population may be due to poor germination of mutant seeds. It is also possible that seedlings of the mutant genotype had become similar to the normal phenotypically and were not distinguishable

(Stephansen and Zalik, 1971).

The soluble leaf proteins of the virescent mutant of Gateway barley on polyacrylamide gel electrophoresis showed that fraction 1 protein in the mutant is less in 4 day old seedlings, but it increased with age and at 8 day no difference was observed. At 4 day stage, the mutant was also found to lack some lamellar proteins, but at the 8 day old stage no such differences were noticed (Jhamb and Zalik, 1973). in the normal Gateway barley, microtubules were present in etioplasts and also during formation of the lamellar system. However, in the mutant, the microtubules were observed only after the formation of the lamellar system. It was concluded that the microtubules might perform a function in orienting the lamellar system of chloroplasts during their development (Jhamb and Zalik, 1975).

Increase in chlorophyll content between 4th day and 8th day roughly paralleled the increase in photosynthetic activities of the mutant plastids. However, when the photoreductive activities were expressed on a per mg. chlorophyll basis, there was a 2 fold increase in their specific activities from 4th to 5th day. Thus the initial increase in the photoreductive activity expressed on a chlorophyll basis could not have been due to an increase in chlorophyll content. It might have been due to the synthesis

of other membrane components which were limiting. The limiting components could be a membrane protein *or* some other factor required for proper functioning of photosynthetic electron transport. It was concluded that chlorophyll content did not limit photoreductive activity at the earliest stage of development (Horak and Zalik, 1975).

Studies on carboxylase enzyme activities revealed profound differences between normal and mutant Gateway barley seedlings grown in light for 4 days. Normal seedlings at 4 day old stage showed 11 times more RuBP-carboxylase activity and 18 times higher activity per gram fresh weight, as compared to the mutant. In the normal seedlings, PEP carboxylase represented only 8% of the sum of the two carboxylase activities, whereas in the mutant seedlings it accounted for 70% of the carboxylase activity. The high PEP carboxylase activity in young seedlings of the mutant grown in light, suggests an adaptation within the mutant during the period when the amount of RuBP-carboxylase is low (Barankiewicz *et al.*, 1979).

Acyl lipids were also found to be deficient in the virescent seedlings of the barley mutant (Thomson and Zalik, 1981). Further, it was shown that the addition of 5-ALA increased the chlorophyll content to the same extent in both normal and mutant seedlings, indicating that the pigment

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deficiency is not due to blockage in the biosynthetic pathway of chlorophyll. This deficiency is suggested to be one of the several pleiotropic responses resulting from the failure to synthesize a specific gene product that is required for normal chloroplast formation. This reflects a close co-ordination of the synthesis of macromolecules and plastid development and provides evidence that lipid components other than pigments may also play an active role in the assembly of the photosynthetic apparatus.

During greening, the protein content per plastid did not vary between normal and mutant barley seedlings. However, a difference in the SDS-PAGE polypeptide profile was observed between normal and mutant seedlings. High molecular weight proteins of 96 kD and 66 kD were found to decrease, whereas, 34 kD, 27 kD and 22 kD proteins were found to increase in relative quantity as a function of greening (Kyle and Zalik, 1982).

The fully greened mutant seedlings of barley were found not to be deficient in the light-harvesting chlorophyll-protein complex and in the reaction centers of PSI and PSII. Further, it was reported that PS I associated photochemical activities appeared within the first hour of plastid development and PS II associated activities and oxygen evolution within the next 6 hours. In all cases, it was noticed that the developmental rate per unit protein was

slower in the mutant following 6 days of etiolation, but no such difference between the genotypes could be seen after 8 days. This is due to a decrease in the developmental rate of the wild-type chloroplasts. An increase in photosynthetic unit size associated with plastid morphogenesis was observed to be faster in normal seedlings after 6 days, as compared to mutant seedlings. However, this difference was found to be negligible after 8 days (Kyle and Zalik, 1982). It was concluded that all aspects of chloroplast development are affected in the mutant by an overall reduction in the rate of chloroplast morphogenesis. The mutation therefore, does not affect any single photo-chemical parameter in particular. The virescent mutant of gateway barley, therefore undergoes the normal pattern of proplastid to chloroplast development, but at a markedly reduced rate.

Chloroplasts are the major sites of cellular fatty acid synthesis in green tissue. The acetyl-CoA carboxylase which catalyzes the formation of malonoyl CoA, which in turn is utilized by the fatty acid synthetase complex is found to be maximal during the interval of rapid lipid accumulation and declined sharply at leaf maturity. Quantitatively, the activity was higher in the mutant than in the wild type (Thomson and Zalik, 1981).

In cotton, it was reported that a single nuclear gene mutation leads to the virescence of seedlings. The young

seedlings are distinctly yellow and turn green with age. The yellow leaves of the virescent cotton mutant, show a relatively higher photosynthetic activity on the basis of chlorophyll as compared to that of normal leaves. Further it was noticed that at saturating light intensity, the rate of carbondioxide fixation was 8 fold higher in the virescent leaves as compared to green leaves. It was suggested that the impaired pigment synthesis which could be lethal is offset by a high photosynthetic capacity in the virescent leaves (Benedict and Kohel, 1968).

The level of RuBP-carboxylase in virescent cotton leaves was found to be normal or even higher than that of normal green leaves. However, no significant correlation was observed between the rate of synthesis of chlorophyll and the rate of synthesis of RuBP-carboxylase in the mutant leaves. On a chlorophyll basis, the synthesis of RuBP-carboxylase in the mutant far exceeds that of the normal (Benedict and Kohel, 1969).

In summary, the yellow viresent cotton leaves have a high photosynthetic rate, a low amount of PEP carboxylase activity and a total absence of lamellar aggregation into grana in the chloroplasts (Benedict and Kohel, 1970).

A virescent chloroplast mutation has been described in tobacco (Archer and Bonnett, 1987a). This mutant is unique

in that it is inherited maternally unlike other virescent mutants which follow Mendelian inheritance. This mutant displays a lag in chlorophyll accumulation and granalstack formation in young leaves. Further, this mutant when compared to the normal, shows a much higher reduction in the thylakoid proteins. Stromal protein levels in this mutant are also reduced, although to a lesser extent. Polyacrylamide gel electrophoresis of the thylakoid extracts showed that three polypeptides are specifically decreased in the mutant. Electrophoresis of the thylakoid proteins coded by the chloroplast genome, revealed the absence of a 37.5 kD polypeptide in the mutant (Archer et al, 19B7b).

Rate of chlorophyll synthesis and chloroplast number per cell in the virescent mutant of tobacco were similar to that of the control. Further, carotenoid content in the mutant was found to be sufficient to protect chlorophyll from photo-oxidation. Photosynthetic rates of this tobacco mutant at low-light intensities suggested a reduced ability to collect light. Further, a significant reduction in thylakoids per granum was also observed in virescnt leaves. However, mature virescent leaves with nearly normal chlorophyll content showed normal granal profiles (Archer and Bonnett, 1987a).

In Phaseolus vulgaris, the leaves of young virescent seedlings fix less carbondioxide than those of control

plants. In addition to this, RuBP carboxylase activity was observed to be lower in mutant leaves than that of control leaves. Photophosphorylation per chloroplast, mediated by photosystems 1 and II is reported to be slightly higher in the mutant plastids. Further, it was reported that the virescent mutant showed slight differences in the amount and composition of leaf lipids as compared to normal. Large differencs in RNA content was also noticed between normal and virescent seedlings. Mutant leaves also showed a higher initial content of cytoplasmic ribosomal RNA compared to controls. However, as leaves age, it was observed that ribosomal RNA content is initially similar, but continued synthesis is found to be greater and more prolonged in the mutant (Heyes and Dale, 1971).

In virescent peanut leaves, a 72-hour lag period was noticed before the onset of rapid chlorophyll accumulation. A distinctly lower rate of protein synthesis, and normal rates of nucleic acid synthesis was observed in the mutant. Further, it was shown that during the lag period of chlorophyll accumulation, development of chloroplast grana and the activity of many enzymes of the reductive pentose phosphate cycle, PEP carboxylase and malate dehydrogenase are reduced in the virscent leaves (Benedict and Ketring, 1972).

The temperature-sensitive maize mutant M 11, grown in the dark and at a temperature of 15 ^{o}C, shows an abnormal

ultrastructure of the etioplast. It was observed that the pigment content was reduced, but *in vivo* absorption characteristics suggestd that the normal protochlorophyll (ide)-holochrome was present. It was shown that the mutant M 11 when grown in the light at 15°C showed abnormal ultrastructure of the plastids, extreme reduction in pigment content and marked deficiency in ribosomes. It was suggested that in the mutant at 15°C, plastid membrane was extremely sensitive to light (Millerd *et al*, 1969).

The role of temperature in regulating the expression of virescent trait has been studied in five non-allelic mutants of maize (Hopkins and Walden, 1977). These are VV, V 16 V 16, V 3 V 3, V 12 V 12 and V 18 V 18. The mutant VV was found to be insensitive to temperature. The other four mutants, however, are characterized by a discrete threshold temperature below which greening will not occur. The threshold temperatures are found to be specific and range from 20-25°C. Subthreshold temperatures were found to lead to lethality due to endosperm depletion. Rate of greening was found to increase with increasing temperature, above the threshold temperature. It was suggested that greening perhaps represents a loss of temperature sensitivity as the seedling matures. It was found that loss of chlorophyll in the mutant V 16 V 16 at 20°C is accompanied by a corresponding decrease in carotenoids. On the other hand, virescent seedlings

accumulate only trace levels of protochlorophyll in darkness.

However, under intermittent or continuous low-light intensity at 2u °C, the virescent seedlings accumulate only trace levels of chlorophyll. Seedlings etiolated at 28 °C were found to synthesize chlorophyll in light at 20 °C and this chlorophyll was stable in light for atleast 48 hours (Hopkins, 1982). These observations suggest that photo-oxidation of chlorophyll is probably not a significant factor in the dramatic failure to accumulate chlorophyll at temperatures below 25 °C in virescent -1b seedlings. The genetic lesion perhaps causes a temperature-sensitive block at an earlier point in the biosynthetic pathway leading to protochlorophyll. V V seedlings when grown at 16 1b temperatures below 25 °C, show aberrant plastids, about 2/3 size of the normal and contain aberrant thylakoids and only trace amounts of 70S ribosomes and 23S and 1bS plastidic rRNA (Hopkins and Elfman, 1984).

High flourescence yield of chlorophyll has been used as a rapid screen for photosynthetic mutants of maize. Four mutants of v-424 are reported to be allelic and virescent and displayed higher levels of leaf chlorophyll flourescence during greening. Fully greened mutants had normal leaf chlorophyll flourescence yield and normal levels of light-harvesting complexes (Polacco et al , 1985).

The high chlorophyll flourescence (hcf) mutations *are*

nuclear mutants which are deficient in photosynthetic electron transport but not in the accumulation of chlorophyll. The high chlorophyll flourescence observed in these mutants indicates an inefficient use of harvested light energy. These mutations lead to a reduction in the component polypeptides of one or more of the chloroplast electron transport or ATP-generating complexes. Chloroplast RNA's are not altered in these mutants, indicating that the nuclear mutations lead to loss of chloroplast-encoded polypeptides by causing a post-transcriptional block. However, the mutant hcf-38, accumulates aberrant amounts and sizes of certain chloroplast RNA's implying that this locus may function in chloroplast transcription or RNA processing. The hcf mutations are typically pleiotropic lesions at a single locus resulting in the loss of a set of functionally related proteins (Barkan et al, 1986).

Mutants that affect synthesis of photosynthetic pigments, chloroplast organization and electron transport systems are fairly common in higher plants. The mutant plants fix as much carbondioxide per unit leaf area as wild type controls, at saturating light intensities. Since the chlorophyll content in these mutants is less than that of wild type, carbondioxide fixation expressed on chlorophyll basis is higher than in normal plants. This shows that the rate limitation on carbondioxide fixation is not imposed by chlorophyll content.

Investigation of the mutants by biochemical techniques and electron microscopy show a striking developmental interdependence of the structural elements of chloroplasts and the photosynthetic pigments. The earliest studies on this aspect were conducted with certain bariey mutants (von Wettstein and Eriksson, 1964). Lethal or semi-lethal mutants that are white (albino), yellow (xantha) or pale green (viridis) are the most common class of mutants in higher plants. Though these mutants are referred to as chlorophyll mutants, only a minority actually represent blocks in chlorophyll synthesis and the others result from blocks in the formation of accessory pigments or the structural elements of chloroplasts, whose normal development is essential for stability of the photosynthetic pigments.

The chlorina-2 mutant of barley completely lacks chlorophyll-b and is smaller than normal throughout its growth period. It contains lesser amounts of reducing sugars and sucrose. The photosynthetic and respiratory rates are found to be equal to that of normal plants. Hill-reaction in the chlorina-2 mutant of barley is found to be normal under high-light intensities. But, at lower intensities of light, the mutant carries out the Hill-reaction less effectively. This shows that chlorophyll-b may not essentially have a photochemical role. However, it was suggested that chlorophyll-b perhaps affects lamellar pairing

since chloroplasts of this mutant reveal fewer grana.

The investigations of chloroplast mutants, particularly the barley mutants, have shown the interdependance of the processes of lamellar organization and pigment-synthesis, leading to the formation of normal chloroplasts. Structural elements in chloroplasts are not asssembled properly in the absence of photosynthetic pigments and conversely, a given structural arrangement is apparently necessary for photostabililty of the chlorophyll pigments.

In maize, early chloroplast development is normally accomplished while leaf tissue is tightly rolled within the leaf sheath. This suggests that the normally late appearance of light-harvesting complex is not obligatory for thylakoid differentiation. Mutants have been described in maize where loss of PS II or the cyt.f/b complex does not block assembly

of other fully active complexes into the thylakoids. It is probably advantageous for developing chloroplasts to delay assembly of the major light-harvesting apparatus until electron transport is adequately high since excessive photon capture may lead to irreversible damage. A barley mutant has been described which generates thylakoids with high levels of LHC-II, irrespective of the developmental stage of the chloroplast and this mutant dies at an early seedling stage.

In the V-424 mutant described earlier, the thylakoid

proteins coded for by the chloroplast genome were present in decreased amounts, while the amount of nuclear-coded light-harvesting chlorophyll a/b binding protein (cab-protein) was similar to that of the control strain. It was found that no thylakoid polypeptides were missing in the mutant, but the PS II reaction centre protein and the 34 kD atrazine-binding protein levels were only 24% and 50% of control levels respectively. DNA restriction enzyme analysis showed that the chloroplast genome is altered in the Vir-C mutant, supporting the genetic evidence which showed that the mutation is inherited in a non-Mendelian fashion.

In contrast with many photosynthetic mutations, the virescent mutation is not lethal and virescent plants survive to flower and produce seed. Despite the number of virescent mutations that have been described, the primary effect of the mutation is unknown. The phenotype suggests a temporal aberration in some factor governing chlorophyll content, such as the ability to synthesize, protect or accumulate chlorophyll. The variety of ways by which chlorophyll can be influenced may explain why the virescent phenotype is produced by a number of distinct mutant loci within a given species. In maize, ten mutant loci on eight different nuclear chromosomes each produce the virescent phenotype. Few virescent mutations which are maternally inherited are also reported. The existence of both nuclear and cytoplasmic

mutations producing the same phenotype supports the view that virescent mutants comprise a diverse group in which each distinct mutant locus disturbs a specific factor regulating chlorophyll content.

Most nuclear virescent mutants have decreased carotenoid levels in the young leaves. Typically, the carotenoid content in virescent leaves decreases about two fold, while the chlorophyll content decreases three to five fold. Although, the total amount of carotenoid pigments decreases, the ratio of carotenoids to chlorophyll actually increases in virescent mutants and appears to be ample for chlorophyll protection. Poor development of the grana is the most frequently studied structural aberration in virescent mutants. An interesting exception is one virescent mutant of maize (Chollet and Paolillo, 1972). Low chlorophyll content in this mutant is associated with large grana and a poorly developed fretwork of stromal thylakoids. Normal thylakoid structure developed as chlorophyll accumulated.

Photosynthetic rates are found to be different in different virescent mutants of maize. Thus, virescent mutants, although similar in phenotype have varied effects on photosynthesis. These effects may reflect alteration of a different photosynthetic component in each virescent mutant. If several different proteins are required for the normal

sequence of thylakoid stacking, defects in any one of them could lead to a reduction in thylakoid stacking and an inability to accumulate photosynthetic pigments. Specific effects on photosynthetic activity would depend on the role of the defective protein. The virescent phenotype may result from mutations within the structural gene of such a protein. Normal phenotype is recovered when a gene product of similar function is synthesized later in leaf development. Alternatively, virescent mutations may be regulatory mutations controlling the timing of gene expression and, as such may provide a genetic tool to understand the regulation of chloroplast development.

The plastid ribosome deficiencies in virescent maize are not photosensitive, but more directly related to the genetic lesion. A further correlation between virescence phenomenon and plastid rRNA in V_{16} V_{16} seedlings is indicated by the observation that chlorophyll and chloroplast ultrastructure are stable for upto two weeks after green seedlings are transferred to 20 ^{o}C in the light. Apparently, it is the formation and not stability or accumulation of chloroplast ribosomes in actively expanding tissue that is regulated by the virescent gene. The mutant V_{16} V_{18} however, shows an unexpectedly high rRNA content.

Genes coding for chloroplast rRNAs and for some of the

chloroplast ribosomal polypeptides are located in the chloroplast genome, while chloroplast RNA polymerase, aminoacyl t-RNA synthetases and the majority of chloroplast ribosomal polypeptides appear to be coded in the nucleus. Virescence represents nuclear control at an early step in plastid development and a pleiotropism that includes pigment synthesis. The virescent trait results from modification of genes that normally regulate chloroplast ribosome biosynthesis. It is possible to speculate that virescent trait reflects a temperature-sensitive transcription or translation of one of the critical nuclear-coded polypeptides.

ETCHED MUTANT

The genetics and phenotypic behaviour of a number of virescent mutants of maize have been described (Neuffer et al., 1968; Coe et. al, 1983). The etched mutant is an interesting endosperm mutant and a seedling mutant for virescence. This mutant was originally isolated from irradiated maize genotypes by Dr.L.J. Stadler (Stadler, 1940).

The et allele in homozygous recessive condition causes a pitted and scarred endosperm and also gives rise to virescent seedlings (Coe and Neuffer, 1977). Fig:1 shows a selfed etched ear with etching clearly seen on both coloured and

colourless kernels. The et gene has been mapped *on* the long arm of chromosome *3,* at a map position of 1b1. Earlier studies on the genetics of the etched mutant suggested that the et allele shows variable penetrance and expressivity (Rhodes, 1952).

The virescent seedlings show albino leaves during early stages of development and then turn normal green by about 10 days in bright-light and at a temperature of 25 ± 2 °C (Ramesh et al, 1984). This phenomenon of virescence is found to be light and temperature sensitive. Virescence is expressed in bright-light conditions as well as field conditions. Fig:2 shows 6,8 and 10 day old normal and virescent seedlings grown under bright-light.

Ultrastructural studies on the etched mutant revealed a very poor development of the lamellar system in the chloroplasts of 6-day old leaves of virescent seedlings. At the 10 day old stage, when the virescent leaves are indistinguishable from the normal, a normal chloroplast ultrastructure is exhibited by the mutant leaves (Ramesh and Reddy, 1985).

Standard in vitro procedures as well as photo-acoustic spectral analysis revealed significant quantitative differences in chlorophyll and carotenoids during the greening process in light (Ramesh et al, 1984). Studies on the pigment composition between light-grown normal and mutant

Fig. 1: A selfed etched ear (et et) showing the segregation of coloured and colourless kernels.

Fig. 2 : b, 8 and 10 day old normal (right,) and virescent (left) seedlings under bright-light conditions.

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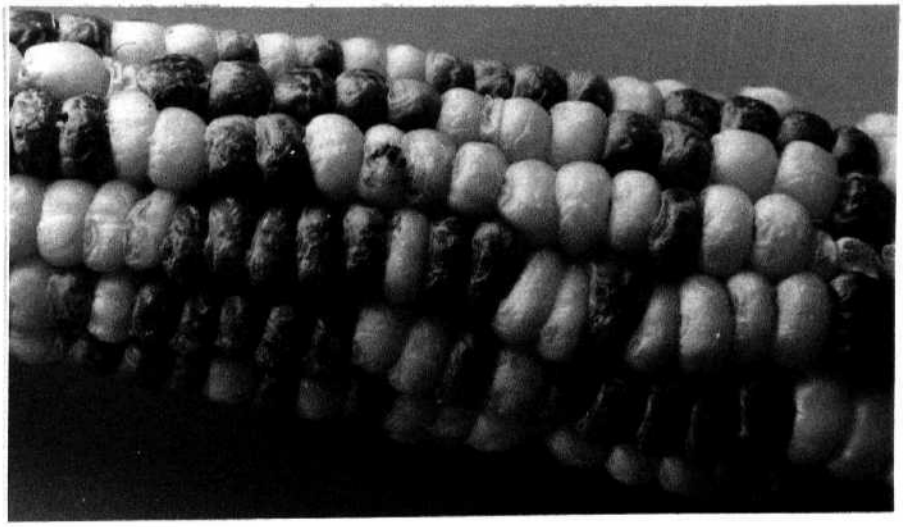

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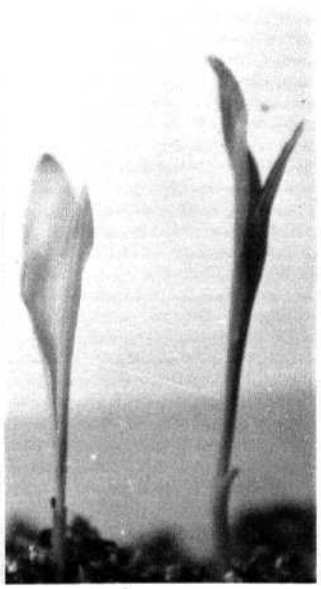

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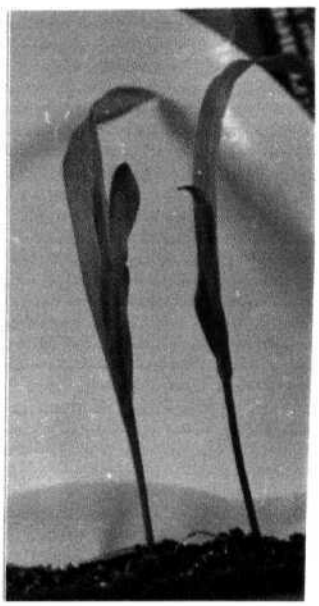

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seedlings at 6-day old stage by thin layer chromatography, revealed no qualitative differences in the pigment composition (Santha Kumari, 1984).

Solubilized thylakoid membrane fraction of mutant leaves revealed significantly lower levels of chlorophyll pigments ana protein, than that of normal upto the 8th day after germination. However, by the 10th day, both normal and mutant seedlings show about equal amounts of pigment and protein.

SDS-PAGE of solubilized thylakoid membrane extracts revealed no qualitative differences in the chlorophyll-protein complexes. However, upto the 8th day after germination, these complexes were found to be less intense in the mutant as compared to the normal. At the 10th day, the mutant and normal seedlings show the chlorophyll-protein complexes with almost equal intensity (Sangeetha et al, 1986).

3. GENETIC ANALYSIS OF THE *et* ALLELE

3.1 INTRODUCTION :

Dr. L.J. Stadler (1940) had identified the *et* mutation for the first time from the progeny of irradiated maize genotypes. Incidentally, this was the first radiation-induced mutants in maize as well as in higher plants. The identification of this mutant was firstly on the basis of kernel phenotype and secondly on the early seedling phenotype. He reported that the *et* allele, in homozygous recessive condition causes a pitted and scarred endosperm and virescence in seedlings. Early 'linkage analysis by Stadler lead to the localization of the allele on the long arm of 3rd chromosome linked to the *a* locus, a gene involved in anthocyanin biosynthesis. A decade later, Rhodes (1952) noted deviations from the expected Mendelian segregations amongst the progeny of *et et* kernels. It was said that the *et* allele shows variable penetrance and expressivity. In the last few decades or so not a single publication appeared on this mutant. It was known by then that the etching of the kernel and the virescence of the seedlings are inseparable by genetic means.

The present study deals with a detailed genetic analysis of the *et* allele in terms of its phenotypic segregations and expression. It is shown here that the progeny populations of selfed etched plants do not show any abnormal ratios and the deviations originally reported by others may be due to the exceptional kernels having almost

undetectable levels of etching i.e., *et* allele shows highly variable expressivity.

3.2 MATERIALS AND METHODS :

3.2.1 *Crosses* : The isogenic maize lines of homozygous normal (*et*$^{+}$ *et*$^{+}$) and mutant (*et*_*et*) genotypes are in the background of line R168. The homozygous stocks were maintained by repeated selfing and sibbing. The heterozygotes (*et*$^{+}$ *et*) were made by reciprocal crosses between the homozygous plants. F_2 progeny were obtained by selfing *et*$^{+}$ *et* heterozygotes and the segregating ears were harvested either at maturity or on specified days after pollination. Test-crosses were also performed to obtain heterozygotes and homozygous kernels on the same segregating test-cross ears.

Each plant was designated with an identification number comprising the year, season, range, row number and plant number. *for* eg : - 1985 R-0122-3, indicates that the plant was grown in the year 1985, in the Rabi season (Winter), in range 1 and row number 22, and is the third plant in the row. All kernels used for a single row were from the same ear and had the same phenotype. Contamination was prevented using silk bags and tassel bags. Pollen bags were put 12 hours in

advance. For uniform pollination, silks were cut 12 hours in advance. Standard corn pollination methods were used.

3.2.2. Phenotypic scoring : The "etched scale": The phenotypic variation among the progeny of seifed ears of *et* *et* plants has been noticed both in the extent of etching upon the surface of the kernel and also in the depth of etching. An "etching-scale" was made from selfed etched kernels for the purpose of classification and analysis of expressivity.

A two-directional scale of etching was made i.e., the letter scale from 'a' to 'e' shows the gradation of the extent of etching on the kernel surface with 'a' at the minimum of the scale and 'e' at the maximum. The numerical scale is from '1' to '3', where the gradation is made depending on the depth of the cracks observed. The '1' type shows only superficial cracks, while the '3' type shows deep cracks in the endosperm. In the preparation of the scale, progeny kernels of similar size from individual ears were selected.

3.3 RESULTS AND DISCUSSION A number of controlled crosses between desirable genotypes were made to test the inheritance and expression of the *etched* allele.

Table 3.3.1 gives the results of segregating pattern of selfed heterozygote *et* $^{+}$ *et* stocks. A very large number of crosses were made but only a representative few crosses are listed in the table. The results clearly show a 3 : 1

Table 3.3.1 : Test of inheritance of the *et* allele as shown by the cross of a et / a et x a et / a et

S.No	Pedigree	Kernel phenotype	Progeny phenotype		Total number of kernels	χ^2 3:1
			Normal	etched		
1	85R-0130-3	colour-less normal	86	25	111	0.362
2	85R-0133-3	,,	78	22	100	0.480
3	85R-0133-10	,,	56	15	71	0.566
4	86R-0179-2	,,	78	24	102	0.117
5	86R-0180-9	,,	89	28	117	0.070
6	86R-0180-12	,,	158	53	211	0.001
7	86R-0182-5	,,	98	28	126	0.517
8	87R-0421-7	,,	43	12	55	0.296
9	87R-0424-1	,,	82	26	108	0.049
10	87R-0424-12	,,	108	32	140	0.335
11	87R-0425-1	,,	46	12	58	0.574
12	87R-0428-2	,,	116	36	152	0.140
13	87R-0428-6	,,	42	12	54	0.221

χ^2 values were found to be non-significant at 5% level of confidence in all cases.

segregation. However, a wide variation of etched phenotypes was noticed among the et et kernels which will be dealt with in a later section. Fig: 3 shows a selfed ear segregating for normal and etched kernels in a 3:1 ratio.

Table 3.3.2 shows the results of test-crosses of the heterozygous plants (et/+ et). The progeny kernels segregate in ratios very close to 1 normal : 1 etched. The values calculated show that the deviation is not significant. Fig: 4 shows a test-cross ear segregating for normal and etched kernels in a 1:1 ratio. These results along with the observations that etched plants on selfing give rise to only etched progeny, unambiguously show that etched trait is inherited as recessive.

We have also analysed the mutant kernel viability, the percentage of germination and the fertility of the plant under field conditions. Further, other parameters like the weight of the kernel, height of the plant were studied. No significant difference was noticeable in any of the above parameters analysed.

Table 3.3.3 gives the weights of mature kernels, both normal and mutant. Single kernel weights, 4-kernel weights and 10-kernel weights were compared and the results do not show any significant deviation of the mutant from the normal. Tables 3.3.4 A-J give the results of selfing of plants grown from classified etched kernels on the basis of "etched scale". Fig: 6 shows the kernels classified as per the

Table 3.3.2 : Segregation of *et et* kernel phenotypes on **testcross ears.**

The cross of *a et* / *a et* + X *a et* / *a et*

S.No	Pedigree	Kernel phenotype	Progeny phenotype: Normal	Progeny phenotype: etched	Total number of kernels	χ^2 1:1
1	86R-0165-4	colourless etched	40	29	69	1.75
2	86R-0166-10	,,	65	53	118	1.22
3	86R-0168-9	,,	86	80	166	0.21
4	86R-0170-5	,,	34	29	63	0.39
5	86R-0179-2	colourless normal	68	59	127	0.63
6	86R-0181-5	,,	48	41	89	0.55
7	86R-0181-6	,,	71	60	131	0.92
8	86R-0184-6	,,	126	108	234	1.38
9	86R-0184-7	,,	83	71	154	0.93

χ^2 values are observed to be non-significant at 5% level of confide

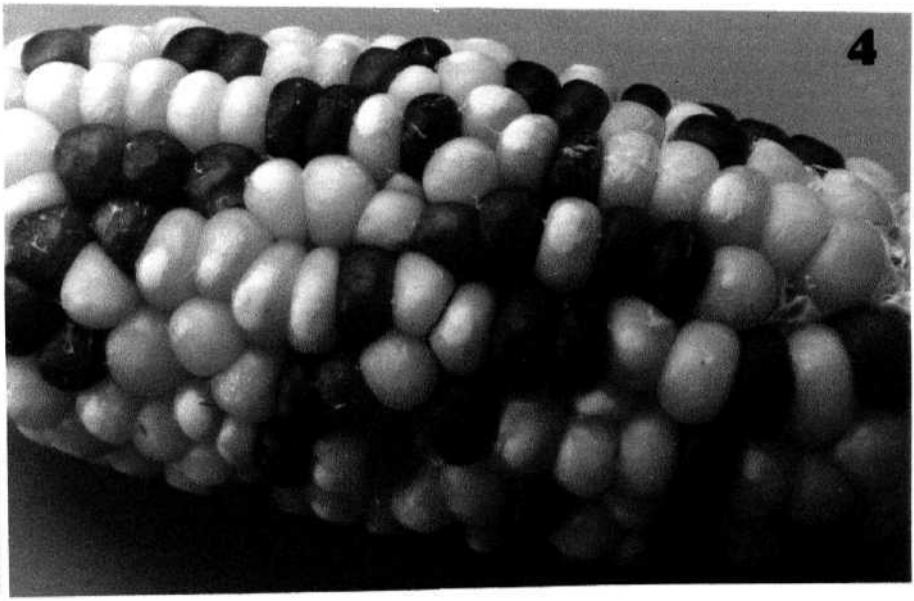
Table 3.3.3: Weights of mutant and normal kernels from segregating selfed ears of heterozygous plants *a et+* / *a et*

S.No	Pedigree	Number of kernels	Mean of Mature kernel weights (gms)	
			Normal	Etched
1	86R-0179	Single kernel weights		
2	86R-0180	,,		
3	86R-0181			
4	87R-0421	,,		
5	87R-0424	,,	0.171±0.014	0.164±0.015
6	86R-0182	4-Kernel weights		
7	87R-0428	,,	0.614±0.017	0.605±0.025
8	86R-0179	10-Kernel weights		
9	86R-0180			
10	86R-0181			
11	87R-0421	,,	1.875±0.273	1.879±0.170
12	87R-0424			
13	87R-0428			

Fig.3 : A selfed ear ($\frac{+}{et}$ $\frac{\otimes}{et}$) showing normal and mutant kernels in a 3 : 1 ratio.

Fig.4 : Test-cross ($\frac{+}{et}$ $\frac{}{et}$ x $\frac{}{et}$ $\frac{}{et}$) ear showing 1 : 1 segregation of normal and *etched* kernels.


3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etching scale. The progeny kernels were classified as per the scale. The results clearly revealed the extensive variation in the phenotypic expression of the et allele. Another important observation is that the progeny of such a selfed plant exhibit a wide array of etched phenotypes. For instance, a plant grown from 1 'a' type (superficial and very little etching on the kernel surface) on selfing gives rise to progeny of almost all types on the scale i.e., 1 'a' to 3 'c'. Thus, it is rare to get a true breeding type etched line. This corraborates the earlier observation that the et allele exhibits variable expressivity and the genetic background does not seem to influence the expression. No preponderance of any one specific type of etched phenotype was noticed over the others. A careful analysis of the segregation data reveals that there is no significant variation in the penetrance of the etched trait.

It can be concluded from our genetic analysis that the et allele is inherited as a Mendelian recessive. The results of reciprocal crosses further demonstrate that the mutation is nuclear in origin. A 100% penetrance and variable expressivity of the et allele has been demonstrated by the results of our genetic analysis. The observations on the inheritance of the virescence phenotype agree with the earlier observations that the two phenotypes are genetically inseparable.

Table:3.3.4 A-J Test for penetrance and expressivity of the*et* allele among the progeny kernels of selfed *et* *et* plants.

Table: 3.3.4 A

Pedigree	Parental phenotype	Total progeny	Kernel phenotype														
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
86R-0166-10	1b etched	61	6	-	15	3	1	-	2	8	-	-	-	-	-	26	-
	3c etched	65	33	8	1	3	-	-	4	-	6	-	1	-	9	-	-

Table:3.3.4 B

Pedigree	Parental phenotype	Total progeny	Kernel phenotype														
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
86R-0167-3	3d etched	119	3	1	9	11	-	-	46	2	16	-	11	18	-	-	2
	1e etched	128	22	-	56	18	-	11	-	2	-	6	-	9	3	-	1

Table:3.3.4.C

Pedigree	Parental phenotype	Total progeny	Kernel phenotype															
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
86R-0167-9	1d etched	98	6	-	11	10	41	-	-	-	8	3	15	3	-	-	1
	2c etched	81	15	-	-	4	1	1	3	-	2	38	-	-	-	16	1
	2d etched	52	21	18	-	3	4	1	1	1	-	-	3	-	-	-	-
	3a etched	60	-	-	20	-	16	3	2	8	5	1	1	1	-	2	1
	3e etched	110	-	7	6	64	9	2	7	-	-	2	6	-	5	1	1

Table:3.3.4 D

Pedigree	Parental phenotype	Total progeny	Kernel phenotype															
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
86R-0170-8	1a etched	61	23	1	1	-	-	11	3	5	8	2	1	1	1	4	-
	3c etched	79	6	8	31	9	1	-	-	-	-	16	-	-	-	-	8
	4b etched	91	1	1	10	-	-	6	1	49	-	12	3	3	5	-	-

Pedigree	Parental phenotype	Total progeny	Kernel phenotype														
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
86R-0168-10	2a etched	96	54	-	6	-	-	11	-	12	-	-	6	1	5	1	-
	3b etched	118	2	5	10	2	20	4	3	8	20	15	-	-	6	12	11
	1c etched	148	59	-	6	3	4	18	4	-	-	5	7	-	15	11	16
	3c etched	114	14	7	3	11	14	-	12	13	17	-	-	-	-	19	4

Table : 3.3.4 F

Pedigree	Parental phenotype	Total progeny	Kernel phenotype														
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
86R-0170-12	3a etched	192	123	1	1	-	-	11	6	-	-	-	-	42	2	6	-
	2b etched	152	-	-	4	-	-	1	1	22	45	-	-	-	42	29	8
	1d etched	154	1	11	10	-	-	-	1	-	-	63	3	-	12	53	-

Pedigree	Parental phenotype	Total progeny	Kernel phenotype														
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
87R-0422-2	1a etched	197	19	-	53	28	-	14	8	37	19	-	4	2	6	7	-
	2a etched	70	1	1	16	9	-	7	8	-	-	18	3	-	4	3	-
	1c etched	216	1	8	46	52	19	-	29	25	-	7	5	20	-	3	1
	2c etched	96	-	8	19	1	3	1	36	16	9	1	-	2	-	-	-
	3d etched	92	14	-	8	32	1	3	11	-	6	1	-	1	12	3	-

Table: 3.3.4 H

Pedigree	Parental phenotype	Total progeny	Kernel phenotype														
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
87R-0426-4	1a etched	109	12	2	28	52	-	3	1	3	-	-	-	1	-	6	1
	2c etched	98	3	6	46	2	8	-	8	12	-	-	7	1	5	-	-
	2d etched	139	2	-	9	46	-	1	5	18	8	-	6	-	30	11	3

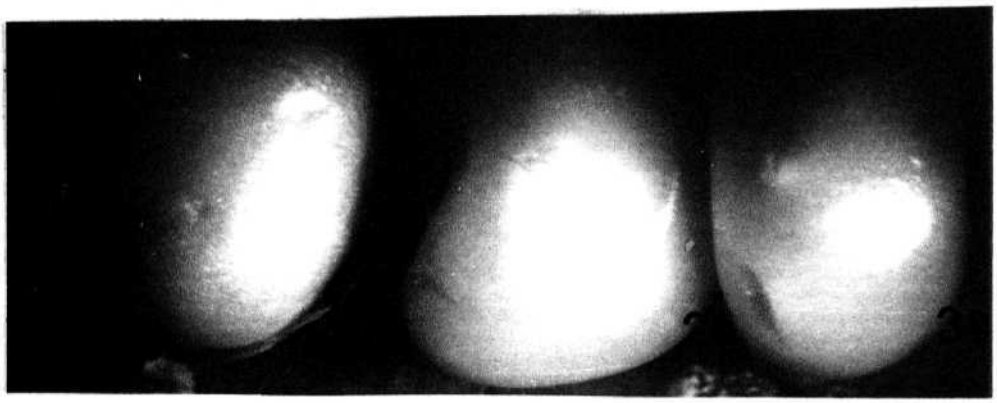
Table: 3.3.4 I

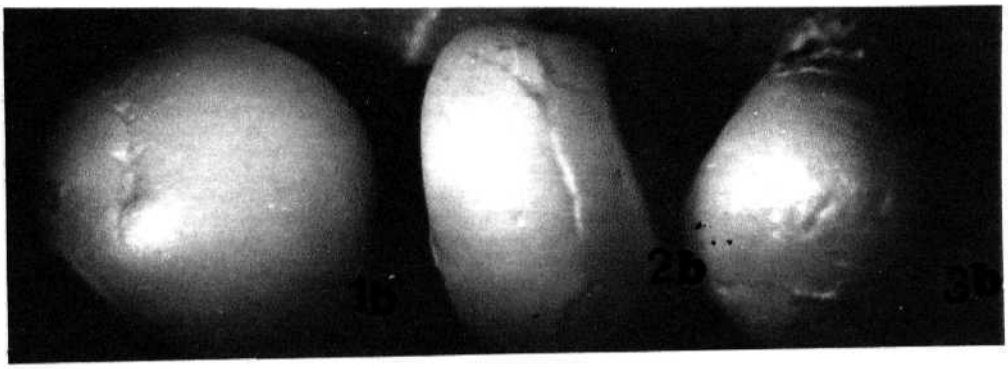
Pedigree	Parental phenotype	Total progeny	Kernel phenotype														
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e
87R-0426-9	2b etched	148	3	5	12	6	20	8	14	18	1	1	7	1	9	40	3
	3b etched	97	8	1	1	4	8	6	56	3	-	-	9	-	-	-	1
	3d etched	108	16	2	9	1	3	8	-	-	15	-	11	-	41	-	2
	3e etched	131	1	-	1	-	11	5	8	-	76	2	9	1	14	3	-

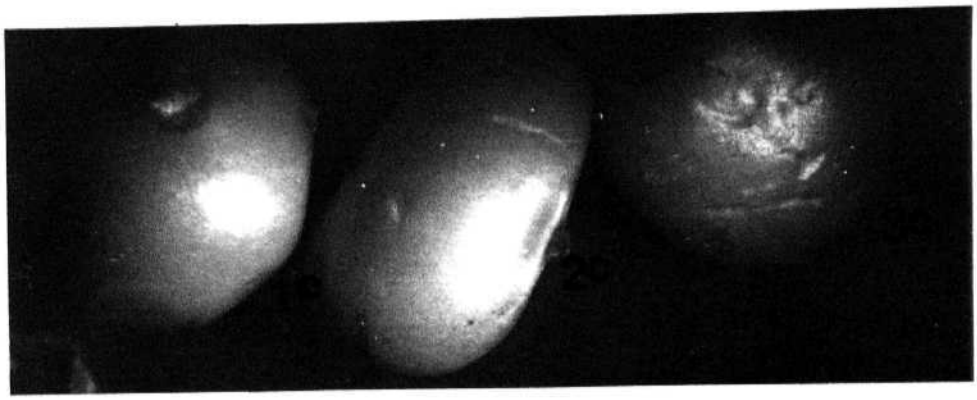
Table:3.3.4 J

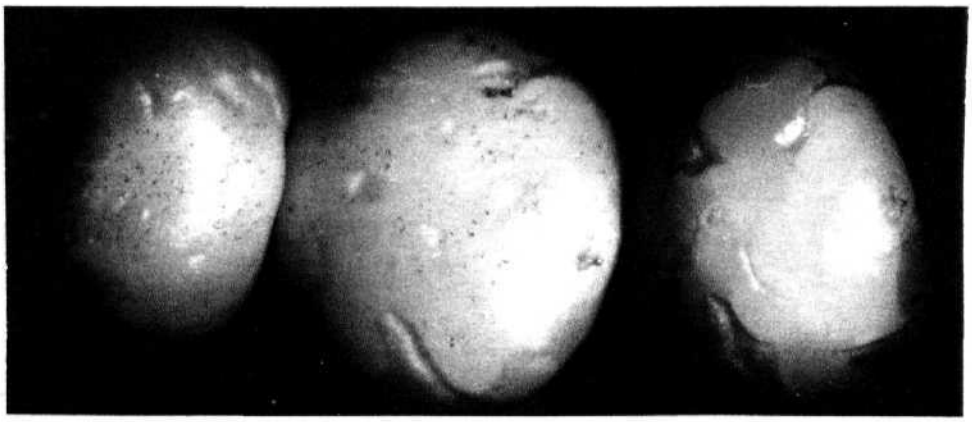
Pedigree	Parental phenotype	Total progeny	Kernel phenotype															
			1a	1b	1c	1d	1e	2a	2b	2c	2d	2e	3a	3b	3c	3d	3e	
87R-0430-2	3b etched	127	12	21	9	13	-	18	6	18	3	5	1	14	7	-	-	
	1c etched	77	23	9	1	-	-	16	8	6	3	7	4	-	-	-	-	
	3c etched	86	2	6	28	8	-	-	6	-	11	1	8	-	16	-	-	
	3d etched	98	-	-	6	-	8	16	1	-	32	-	-	-	-	29	6	
	3e etched	63	1	1	18	-	-	1	-	-	-	11	-	-	23	8	-	
	2e etched	116	3	1	9	39	6	-	15	16	2	12	-	5	-	8	-	
	3e etched	93	15	3	-	1	6	-	1	8	20	33	3	-	-	1	2	

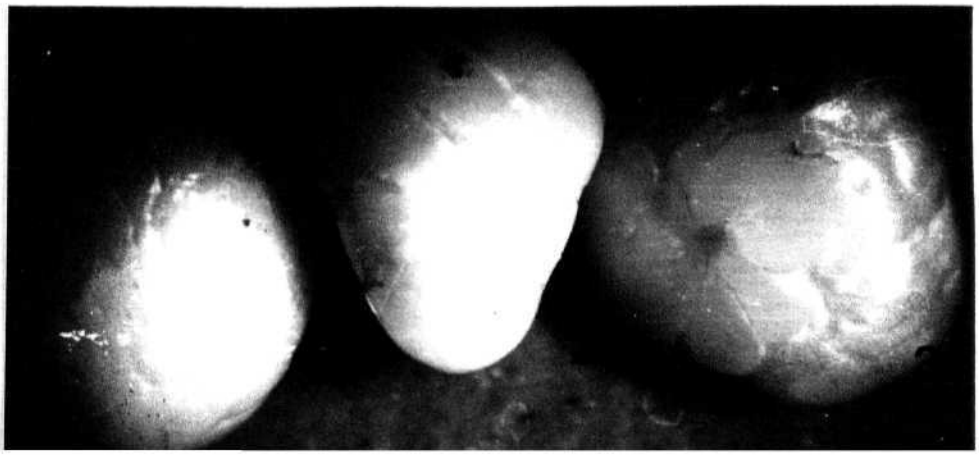
Fig.5 : The "etched scale"











4. SCANNING ELECTRON MICROSCOPIC ANALYSIS OF THE *ETCHED* MUTANT

4.1 INTRODUCTION:

The *etched* mutation as described earlier, affects both kernel and seedling phenotypes. This chapter deals with a detailed analysis of the aberrant kernel phenotype of the *etched* mutant using scanning electron microscope. However, a brief description of kernel ontogeny and starch metabolism is given below as it is relevent to the present work.

The endosperm which is produced as a result of fusion between one male generative nucleus and two polar nuclei, is the storehouse of large quantities of starch. The endosperm nourishes the embryo during early stages of development, and the plantlet after germination (Brink and Cooper, 1947).

In the cereals, the endosperm develops unilaterally, so that in the mature seed i.e., caryopsis it resides completely on one side of the embryo. Endosperm formation begins a few hours after fertilization, with the embryo sac rapidly

becoming filled with endosperm nuclei while the embryo has reached only the 10 to 24 cell stage. During its development, the endosperm changes tremendously in size (Randolph, 1936). The endosperm develops an outer aleurone layer which develops protein bodies called aleurone grains. Cells of aleurone layer remain alive unlike other endosperm cells of cereals which are dead cells packed with starch and to a lesser extent protein (Jacobsen et al., 1979).

Starch begins to accumulate in maize two weeks after pollination. Except for the pericarp, the young caryopsis lacks starch one week after pollination. About two weeks after pollination, starch is found in the distal marginal cells of the endosperm (Bernstein, 1943;.

Starch is laid down in discrete sub-cellular bodies, the starch grains. They have a characteristic appearance for each species. Starch grain shape is determined to a large extent by amylose content. Higher amounts of amylose are observed to lead to round grains. In maize, the grain shape is predominantly angular. A normal kernel of maize consists of about 74% starch, 4% lipid and *ii%* protein. Endosperm of

maize consists of 88% of the kernel starch whereas the embryo consists of only 9% of total starch (Earle et al., 1956). The starch grains display a great range of size and shape in cereals.

The differentiation processes such as starch synthesis (Shannon, 1974;, protein body formation (Duvick, 1961) and storage of ^{14}C photosynthetic products (Shannon, 1974) begin near the apex of the endosperm and proceed downward. Storage of starch and protein is maximised when these processes are coordinated such that the endosperm cells are successively filled to capacity from top to bottom.

A developing kernel of maize is composed of cells of varying physiological ages and there appears to be a major gradient in cell development from cells located in the basal endosperm region to those in the central crown region and a minor gradient from the periphery towards the centre of the endosperm. As a consequence, at any given time during the development of the kernel, there is a variability between cells as to their starch content. The initiation of starch accumulation proceeds basally and centripetally in the

endosperm cells next to the embryo. Therefore, it was proposed that starch biosynthesis is of progressive type and occurs in waves during kernel development (Lampe, 1931; Boyer et al, 1977).

Several mutant genes are known in maize that affect starch synthesis and thus endosperm development. A very well known case is that of the waxy gene causing very little amyiose production. The effect of the waxy gene is shown to be extended to the microspore (Brink and MacGillivray, 1924 ; Demerec, 1924). it was later shown that the mutant gene had the same effect on the starch of the female gametophyte (Brink, 1925). The mutant gene amylose-extender, which increases the proportion of amylose of the endosperm has the same effect in the pollen (Banks et al, 1971). The sugary mutant leads to a change in the carbohydrate storage products of the endosperm. The principal storage product in the mutant is the water-soluble polysaccharide, phytoglycogen (Sumner and Somers, 1944).

The defective kernel mutants of maize wherein both the endosperm and embryo are abnormal, are retarded in the

embryonic development (Mangelsdorf, 1926). The germless mutants wherein only the embryo is defective provide excellent material for the genetic analysis of embryogenesis (Neuffer and Sheridan, 1980). For the study of embryo-endosperm interaction, defective kernel mutants are ideal. Analysis of the defective kernel mutants revealed that the developmental fates of the two tissues, endosperm and embryo appear to be largely independant (Sheridan and Neuffer, 1981).

The mutants in which the endosperm is defective become distinguishable at an early point in kernel development. Since the etched kernels become distinguishable at an early stage of kernel development, the mutant kernels and endosperm can be readily studied over a large period of their developmental cycle.

In the present study, we have looked at the structural aberrations on the endosperm of et et kernels using Scanning Electron Microscopy. We also compared the SEM data from other organs like embryo and pollen of both the mutant and wild type. This was done to test whether et mutant is tissue specific or general. Further, this study is aimed at finding

out whether the expression of *et* allele is transient or total constitutive during the endosperm development and maturation.

4.2 MATERIALS AND METHODS:

4.2.1 *Scanning electron microscopy (SEM) of endosperm*: Mature kernels of normal (et^+ et^+) and mutant *et et* genotypes were soaked for an hour in double distilled water. After removing pericarp, the endosperms were fixed on specimen stubs with electroconductive silver paint and coated with gold for 1u min. in JEOL FC-1100 ion sputter. "I he specimens were observed under a JEOL-35 Scanning Electron Microscope at a voltage of 15 KV.

4.2.2 *Isolation of starch grains and preparation for Scanning* fc lectron Microscopy: Normal (*et* $^+$ *et* $^+$) and mutant (*et et*) kernels were soaked in water for about 6-8 hours. The kernels were then ground in 75% ethanol . The suspension was heated for 30 min. in a boiling water bath and centrifuged at 10,000 g for 15 min. The resulting starch was suspended in a small volume of 7% glutaral dehyde in 0.02 M phosphate buffer (pH 7.2) for about one hour. It was then centrifuged for b min at 5,000 g. The pellet was then washed in distilled

water 6-7 times. The precipitate was suspended in ethanol and the suspension was taken and fixed on specimen stubs with electroconductive silver paint and coated with gold for 1u min. in a JEOL FC-1100 ion sputter. The specimens were observed under a JEOL-35 Scanning Electron Microscope at a voltage of 15 KV.

4.3 RESULTS AND DISCUSSION:

The embryo and endosperm of the kernel, and pollen grains of normal and mutant genotypes were studied by scanning electron microscope. Starch grains isolated from normal and mutant kernels were also examined.

The surface scans of the embryo of normal and mutant genotypes are shown in Fig.6a and Fig.6b. As can be observed from the figures, the embryo of $\underline{et}^{+}\ \underline{et}^{+}$ and $\underline{et}\ \underline{et}$ kernels do not show any detectable differences indicating that the *et* mutation does not affect the embryo phenotypically.

Pollen grains of $\underline{et}^{+}\ \underline{et}^{+}$ and $\underline{et}\ \underline{et}$ genotypes were also examined and are shown in Fig.7a and Fig.7b. As is revealed

Fig.6 : Surface scan of the embryo of
(a) normal kernel (b) mutant kernel.

Fig.7 : Surface scan of a pollen grain of
(a) normal genotype (b) mutant genotype.

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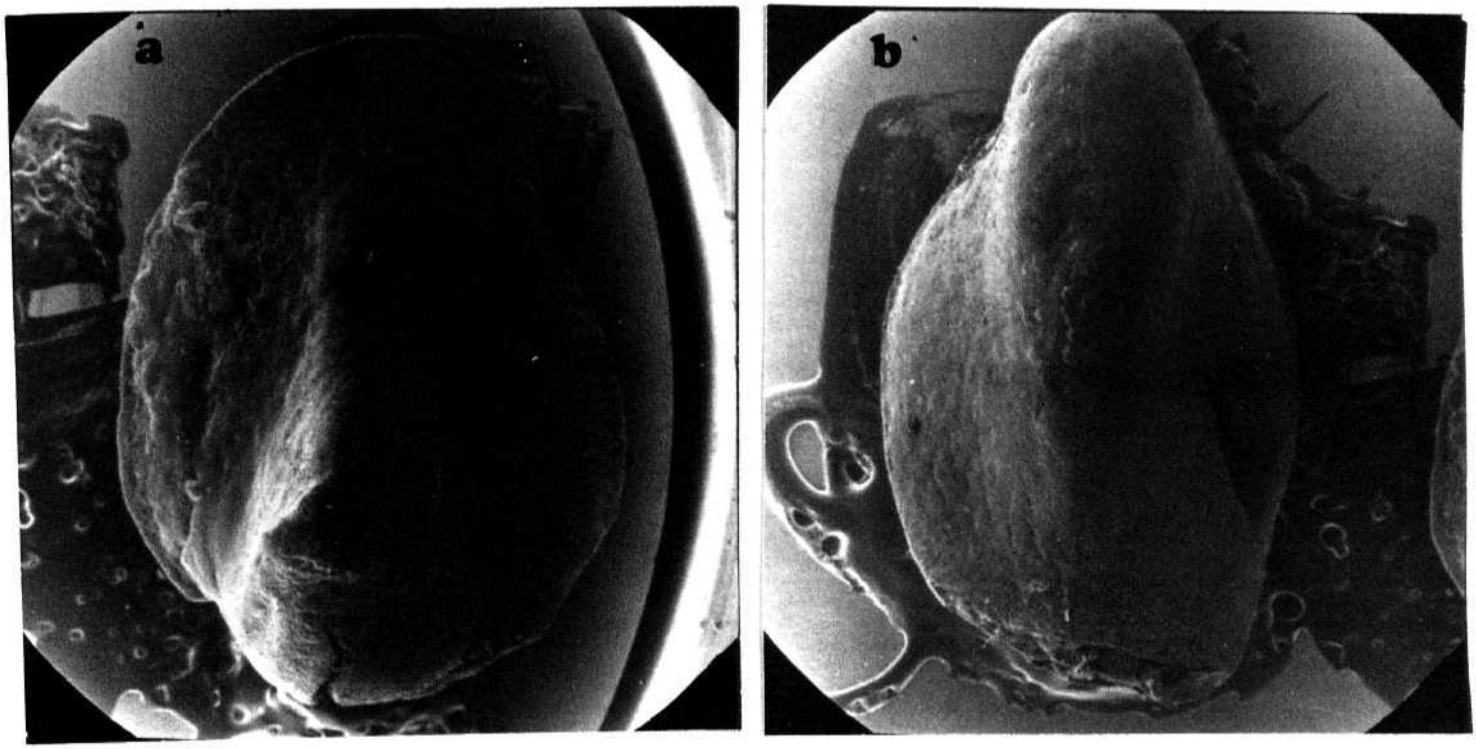

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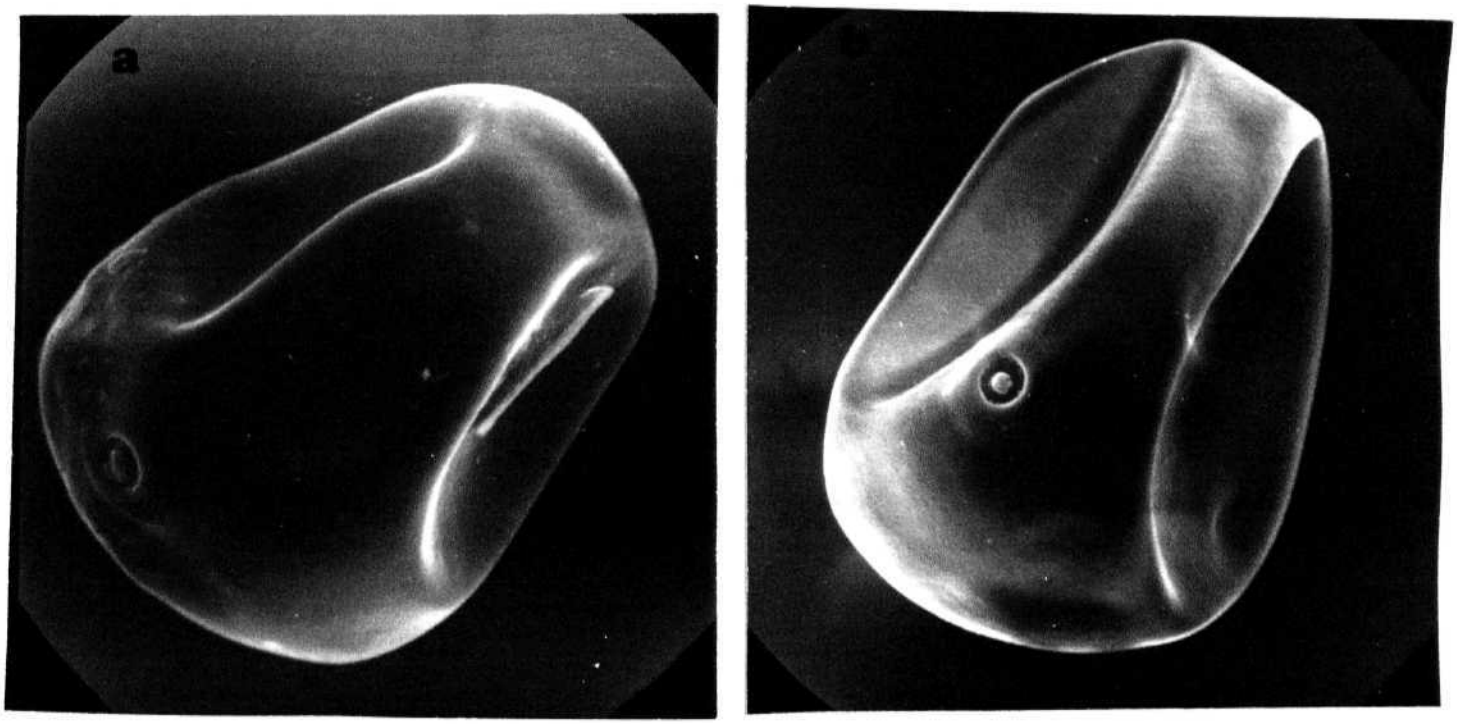

by the surface scans, the pollen grains appear similar in both genotypes.

On the other hand, the surface scans of the endosperm of *et et* kernels clearly reveal cracks or scars on the surface. Fig.8a and Fig.8b show the normal and mutant endosperm surface as revealed by scanning electron microscope at a magnification of 300x. In order to gain an insight into the endosperm lesions caused by *et* allele, endosperm transverse sections were taken at different levels and observed under scanning electron microscope.

The phenotypic variations noticed in the extent and depth of etching of kernels is substantiated by observations under SEM. Fig.9a shows the SEM scan of the endosperm of *et et* kernels. The depressions or cracks noticed on the surface are also noticeable in the sections of the endopserm at various levels as revealed by Fig.9a. Fig. 9b shows a **scan of** the magnified portion of the crack in the endosperm shown in Fig 9a. Starchless cells are clearly noticed along the area of the crack. Fig.10a and Fig.10b show the SEM photomicrographs of *et*$^{+}$ *et*$^{+}$ and ~~*et*~~ *et* kernels at a

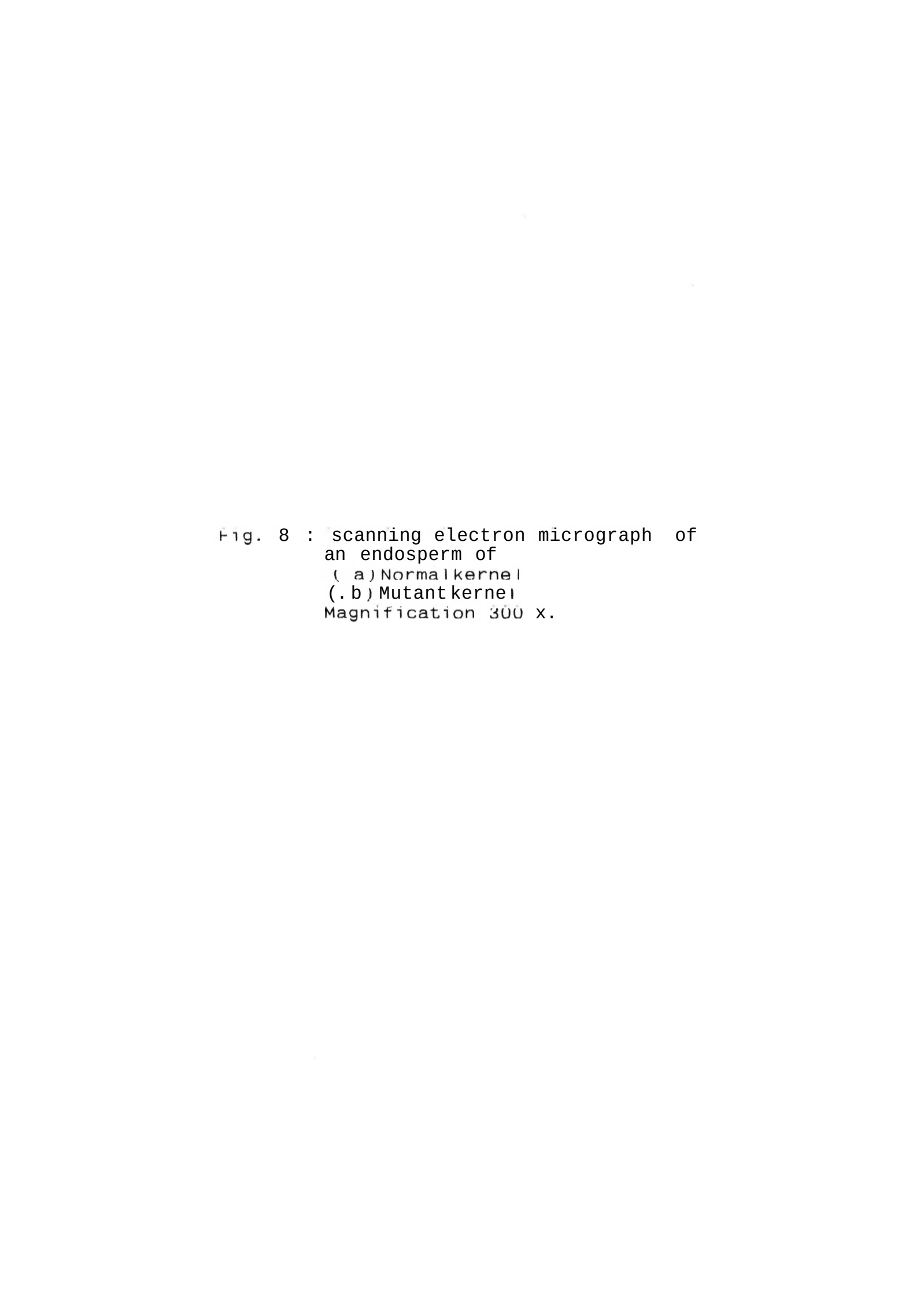

Fig. 8 : scanning electron micrograph of an endosperm of
(a) Normal kernel
(b) Mutant kernel
Magnification 300 x.

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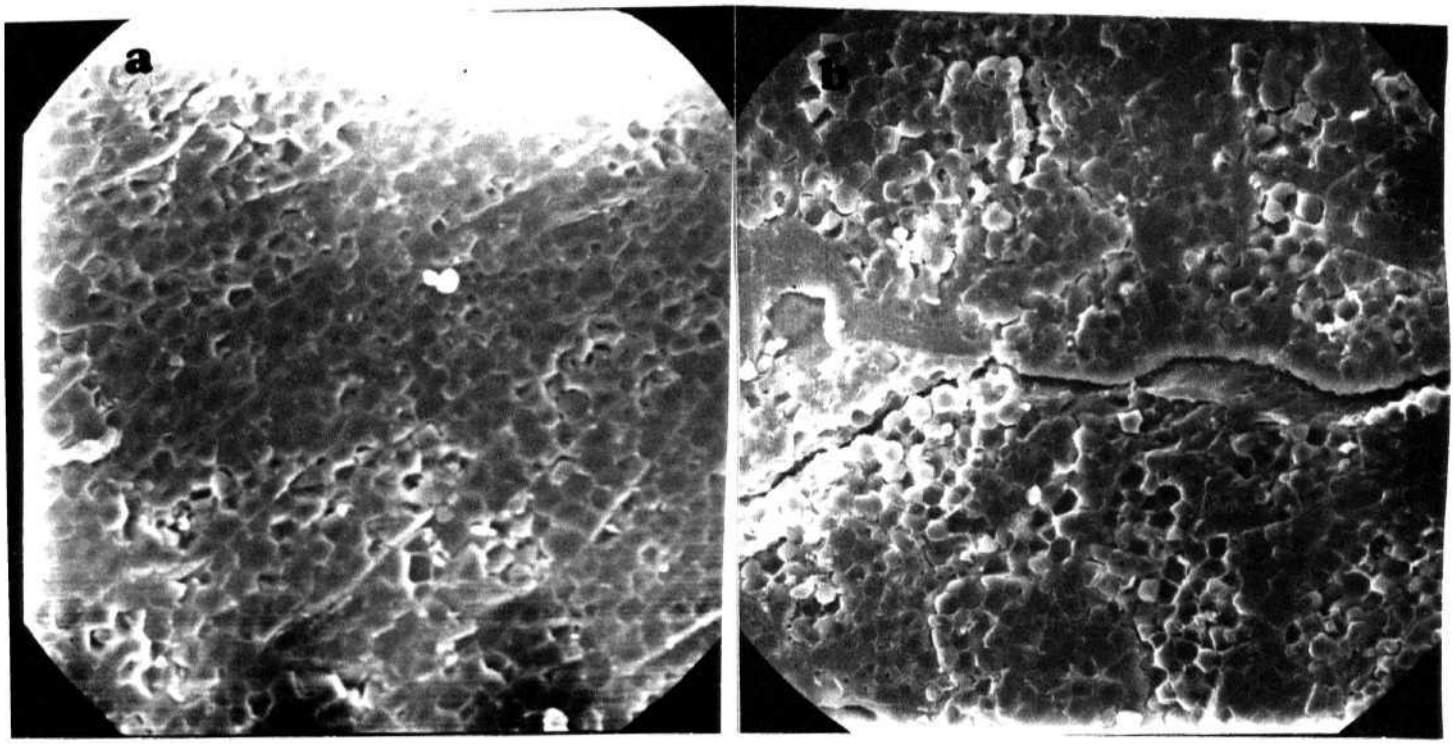


magnification of 1000 X. Fig.10b shows areas of depressions in which no cells are observed. On the other hand, no depressions are noticed in the endosperm of normal kernels at any magnification.

Fig.11a and Fig.11b show the starch grains isolated from mature $\underline{et}^{+}$ $\underline{et}^{+}$ and $\underline{et}$ $\underline{et}$ kernels at magnification of 1000 X. The starch grains of normal and mutant kernels at a magnification of 6000 X, are shown in Fig.12a and Fig.12b. As is evident from the figures, the starch grains in the mutant kernels do not differ from normal kernels in size or in shape.

The results of the scanning electron microscopic analysis further confirm the variable expressivity of the *et* allele. The variation in the expressivity of the et allele was reported earlier (Rhodes, 1952). The cracks or discontinuities noticed in the endosperm were suggested to be due to sectors of starch-less cells among normal cells .

The apparent variation observed in the etching of the endosperm, may be due to the time at which the et allele expression is switched on or off during the development of

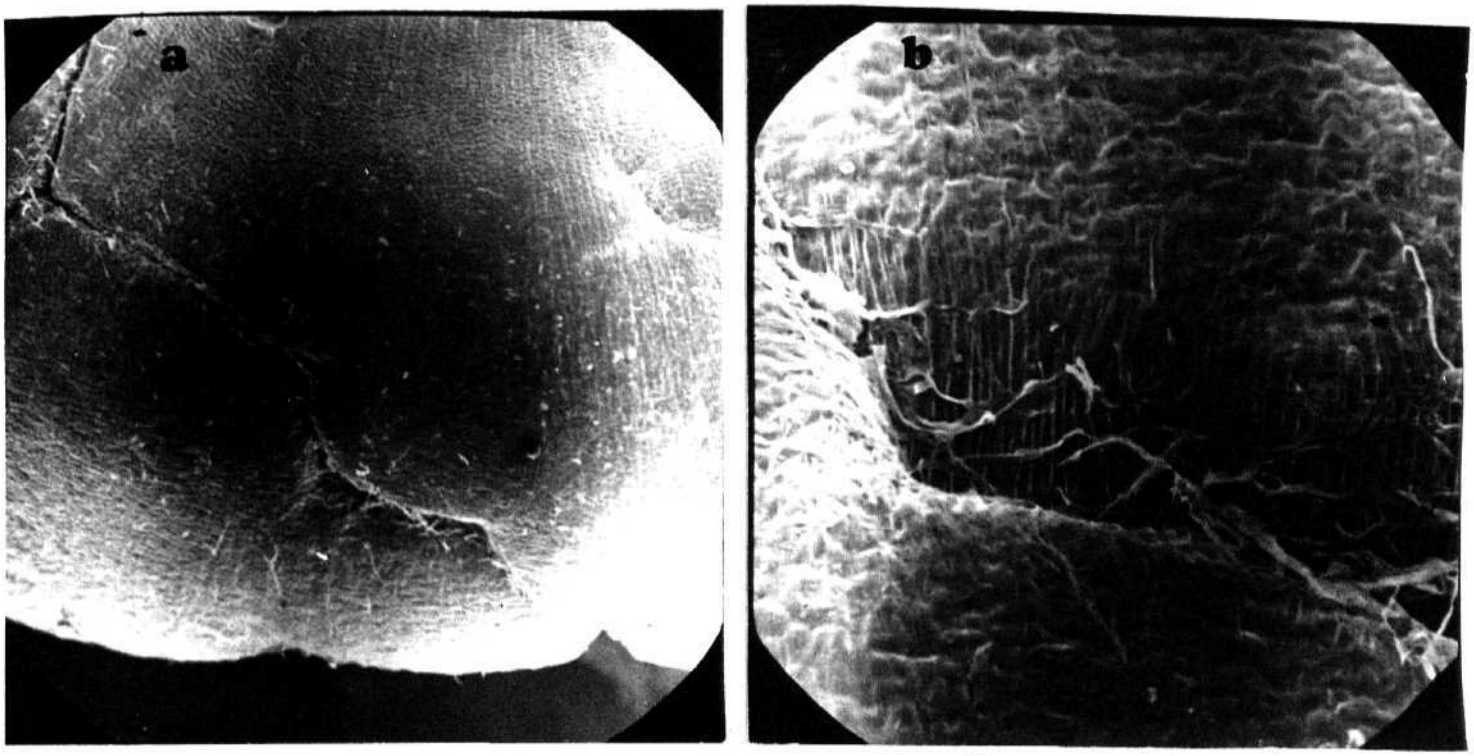
Fig.9 : (a) Scanning electron micrograph of an *etched* endosperm showing a deep scar.

(b) Scanning electron micrograph showing starch-less cells in the deeply-cracked area of an etched endosperm.

Fig.10 : (a) Scanning electron micrograph of the endosperm of *et* *et* kernel at a magnification of 1000x.

(b) Scanning electron micrograph (1000 x) of the endosperm of *et* *et* kernel showing a depression area with no cells.

9



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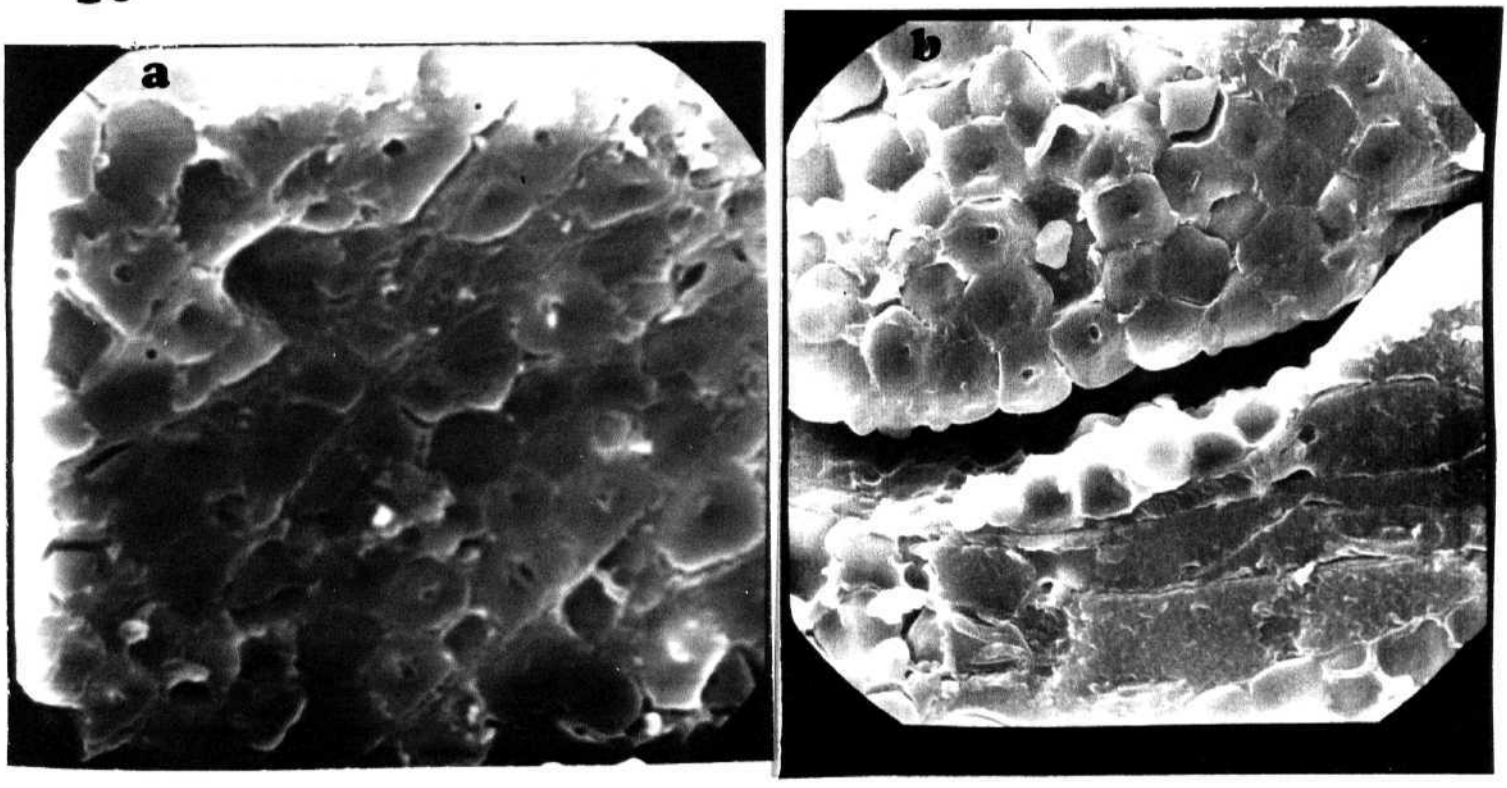

Fig. 11 : Starch grains at a magnification of 1000 x.
(a) Normal (b) Mutant

Fig.12 : Starch grains at a magnificatin of 6000 x
(a) Normal (b) Mutant

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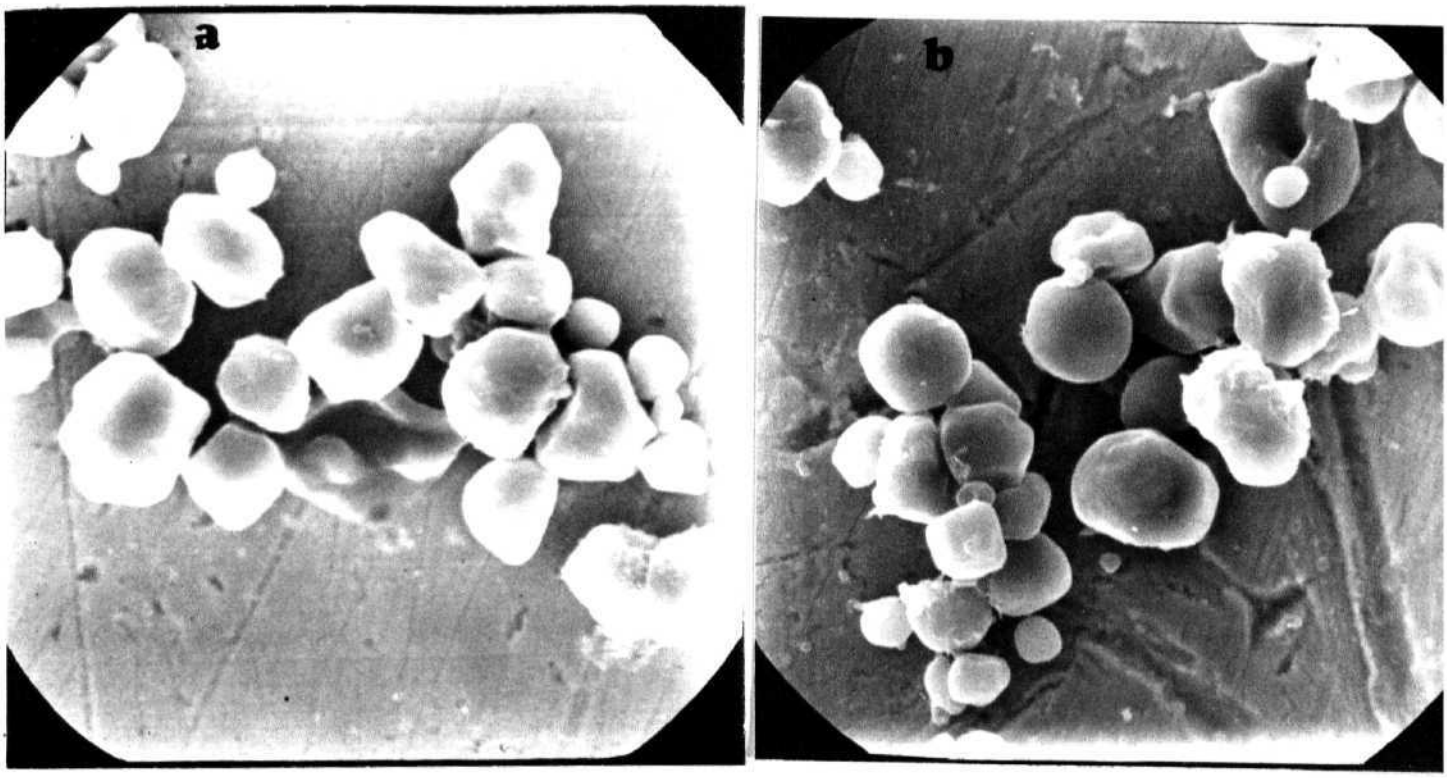

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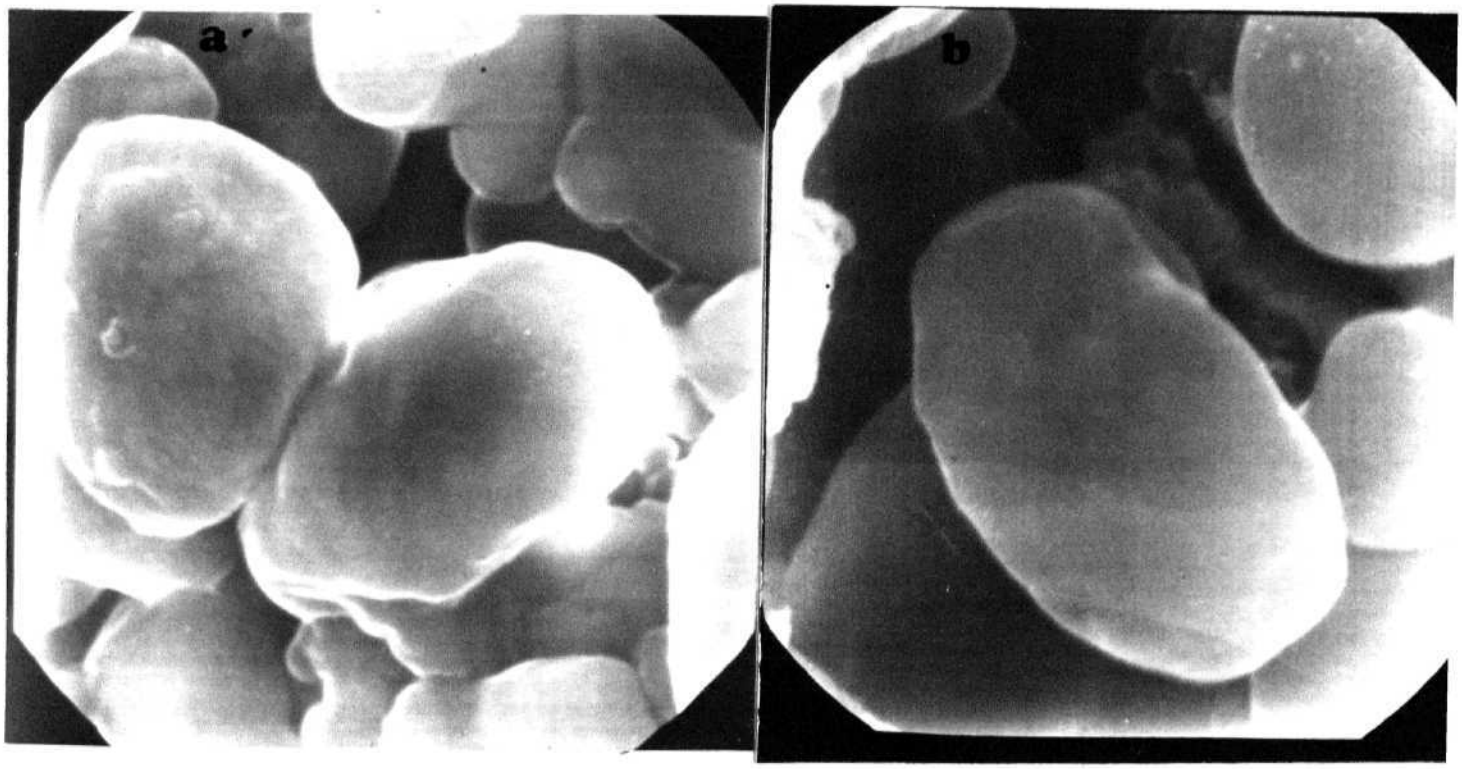


the kernel. The et et kernels can be easily distinguished phenotypically from normal kernels, quite early during kernel development. It can be argued that the cells in which the et gene is active can neither synthesize nor accumulate starch granules unlike the normal et+ et+ cells. Accordingly cells *or* cell-lineages of such progenitor et et cell will have no starch at all. Such areas in the endosperm constitute discontinuities. The streaks of such broken cells observed by SEM in et et kernels support this cell-lineage pattern of et gene expression.

The et gene expression is transient during kernel development, because none of the gaps are extended throughout the kernel. Thus, if starch synthesis continues in cells in which et gene activity ceases, such cells and their lineages will then be phenotypically normal. The result is a dispersion of etched areas on a normal background area of the kernel. However it is not clear as to what determines the extent of transient expression of the et allele in endosperm cells. It is clear that et allele has no apparent effect on the size and shape of starch grains. The results of Scanning electron microscopic analysis confirm the conclusion that the et effect is highly tissue specific in maize.

5. EFFECT OF *et* MUTATION ON STARCH METABOLISM IN KERNELS AND SEEDLINGS

5.1 INTRODUCTION

The seed occupies a critical position in the life-cycle of higher plants. It sustains the young plant in the early stages of growth before it becomes an independent and autotrophic organism able to use light energy for its growth and development. Seeds contain carbohydrates, fats and oils, and proteins as a source of food reserves to support germination and early seedling growth.

Carbohydrates, mainly starch, predominate in cereal endosperms. starch, the main organic reserve or cereal grains consists of two polymers of D-glucose, one linear and the other branched. The linear polymer, amylose, is formed from glucose units joined by α-1,4 glucosidic linkages. The branched polymer amylopectin consists of many amylose chains linked by α-1,6 bonds. Normal maize contains about 25% amylose and 75% amylopectin.

The starch reserves in maize are reported to be present in different proportions in different tissues (Earle *et al.*,

1956). Further, there are protein-rich and starch-rich regions in the endosperm called the horny and floury endosperm respectively (Weber, 1980).

Sucrose, which is the sugar translocated from the mother plant to the kernel, is the substrate for starch formation. Sucrose is converted to fructose and UDP glucose by sucrose synthetase. Both fructose and UUP glucose are then converted to glucose-1-phosphate, which is then converted to AUP glucose by AUP glucose-pyro-phosphorylase. The AUP glucose donates its glucose to a glucose primer, thus increasing the chain length by one unit. The enzyme involved is AUP-glucose-starch synthetase. The Q-enzyme introduces α-1,6 branch points.

The mobilization of stored reserves in the storage organs is a post-germinative event. The amylose and amylopectin in the native starch grains are first hydrolyzed by α-amylase which breaks the α-1,4 glycosidic linkages between the glucose residues randomly throughout the chain. The released oligosaccharides are further hydrolyzed by β-amylase until glucose and maltose are produced.

$$\text{Amylose} \xrightarrow{\alpha\text{-amylase}} \text{Glucose + Maltose}$$

α-amylase cannot hydrolyze the α-1,6 branch-points of amylopectin and hence highly branched cores ot glucose units, called limit dextrins are produced.

Amylopectin —α-amylase——> Glucose + Maltose + limit dextrin.

β-amylase cannot hydrolyze native starch grains. It can cleave successive maltose units from the non-reducing end of large oligomers, released by prior α-amylolytic attack.

Amylose —β-amylase——> Maltose

Amylopectin —β-amylase——> Maltose + limit dextrin.

Both α and β amylases are present in higher plants. Germinating cereal grains are known to produce and secrete these starch-hydrolysing enzymes. The sole physiological function ot these enzymes is reported to be the digestion ot starch reserves ot the storage tissues. The first genetic analysis ot amylase isozymes in plants was reported by Scandalios (1966), using certain inbred lines ot Zea mays. Studies in maize and barley (Frydenberg and Nielsen, 1965) strongly suggest a simple Mendelian control ot the amylases in higher plants. The two major amylases are each controlled

by an independent gene, each having a pair or co-dominant alleles (Chao and Scandalios, 1969; Scandalios et al, 1974).

Studies on maize kernels during germination revealed that both amylases are abundant in the endosperm but their expression differed considerably during different stages. Based on genetic analysis, a separate origin for each of the amylases has been reported (Bernstein, 1943). The α-amylase is reported to be originating from the embryo i.e., the scutellum whereas the β-amylase from the endosperm. However, recent studies gave evidence suggesting strongly that α-amylase is synthesized independantly in both the scutellum and endosperm (Chao and Scandalios, 1969; 1971). Chao and Scandalios (1972) negated the earlier finding that β-amylase originates in the endosperm.

The studies on developing maize endosperms revealed very low levels of β-amylase and total absence of α-amylase activity. very little amylolytic activity is observed in the endosperm of the dormant maize kernels. During germination, however, the amylase activities increase in the endosperm.

in this study, attempts are made to investigate the effect of et mutation on starch metabolism during germination

and early seedling growth. This is because the phenomenon of virescence is a post-germinative event and lasts till early stages of seedling growth. therefore, an analysis or the starch accumulation and degradation processes in the mutant could give valuable information to an understanding of the phenomenon ot virescence.

5.2 MATERIALS AND METHODS

5.2.1 Germination of Kernels: The mature kernels ot normal ($\overline{et}^{+}\ \overline{et}^{+}$) and mutant ($\overline{et}\ \overline{et}$) genotypes, were soaked in tap-water for 8-10 hrs. The kernels were surface-sterilized in sodium hypochlorite tor about 10 min. and washed twice with double-distilled water. The kernels were then kept tor germination in vermiculite in plastic trays.

The temperature was maintained at 25 ± 2 °C. Light was provided by fluorescent lamps with an intensity ot [3(8.362 * 10^{-9}) mole m^{-2} sec^{-1}. The illuminance was measured with a Li-Cor Model photometer with a radiometric sensor

5.2.2. Determination of Starch content

a. Kernels: Developing kernels (20, 30, 40 DAP.) mature kernels (dry kernels) and germinating kernels (3,4 and 8 DAG)

were used for starch estimations. Known quantities of kernel samples were ground in 75% ethanol (3 ml/gm) using a mortar and pestle. The resulting suspension was heated for 30 min. in a boiling water bath and centrifuged at 10,000 g for 15 min. The resulting starch precipitate was suspended in 0.2 N potassium hydroxide and boiled for 30 min. and subsequently neutralised to pH 6.5 with glacial acetic acid. The suspension was incubated with amyloglucosidase (E.C. 3.2.1.3, Sigma) at 37° C for 18 hrs. After centrifugation of the suspension, the supernatant was used for glucose estimation.

b) Leaves: Leaves from 6,8 and 10 day old and normal mutant seedlings were harvested and their starch content was estimated as per the procedure of Mac Rae (1971). The leaves were ground in boiling 80% ethanol and centrifuged at 2000 g for 10 min. Glucose, sucrose, certain oligosaccharides and short chain glucose polymers were removed from the leaf tissue with hot alcohol. This alcohol extraction procedure was repeated twice. The pellet was suspended in distilled water and incubated at 100° C in a boiling water bath for 30 min. This gelatinised starch suspension was treated with amyloglucosidase in the presence of sodium acetate-acetic acid buffer, pH 4.6, and incubated at 37° C for 36 hrs.

Amyloglucosidase hydrolyses starch to glucose, and the hydrolysate was deproteinised (1 ml aliquot was taken and it was de-proteinised by adding b ml water, 2 ml of 0.3 N sodium hydroxide and 2 ml of fa% zinc sulphate solution). After de-proteinisation, the released glucose was estimated by a modified photometric adaptation of the Somogyi method called the Nelson's method (Nelson, 1944,). The details of the procedure and reagents required are given below.

copper Reagent A:

25 gms of sodium carbonate (anhydrous), 25 gms of Rochelle salt (sodium, potassium tartarate), 20 gms of sodium bicarbonate and 200 gms of anhydrous sodium sulphate were dissolved in about 800 ml of water and diluted to 1 litre. This solution was filtered and stored above 20° c.

Copper Reagent *ti:*

15% Copper sulphate containing one or two drops of conc. sulphuric acid per 100 ml.

Arsenomolybdate colour reagent:

25 gms. of ammonium molybdate was dissolved in 450 ml of

distilled water. 21 ml of conc. sulphuric acid was added. 3 gms of sodiumbi-arsenate dissolved in 25 ml of water was mixed and placed in an incubator at 37° C for 24 to 4a hrs. This reagent was stored in a glass-stoppered brown bottle.

To 1 ml of a deproteinised aliquot, 25 parts of copper reagent A and 1 part of copper reagent B were added. The solutions were mixed and heated for 20 min. in a boiling water bath. The tubes were then cooled and 1 ml of arsenomolybdate reagent was added. The colour develops very rapidly and will be completed by the time thorough mixing and evolution of CO_2 was completed. The solution was then diluted to 25 ml and read in a spectrophotometer at 520 nm. The optical density of the colour developed was proportional to the glucose present.

Glucose factor for starch:

Hydrolysis of pure starches by the purified amyloglucosidase preparation yielded 1.09b ± 0.005 gms glucose per gram starch, thus giving the glucose to starch conversion factor of 0.91 (Pucher et al, 1948).

The starch content of the tissue was calculated as

b(

$$\frac{\text{Glucose conc. determined (gms/ml)} \times \text{Volume of the hydrolysate (ml)} \times 10 \times 0.91}{100 \times \text{Wt. of starting tissue (mg)}} \times 100\%$$

5.2.3 Estimation of amylases in germinating Kernels and leaves

a) Total amylases: Normal ($et^+ et^+$) and etched ($et\ et$) kernels 3, 4 and fa days after germination and leavaes of b, 8 and 1U day old seedlings were used for amylase estimations.

A Known quantity of kernel or leaf material (u.b - 1 gm) was homogenized in z ml of chilled extraction butter (20 m. moles sodium citrate) pH 6.1. The homogenate was diluted to 7 ml with extraction butter and then spun at 3U,uuu g for 3D min. All steps were performed at u-4 °C. The total amylase activity was assayed in a reaction mixture (4 ml) containing 240 μ moles of sodium-citrate butter, pH 6.1, 8 mg soluble starch, 6 μ moles sodium flouride and 50 μ l of enzyme extract. Aliquots of buu μl were withdrawn at u, 30 and bu min and the reaction was stopped by adding 500 μl of 3, *b* dinitrosalicylic acid reagent (Bernfield, 1955). [3,5 dinitro-salicyclic acid reagent: To 20 ml of 2 N sodium hydroxide, bu ml of distilled water and 1 g of dinitrosalicylic acid was added. it was dissolved on a

magnetic stirrer cum hot-plate without boiling. 30 gms of sodium potassium tertarate was added and the volume was made upto 100 ml with distilled water. The reagent was stored in a brown bottle protected from light). The solution was then boiled for 5 min in a boiling water bath. After cooling, the mixture was diluted with distilled water to a final volume of 6 ml. The increase in the amount of reducing sugars was determined colorimetrically at 540 nm using maltose as the standard.

b) Estimation of α-amylase: α-amylase was assayed by the above mentioned reaction conditions except β- limit dextrin was the substrate instead of soluble starch.

c) Estimation of β-amylase: The procedure was essentially same as above except that the enzyme extract was preincubated with 5 mM EDTA for 48 hrs.

Micromoles of maltose liberated/second was calculated. The enzyme activity was expressed as n kat/mg. seed and n kat/gm leaf. Protein estimations were according to the procedure of Lowry (Lowry et al., 1951).

5.2.4 Electrophoretic analysis of isozymes of amylases

in germinating kernels and leaves:

Electrophoresis of the total amylase extract was performed in 7% polyacrylamide gel, according to the procedure or Davis at 4°C (Davis, 1964).

Solutions for electrophoresis:

Sol .A : 1 N HCl 48 ml

Tris 36.6 gms pH 8.9

TEMED 0.23 ml

Volume made upto 100 ml with distilled water.

Sol .B : 1 N HCl 48 ml

Tris 5.98 gms pH 6.7

TEMED 0.46 ml

Volume made upto 100 ml with distilled water.

Sol. C : Acrylamide 28.0 gms

Bis-acrylamide 0.73 gms

Volume made upto 100 ml with distilled water.

Sol. D : Acrylamide 10.0 gms

Bis-acrylamide 2.5 gms

Volume made upto 100 ml with distilled water.

Sol. E : Riboflavin 4 mg

Volume made upto 100 ml with distilled water.

Sol. F : sucrose 40 gms

Volume made upto 100 ml with distilled water.

Sol. G : (To be prepared tresh).

Ammonium per sulphate - 0.14 gms

Volume made upto 100 ml with distil led water.

Preparation of gel for electrophoresis or enzyme extract:

Running gel : 1 part B solution
(Large pore solution) 2 parts D solution
1 part E solution
4 parts F solution

Stacking gel : 1 part A solution
(Small pore solution) 2 parts C solution
1 part distil led water
4 parts G solution

Preparation of reservoir butter: (10 x)

Iris 6.0 gms

Glycine 28.8 gms

Volume made upto 1 litre with distilled water.

pH or the reservoir butter = 8.3.

Electrophoresis was carried out for about 5-6 hrs at 4°C. A constant current or 10 mA/slab was used.

5.2.5 Staining of amylases in polyacrylamide gels:

The amylases in native gels were visualized using a negative staining method (Work and Work, 1972). The gel was incubated in the extraction buffer (Sodium citrate buffer, pH 6.1) at 30°C for 30 min and then transferred to a tray containing amylose solution (4 mg amylose in 1 ml extraction buffer, pH 6.1) and incubated for 1 hr at room temperature. The gel was then thoroughly washed in the extraction buffer, to remove the adhering starch on its surface. The gel was then stained with iodine solution (24 mM potassium iodide and 1 mM iodine). After a few minutes clear white transparent bands against a blue background were visualized.

5.3 RESULTS AND DISCUSSION:

Starch content was estimated in immature developing kernels, mature kernels and in germinating kernels and seedlings of both $\underline{et}^{+}\ \underline{et}^{+}$ and $\underline{et}\ \underline{et}$ genotypes. The levels of amylolytic enzymes were also estimated in germinating kernels

and seedlings of normal and etched stocks.

The starch levels of immature developing kernels (20, 30, and 40 days after pollination) of normal and mutant genotypes are given in Table 5.3.1. It is clear that the et et kernels accumulate greatly reduced levels of starch (more than 50%) at 20 DAP stage as compared to normal. At the 30 DAP stage, the *et* et kernels show about 50% decrease in starch content as compared to that of normal. 40 DAP kernels also show a similar difference in starch content.

The starch content of mature and germinating kernels (0, 3, 4 and 5 days after germination (DAG)) of $\frac{+}{et}$ $\frac{+}{et}$ and et et genotypes are given in Table 5.3.2. The mature et et kernels accumulate significantly lower levels of starch than that of normal. This difference in starch content is also seen in germinating kernels of 3 DAG stage. On the contrary, the $\frac{+}{et}$ $\frac{+}{et}$ and et et kernels of 4 DAG and 5 DAG stages, do not show significant differences in the starch content. Further, it was noticed that the mutant kernels consistently show the same amount of starch during germination (3 to 5 DAG), whereas normal kernels show a decrease in the starch levels during the same period.

Table - 5.3.1 : Starch content of immature developing kernels

S.No	Days after pollination	Starch content (% fresh weight)	
		Genotype	
		$\frac{+}{et}$ $\frac{+}{et}$	$\frac{et}{et}$
1	20	40.8 ± 3.68	18.6 ± 2.61
2	30	58.3 ± 1.23	30.8 ± 1.85
3	40	66.3 ± 1.06	39.6 ± 2.01

fcach value is an average of atleast 6 independent experiments.

Table - 5.3.2 : Starch content of germinating kernels

S.No.	Days after germination	Starch content (% fresh weight)	
		Genotype	
		$\underline{et}^{+}\ \underline{et}^{+}$	$\underline{et}\ \underline{et}$
1	1	68.31 ± 2.60	42.84 ± 1.80
2	3	56.39 ± 1.13	40.22 ± 1.18
3	4	43.11 ± 1.19	39.86 ± 1.12
4	5	42.30 ± 1.30	41.86 ± 1.25

Each value is an average of 5-6 experiments. The kernels were germinated under fluorescent light at 25 ± 2 C.

The starch levels of leaves of virescent et et as well as normal + + et et seedlings at 6, 8 and 10 DAG are given in Table S.3.3. It is observed that the leaves of both et et and + + et et seedlings show similar levels of starch at all the three stages studied. Further, the starch profiles were found to be the same in growing seedlings of both the genotypes.

The amylase enzyme activity as well as its specific activity in germinating 3, 4 and *b* DAG kernels of both genotypes are given in Fig. 13 a. It can be seen that the total amylase levels as well as its specific activity in + + et et kernels increased between the 3rd and 5th day after germination. On the contrary, the total amylase levels and its specific activity decreased during the same period in kernels of et et genotype. An interesting observation here is that the total amylase levels are significantly higher in + + et et kernels during all the three tested stages.

The α-amylase activity and its specific activity in germinating kernels (3,4 and 5 DAG) of normal and mutant genotypes are given in Fig. 14 a. The α-amylase levels of

Table 5.3.3 : Starch content of developing seedlings*

S.No.	Days after germination	Starch content (% fresh weight)	
		Genotype	
		$et^+ \; et^+$	$et \; et$
1	6	1.396 ± 0.055	1.523 ± 0.024
2	8	3.69 ± 0.113	4.47 ± 0.169
3	10	1.85 ± 0.029	2.16 ± 0.065

Each value is an average of atleast *b* experiments.

Leaves were collected from seedlings grown under flourescent light at 25 ± 2° C.

* kernels were not included in the estimations.

+ +
et et kernels was observed to be significantly higher than that of et et kernels at all the three stages studied. However, the overall α-amylolytic pattern was found to be similar in both the genotypes. Similarly, the specific activity profiles were also found to be similar in the germinating kernels of both the genotypes.

The β-amylase activity and its specific activity in germinating normal and mutant kernels of 3,4 and S DAG are given in Fig.15 a. In + + et et kernels, the β-amylase levels increased from 3rd day to 5th day after germination. On the contrary, the β-amylase levels actually decreased during the same period in et et kernels. Further, β-amylase levels were observed to be significantly higher in + + et et kernels of 4 DAG and 5 DAG compared to that of et et. Fig.13 b shows the total amylase and its specific activity profiles in mutant and normal seedlings of 6,8 and 1u DAG. It was observed that total amylase levels were significantly + + higher in normal (et et) leaves than that of virescent (et et) leaves. Total amylase levels in leaves of both the genotypes peaked at 8 DAG followed by a decrease by the 10th day. Similarly, the specific activity of total amylase peaks

at 8th day in et^{+} et^{+} seedlings followed by a decrease at the 10th day. On the contrary, specific activity of total amylase in et et seedlings increased from bth to 10th DAG.

The α-amylase levels and its specific activity in 6,8 and 10 DAG normal and mutant leaves are given in Fig. 14 b. At 6, 8 and 10 days after germination, the α - amylase levels were observed to be higher in normal leaves as compared to the virescent leaves. The α-amylolytic profiles and its specific activity profiles peak at the 8th day in both normal and virescent leaves.

The β-amylase "levels and its specific activity in b,8 and 10 DAG normal and mutant leaves are given in Fig.15 b. β-amylase activity was found to be significantly higher in normal leaves as compared to virescent leaves, at all the three stages studied. β-amylase levels in et^{+} et^{+} leaves were found to peak at the Bth day, whereas, in et et leaves the levels were observed to increase upto the 10th day.

In order to understand if the et allele causes any qualitative changes in the isozymes of amylases, in addition to quantitatively changing the enzyme levels, we studied the

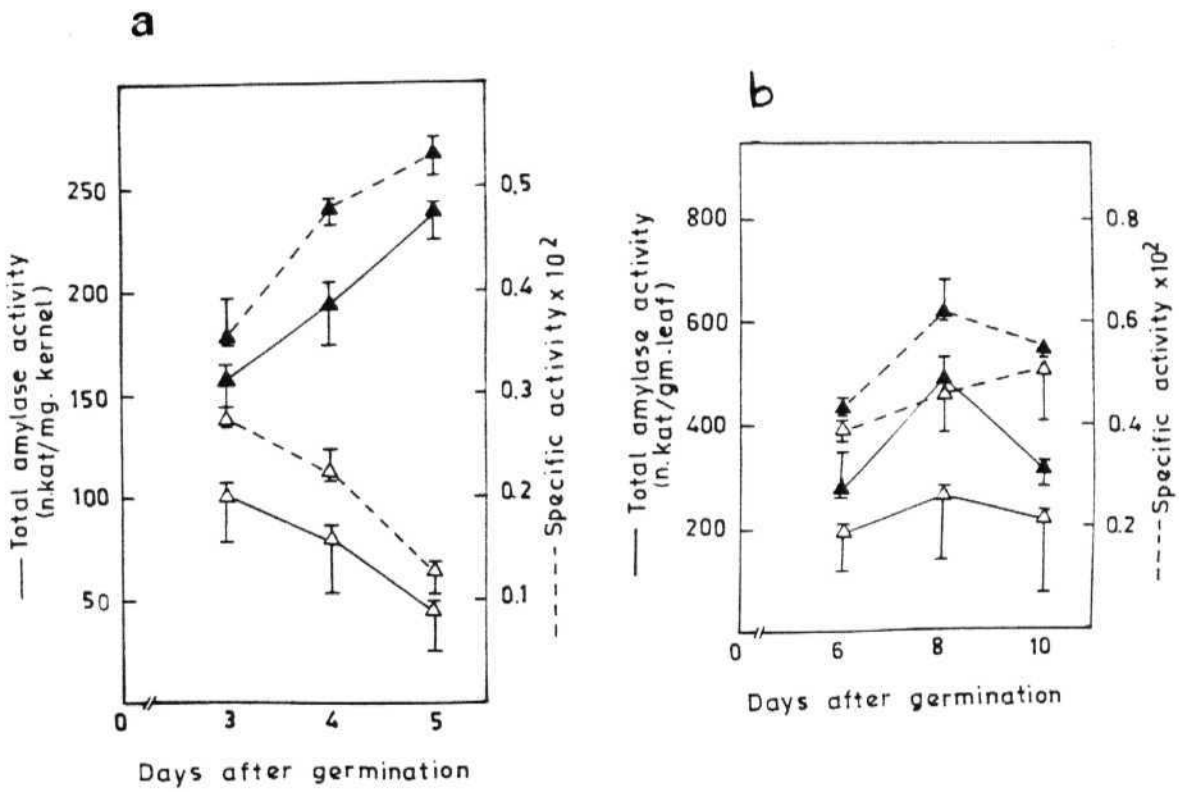


Fig. 13 : Total amylase activity and specific activity profiles in
(a.) Germinating Kernels (b) Seedlings.

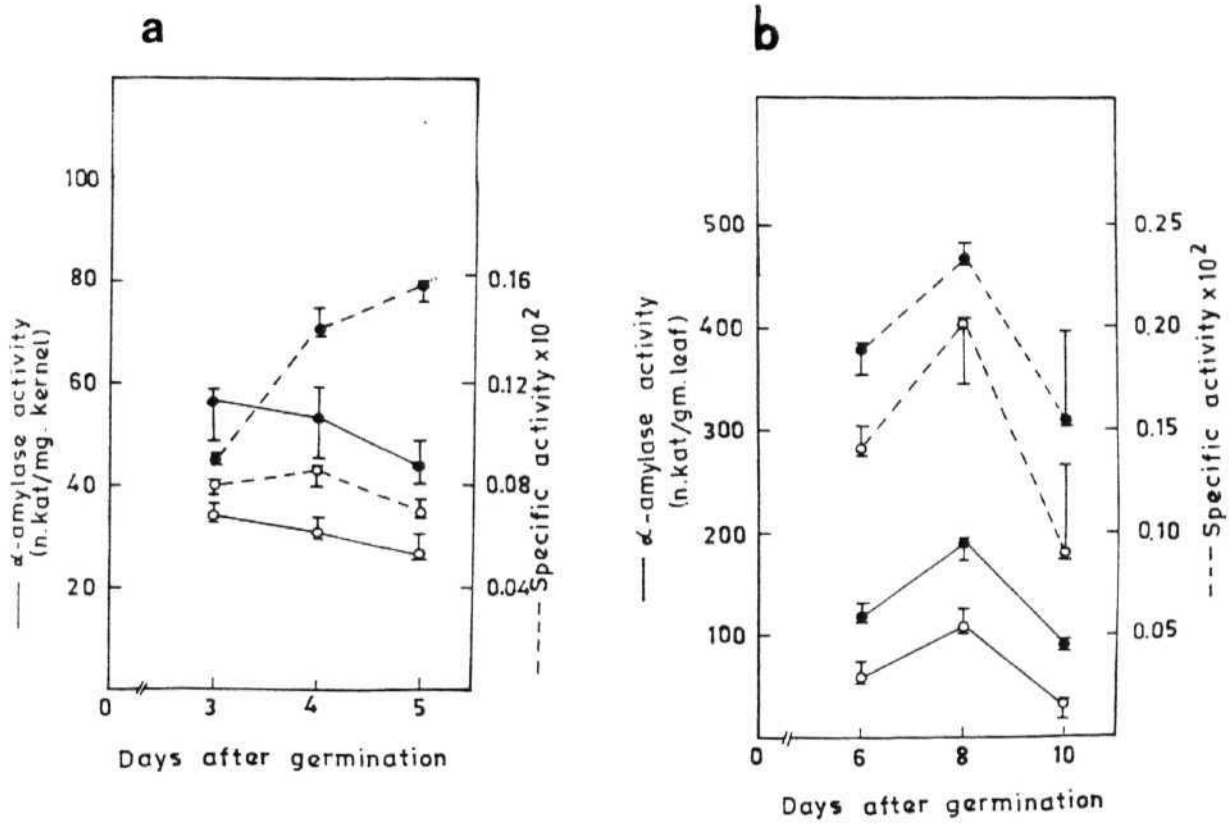


Fig. 14 : α - amylase activity and specific activity profiles in (a) Germinating kernels (b) Seedlings.

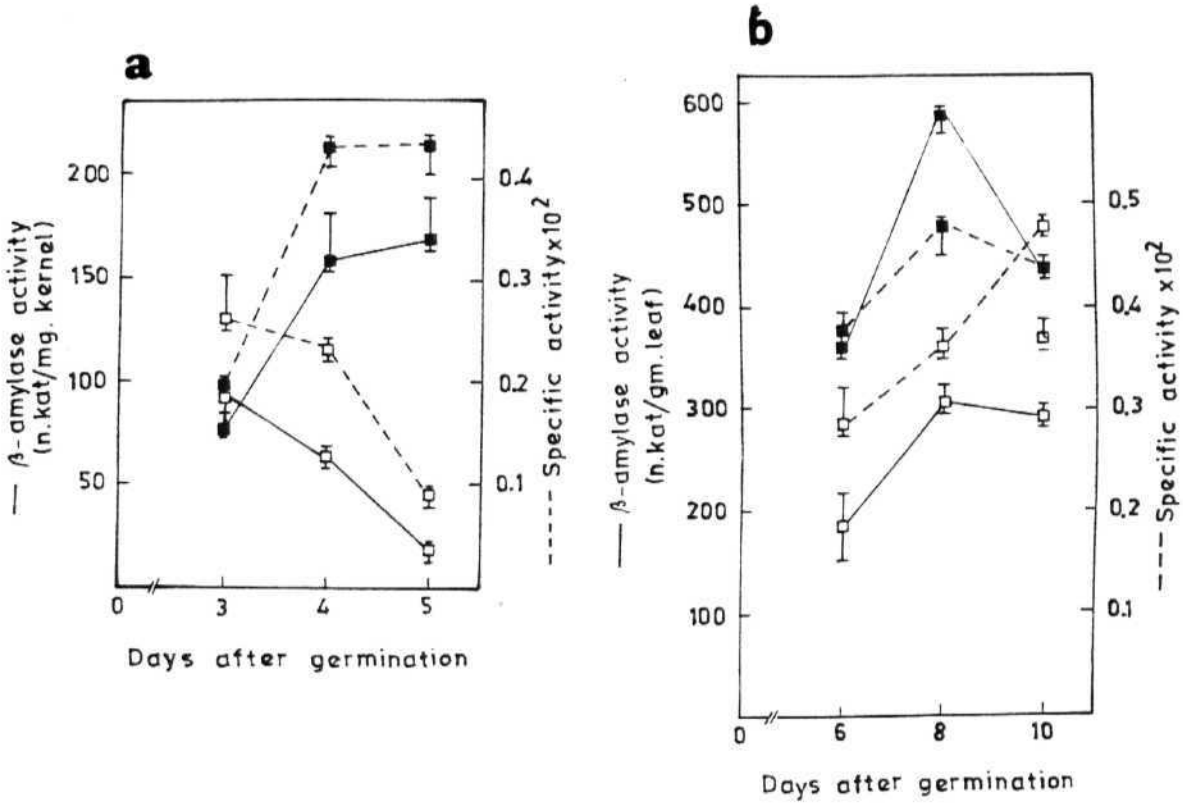


Fig. 15 : β- amylase activity and specific activity profiles in (a) Germinating kernels (b) Seedlings.

electrophoretic profiles of amyiases in germinating kernels and seedlings of normal and mutant genotypes.

Electrophoretic profiles of total amyiases of 3,4 and 5 day old $\frac{+}{et}$ $\frac{+}{et}$ and $\frac{et}{et}$ $\frac{et}{et}$ kernels are shown in Fig.16 . It was found that the zymograms of normal and mutant genotypes do not show any qualitative differences in the isozymes of amyiases, throughout the period of kernel germination.

fclectrophoretic profiles of leaf amyiases of normal and virescent seedlings at 6, a and 10 DAG are shown in Fig. 17 . No difference was noticeable in the zymograms of b, 8 and 10 DAG normal and virescent leaves with respect to the isozymes of amylases. Qualitative and quantitative analysis of amyiases has clearly shown that the et mutation affects only the levels, but not the isozymic pattern of amyiases. It is possible that the starch-hydrolysing enzymes are somehow affected by the structural discontinuity in the endosperm. It is unlikely that the et gene has any direct effect on the expression of genes encoding amyiases.

The amylolytic enzyme activity profiles in the growing seedlings under light, did not show any significant

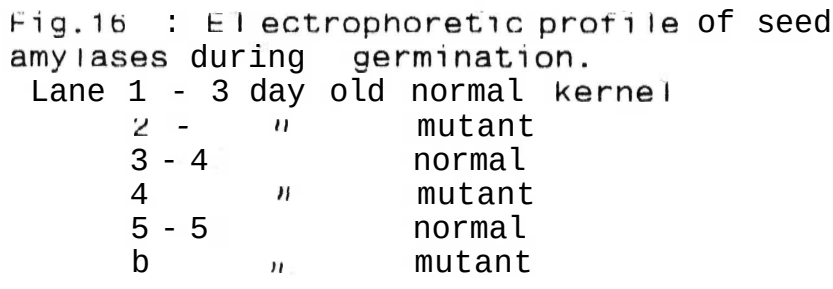


Fig.16 : Electrophoretic profile of seed amylases during germination.
Lane 1 - 3 day old normal kernel
2 - " mutant
3 - 4 normal
4 " mutant
5 - 5 normal
b " mutant

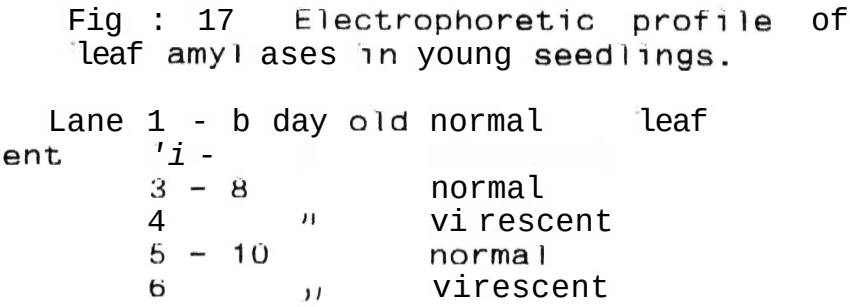
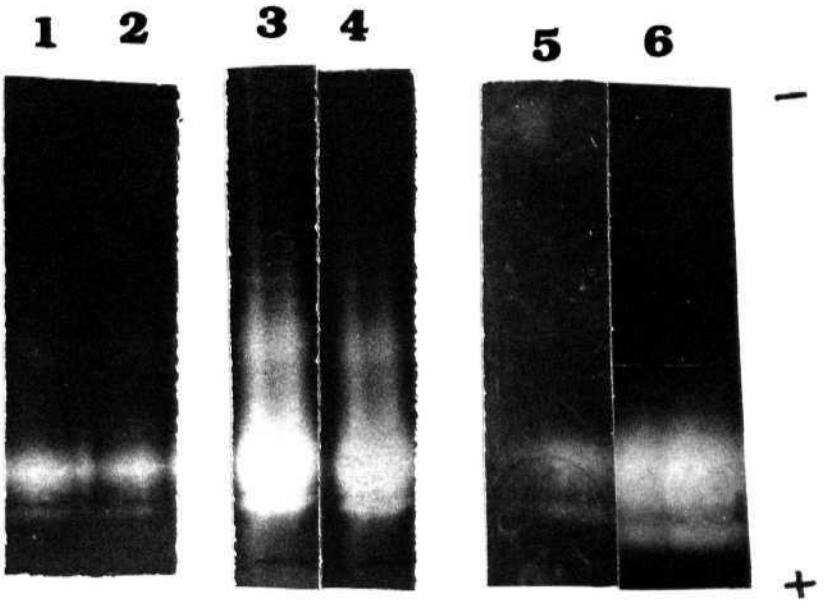


Fig : 17 Electrophoretic profile of leaf amylases in young seedlings.

Lane 1 - b day old normal leaf
" virescent
3 - 8 normal
4 " virescent
5 - 10 normal
6 " virescent

16



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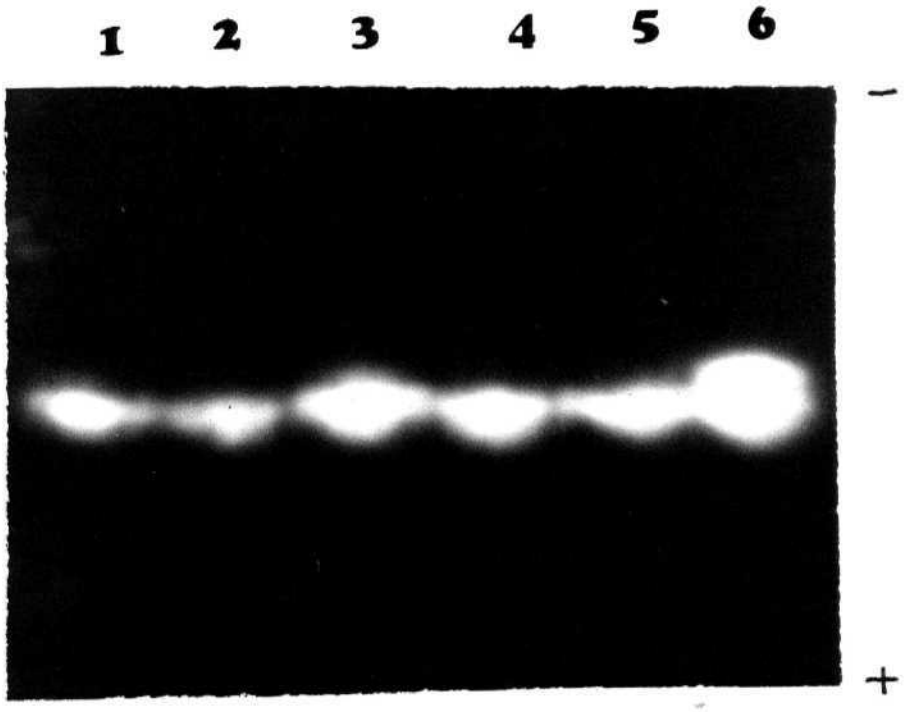


deviations from that of the germinating kernels, indicating that the *et* affect is continued upto 10 DAG (Sangeetha and Reddy, 1988).

Our observations lead us to interpret that the *et* mutation affects starch accumulation in maize endosperm during early stages of development. This was clearly shown by significant reduction in the starch levels in 20 and 30 DAP *et et* kernels. However, at maturity these endosperms show some increase in their starch levels indicating a partial recovery. During germination, the *etched* mutation appears to cause a significant reduction in amylolytic enzyme activities. Further, during later stages of germination, the starch levels in both mutant and normal become almost equal. This does not indicate, however, that starch synthesis is enhanced in *et et* endosperm. It is rather due to increased hydrolysis of starch in *et*$^{+}$ *et*$^{+}$ kernels during germination. It is clear that starch hydrolysis is impaired in *et et* kernels as reflected by very little change in starch levels during early stages of germination.

It is not clear whether this altered starch-hydrolysis is anything to do with virescence. It is possible that rapid

mobilisation of food reserves may act as one of the signals for onset of normal greening. Since *et* mutation affects this process of starch hydrolysis, it may have an indirect affect on greening. These observations lead to the suggestion that the virescence of *et et* seedlings has its origin in the endosperm lesions caused by *et* allele during kernel development.

6. ANALYSIS OF CERTAIN ENZYMES IN THE *ETCHED* MUTANT

6.1 INTRODUCTION:

Isozymic analysis of genetically well defined enzymes served as one of the most effective tools in developmental genetic studies in higher organisms. The earliest systematic study dealing with genetic aspects of isozymes in higher plants was the work on multiple esterases in maize endosperms (Schwartz, 1960). The genetics of other isozyme systems has also been mostly investigated in *Zea mays*. Two of these systems are catalases (Scandalios, 1965 b; Sandalios, 1968), and amylases (Scandalios, 1966a).

Isozymes offer unique advantages to examine gene activity in a changing cellular or tissue environment. Changes in isozyme patterns in samples of a particular tissue, taken in the course of development, reveal the appearance or disappearance of individual isozymes. This suggests that genes involved in the synthesis of isozymes are differentially activated in development. Functional aspects of specific isozymes can be understood by their cellular and sub-cellular localization.

Peroxidases: Peroxidases are a group of isozymes with

similar catalytic properties. They act on a great variety of substrates. They can utilize hydrogen peroxide to oxidize a wide range of hydrogen donors such as phenolic substances, cytochrome C, nitrite, leuco-dyes, ascorbic acid, indole, amines and certain inorganic ions. Peroxidases are believed to have a role in lignification (Siegel, 195b), respiration (Nicholls, 1965) inactivation of catalytic proteins (Sizer, 1953), degradation of anthocyanins (Grommeck and Markakis, 1964) and metabolism of hormones such as gibberellins, cytokinins, ethylene and particularly auxins. Peroxidases are heme-protein enzymes which contain hematin prosthetic groups. Peroxidases are nonspecific with respect to the hydrogen donor, but are highly specific with respect to the peroxide grouping.

Isozymes of peroxidases have been reported in a variety of plants. Peroxidases are also reported to be tissue and organ-specific in several plant systems (Scandalios, 1964). Genetic variants of peroxidase isozymes have been found in pollen and immature endosperm of several inbred maize strains. Electrophoretic analysis of parent and F_1 generation pollen extracts revealed that the variants are

inherited according to simple Mendelian rules and are determined by co-dominant alleles at one locus. Distinct variation in the peroxidase content has been reported in different tissues.

Thirteen distinct peroxidases have been identified in maize, most of them having null or co-dominant variants. Genetic polymorphisms have been shown for nine of the maize peroxidases (Brewbaker and Hasegawa, 1974). None of the maize peroxidases were detected in all tissues studied. No tissue of maize studied so far is found to be free of peroxidase activity. Studies of peroxidases during maize development. shows that essentially all tissues show a general pattern of increasing number and intensity of isozymes (Hamill and Brewbaker, 1969).

Catalases: Catalase is a heme-protein which acts specifically on hydrogen peroxide. Catalases have four iron atoms per molecule attached to the protein and chelated to protoporphyrin IX. It is widely distributed in plants, microorganisms and animals. Its biological role remains unknown although its presence seems to be important as an accessory to the consumption of oxygen.

The distribution of catalase isozymes in various tissues of the maize sporophyte, was investigated (Scandalios, 1968). During the differentiation and development of the maize sporophyte shifts in number and intensity of catalase isozymes have been found in the early stages of development of maize plant (dry kernel to 5-day old seedling). It is reported that there is a shift of one electrophoretic form to another during this stage of development.

Six distinct electrophoretic variants of catalase have been found in maize endosperms. Maize endosperm catalase is suggested to be regulated by six allelic genes, the alleles acting without dominance (Scandalios, 1968).

Catalase in maize is coded by two non allelic loci, cat 1 and cat 2 mapping to two different locations in the maize genome (Roupakias et al, 1980). The catalase genes exhibit a high degree of temporal and spatial specificity in their expression during development of the maize plant (Scandalios, 1983).

In maize scutellum the cat 1 and cat 'l genes are differentially expressed during early seedling growth (Scandalios, 1979). Cat 1 is expressed during kernel

maturation whereas cat 2 is expressed during germination. It was shown that cat 1 is expressed in the developing kernel, dry seed and during the early days post-imbibition. cat 2 production becomes evident on days-2 and -3-post-imbibition. When both genes are expressed a five-banded zymogram pattern is observed due to the random association of the cat-1 and cat-2 subunits and the tetrameric structure of maize catalase (Quail and Varner, 1971; Scandalios, 1965). As development proceeds, cat-1 levels decline whereas cat-2 accumulates resulting in a shift of catalase gene expression.

The expression of the two structural genes is regulated by several factors (Scandalios, 1974). Car-1 is a distinct temporal regulatory gene located 37 map units from the cat-2 structural gene. Car-1 is linked with cat-2 and is located on chromosome 1S. Since the regulatory gene is not located adjacent to the structural gene, its action on cat-2 gene expression may be mediated by diffusible substances (trans-acting) which are produced by the regulatory gene. The other regulatory gene car-2 specifically affects cat-1 protein synthesis.

The subcellular distribution of catalase in the

glyoxysomes suggests a possible physiological role tor the catalytic reaction of catalase. Glyoxysomes are known to contain oxidases which reduce oxygen to hydrogen peroxide. Catalase which normally acts peroxidatically, may then as a safety mechanism, switch to its catalytic reaction to purge the cells of noxious amounts of hydrogen peroxide in the absence of a sufficient supply of hydrogen donors.

RuBP-Carboxylase and PEP-Carboxlase:

The enzyme ribulose-bis-phosphate carboxylase fixes carbondioxide in plants and is the most abundant soluble leaf protein . C plants like maize possess a secondary carboxylation reaction catalyzd by PEP-carboxylase.
4

Rubisco is an aggregate of eight identical 12,000-14,000 mol.wt. small subunits and eight identical 50,000-55,000 mol.wt. large subunits (Kawashima and Wildman, 1970). The small sub-unit is encoded for by a nuclear gene (Kawashima and Wildman, 1972,.). The large sub-unit is encoded by chloroplast DNA and it has also been physically mapped on chloroplast DNA in maize (Coen et al, 1977; Bedbrook et al , 1979; Link and Bogorad, 1yau). By the use of antibodies "labelled with flourescent dyes, it has been shown that Rubisco in C plants is restricted to bundle-sheath

cells. Because of its high sedimentation coefficient and high in-vivo concentration, it is easy to separate Rubisco from other leaf proteins (Goldthwaite and Bogorad, 1971). The gene for large sub-unit is located within a 2500 base-pair DNA fragment which maps approximately 30,000 base-pairs from the 5' end of one of the two sets of plastid rRNA genes and approximately 71,000 base-pairs from the 5' end of the other set of rRNA genes (Bedbrook et al, 1979).

PEP-carboxylase is located in the cytoplasm of mesophyll cells and has a sub-unit molecular mass of 100-103 kDa and is believed to comprise 8-15% of the total leaf soluble protein (Uedan and Sugiyama, 1976; Hudspeth et al, 1986).

In a reaction catalyzed by PEP-carboxylase atmospheric carbondioxide is converted to oxalo-acetic acid. The oxaloacetic acid is then either reduced to malate or transaminated to aspartate. In maize, the aspartate formed is then decarboxylated and the liberated carbondioxide is then fixed through Rubisco in the normal C_3 cycle in the bundle-sheath chloroplasts.

In this chapter, the possible influence of et mutation on the general metabolism of the cell in developing as well

as germinating kernels and seedlings has been investigated. *in* as much as marker enzymes are necessary tor such an analysis, peroxidase and catalase were chosen tor studies on kernel metabolism and Rubisco and PEPcarboxylase tor studies on seedling metabolism. Developmental profiles of these enzyme activities in developing and germinating seeds and seedlings of both et+ et+ and et et have been compared and analysed. Isozyme profiles of these enzymes have also been analysed in both the genotypes.

6.2MATERIALS AND METHODS

6-2.1 Estimation of peroxidases in germinating kernels and 1eaves: Normal (et+ et+) and etched (et et) germinating kernels (3, 4 and 5 day old) and leaves (b, 8 and 10 day old) were weighed and homogenized in 10mM sodium-phosphate buffer, pH 7.0. The homogenate was then centrifuged at 15000 g for 30 min. Supernatant was dialyzed against distilled water for about 12-16 hours at 4°C. Phenolic inhibitors were removed py dialysis and the dialysate was used for peroxidase assay.

Peroxidase activity was determined spectrophotometrically by measuring the increase in absorbance at 25°C in a HITACHI Spectrophotometer using guaiacol as hydrogen donor.

To 2 ml of 10mM sodium-phosphate buffer (pH 7.0), containing 30 μ moles of guaiacol and 40 μ moles of hydrogen perioxide, 60 μl of dialysate was added to start the reaction. optical density was recorded at 47o nm at one minute intervals. Change in absorbance of 0.001/min was taken as 1 unit of enzyme activity. The enzyme activity was expressed as units/mg protein. Soluble protein was measured by the method of Lowry using bovine albumin as standard.

6.2.2 Estimation of catalases in germinating kernels and leaves Mature normal (et$^+$ et$^+$) and mutant (et et) kernels (3, 4 and 5 DAG) and seedlings (6, 8 and 10 day old) were used for catalase estimations. Known quantities of germinating kernels and leaves were homogenized in 10mM sodium-phosphate buffer, pH 7.0. The homogenate is centrifuged at 15000 g for 30 min and the supernatant was used for assay of catalase.

The activity of catalase was determined by direct measurements of the decrease in light absorption in the region 230 to 250 nm caused by the decomposition of hydrogen peroxide by catalase.

At temperatures of about 25 c and less, the optical density of a 1:500 hydrogen peroxide solution was measured at 240 nm in the HITACHI spectrophotometer. The instrument was left set for OD reading and the shutter closed. Known volume of catalase was then added to the peroxide solution and rapidly stirred and a stop watch was started. The slowly changing optical density was read every 10 seconds for the first 30 seconds. Thereafter, the optical density was measured at, 50 and 70 seconds. Protein estimations were done by the Lowry method (Lowry et sal, 1951). Enzyme activities were represented as units/mg protein.

Polyacrylamide gel electrophoresis: Isozyme analysis: Electrophoresis of the total enzyme extract was performed in 1% polyacrylamide gel according to the procedure of Davis, (Davis,1964).

6.2.3 Visualization of peroxidases on gels: Enzyme activity in the gel was revealed by putting it in benzidine solution (1 gm benzidine, 9 ml acetic acid and 3b ml water). It was then left in dark. Equal volume of 3% hydrogen peroxide was added 10 min after the stain was added. Blue bands were observed. The bands turn brown after about 10 min. The gels

were photographed immediately.

6.2.4 Visualization of catalases on gels: The gel was soaked in a solution of horseradish peroxidase in 10 mM sodium-phosphate buffer, pH 7.0 (50 u moles peroxidase/ml buffer; for 45 min. Hydrogen peroxide was then added to a conc. of 5 mM and soaking was continued for 10 min. Higher concentrations of hydrogen peroxide reduce the sensitivity of the activity stain by inactivating catalase and by preventing complete decomposition of hydrogen peroxide at the position of bands of catalase. The gel was then rapidly rinsed in water for few minutes and was placed in a solution of diamino-benzidine (0.5 mg/ml phosphate buffer) until staining was completed. With this modified staining method for catalase only achromatic bands without dark boundaries appeared. Because peroxidases give only dark bands, there was no ambiguity in identifying the isozymes of both catalases and peroxidases.

6.2.5 Determination of RuBP carboxylase activity: Weighed amounts of leaves were ground in a chilled mortar in a buffer solution containing 0.1 M Tris-Hcl (pH 7.8), 10 mM magnesium chloride, 1 mM EDTA, 5mM dithiothreitol and 0.5 gms of fine sea sand (3 ml of buffer solution was used for 1 gm of leaf

material). The homogenate was then centrituged at 10000 g for 10 min and the supernatant obtained was applied to a *v,* x 15 cm column of Sephadex G-25, preequilibrated with 25 mM Tris-Hcl (pH 7.8), 0.1 mM EDTA and 1mM DTT. The eluate was used for enzyme assay.

Assay method: The spectrophotometric method is based on the enzyme catalyzed formation of 3-P-glycerate from ribulose diphosphate in the presence of bicarbonate buffer, magnesium ions and a sulphydryl compound. By addition of ATP and 3-P-glycerate kinase, glycerate 1,3-diphosphate is formed. This is reduced by DPNH to α-glycerophosphate with the aid of glyceraldehyde - 3-P - dehydrogenase, triose - P - isomerase and α-glycerophosphate dehydrogenase(Racker, 1962).

The reaction mixture in a final volume of 1 ml contains the following solutions. 0.05 ml of 1M Tris buffer (pH 7.8); 0.02 ml of 0.006 M nicotinamide adenine dinucleotide reduced form (DPNH); 0.05 ml of 0.1M Glutathione; 0.05 ml of 0.5% Glyceraldehyde-3-phosphate dehydrogenase; 0.02 ml of 0.025% of 3-phosphoglycerate kinase; 0.05 ml of 0.05% α-glycerophosphate dehydrogenase-triose-P-isomerase; 0.02 ml of 0.025 M ribulose diphosphate; 0.06 ml of 0.2 M adenosine triphosphate; 0.02 ml of 0.5 M magnesium chloride; 0.15 ml

of 0.5 M potassium bicarbonate; 0.06 ml of enzyme extract; 0.45 ml distilled water.

After mixing, optical density readings were taken at 34u nms at 1-min intervals for atleast 8 min. After a lag of about 3-4 min maximum rates were achieved.

Enzyme activity was determined using aliquots of crude enzyme preparations at concentration ranges where the enzyme activities measured were proportional to the volume of the preparations.

One unit of enzyme is defined as that amount which catalyzes the cleavage of 1 micromole of ribulose diphosphate per minute under the conditions of the assay. The calculations are based on the assumption that 1 micromole of DPNH gives an optical density of 6.22. Since the cleavage of 1 mole of ribulose diphsophate results in the oxidation of 4 molecules of DPNH, the values for DPNH oxidation are divided by 4. The enzyme activity is expressed in terms of μ. moles CO_2 fixed/min/mg. chlorophyll or protein or gm. fresh weight.

6.2.6 Determination of PEPcarboxylase activity: Known

quantities of leaves (6, 8 and 10 day old mutant and normal) were homogenized in ice-cold extraction buffer containing 50 mM Hepes-NaoH (pH 7.8), 10 mM magnesium chloride, z% bovine serum albumin 25% glycerol and 1mM dithiothreitol. 3 ml of extraction buffer was used for 1 gm of leaf material. Homogenate was then centrifuged at 10000 g for 10 min and the supernatant obtained was used as the crude enzyme extract.

Assay method:

PEP carboxylase is coupled with NAD malate dehydrogenase added in excess into the reaction medium along with NADH.

$$PEP + CO_2 \xrightarrow[\text{carboxylase}]{\text{PEP}} OAA + NADH \xrightarrow[\text{dehydrogenase}]{\text{malate}} Malate + NAD$$

The utilisation of NADH was measured as the decrease in absorbance at 340 nm. The reaction mixture in a final volume of 1 ml contains 700 µl of 500 mM Tricine(PH 7.2). 100 µl of 100 mM PEP, 50 µl of 200 mM magnesium chloride, 100 µl of 4 mM NADH, 5 µl of malate dehydrogenase and 50 µl of enzyme extract. Decrease in optical density was measured at 340 nm for upto 5 min. The decrease in absorbance of reaction mixture/mm was

determined and then the activity in μ moles CO_2 fixed/mg chlorophyll/hr was calculated. Each mole of NADH corresponds to one μ mole of CO_2 fixed.

Activity = Absorbance/min. x 0.1613x60x(20/x) x 1000

0.1613 = To convert the absorbance of NADH into μmoles

60 = min. to hours

20 = dilution of leaf extract in reaction mixture

x = μ gms chlorophyll/ml of original leaf extract

1000 = μ gms to mg chlorophyll.

6.3 RESULTS AND DISCUSSIONS

Peroxidase levels in germinating kernels (3, 4 and 5 DAG) of normal and mutant genotype are given in Table 6.3.1.

At the 3 day old stage, it is observed that the germinating normal kernels show slightly higher activity, as compared to mutant kernels. Similarly, at 4 and 5 DAG stages also, germinating normal kernels show higher activity as compared to kernels of et et genotype.

Activity profiles showed that in the normal kernels, the enzyme activity increases from the 3rd day to the 5th day. Mutant kernels also show an overall increase in peroxidase

Table 6.3.1 : Peroxidase levels in germinating kernels of normal and mutant genotypes

S.No	Genotype	Days after germination	Specific activity
1	$\underline{et}^{+}\ \underline{et}^{+}$	3	1162 ± 18
2	$\underline{et}\ \underline{et}$	3	1093 ± 13
3	$\underline{et}^{+}\ \underline{et}^{+}$	4	1208 ± 8
4	$\underline{et}\ \underline{et}$	4	1059 ± 21
5	$\underline{et}^{+}\ \underline{et}^{+}$	5	1438 ± 16
6	$\underline{et}\ \underline{et}$	5	1355 ± 15

Each value is an average of atleast 6 independent experiments.

activity during that period with the exception that 4 day stage shows a slight decrease.

Peroxidase levels in leaves of 6, a and 10 DAG normal and mutant seedlings are given in Table 6.3.2. In b day old seedlings, *et*$^{+}$ *et*$^{+}$ leaves show slightly lower activity as compared to *et et* leaves. Normal leaves at 8 and 10 DAG stages however show higher activity as compared to virescent "leaves. The peroxidase activity profiles, however follow the same pattern in normal and virescent leaves.

Electrophoretic profiles of peroxidase isozymes of germinating normal and mutant kernels are shown in Fig.18a. Lane 1 shows the isozyme pattern of the extract ot 3 day old kernels of *et*$^{+}$ *et*$^{+}$ genotype. Lane 2 shows the isozyme pattern of the extracts of 3 day old kernels of *et et* genotype. As can be observed five isozymes are present in both *et*$^{+}$ *et*$^{+}$ and *et et* extracts. No difference was noticeable in the number of isozymes ot peroxidase in normal and mutant genotypes during germination of kernels.

Electrophoretic profiles of peroxidase isozymes in 6, 8 and 10 day old leaves are given Fig.19. Lanes 1, 3 and b show the peroxidase isozyme profiles from the extracts of

Table 6.3.2 : Peroxidase levels in leaves of $\underline{et}^{+}\ \underline{et}^{+}$ and $\underline{et}\ \underline{et}$ seedlings

S.No.	Genotype	Days after germination	Specific activity
1	$\underline{et}^{+}\ \underline{et}^{+}$	6	1344 ± 21
2	$\underline{et}\ \underline{et}$	6	1381 ± 16
3	$\underline{et}^{+}\ \underline{et}^{+}$	8	2325 ± 11
4	$\underline{et}\ \underline{et}$	8	2019 ± 9
5	$\underline{et}^{+}\ \underline{et}^{+}$	10	2391 ± 14
6	$\underline{et}\ \underline{et}$	10	2130 ± 18

Each value is an average of atleast b independent experiments.

Seedlings grown in bright-light and at a temperature of 25 ± 2°C.

normal seedlings of 6, 8 and 10 day old stages respectively. Seven isozymes are clearly noticed in the above mentioned lanes. Lanes 7 and y also show the electrophoretic profiles of peroxidase isozymes from dialyzed extracts of normal seedlings. It is observed that though the intensity of most of the bands increase, the pattern remains the same in both dialyzed and un-dialyzed extracts of normal seedlings. Lanes 2, A and 6 show the peroxidase isozyme profiles from the extracts of et et seedlings of b, 8 and 10 day old stages respectively. The total number of isozymes observed remain the same in the mutant seedlings at all the stages studied. Further, dialyzed samples also do not show any qualitative difference in seedlings of various stages as shown in lanes 8, 10 and 11. However, in the mutant seedlings at fc>, 8 and 10 day old stages, one isozyme of peroxidase is noticed to show a faster mobility. This particular isoenzyme is not observed in the normal seedlings at any of the stages studied. Fig.20 clearly reflects the difference noticed in the normal and mutant seedlings with respect to the mobility of this particular isoenzyme of peroxidase. Lane 1 shows the isoenzyme profile of mutant seedlings and lane 2 shows the isoenzyme profile of normal seedlings.

Fig : 18 Electrophoretic profile of seed peroxidase during germination.

Lane 1 - 3 day old normal kernels
2 - " mutant "

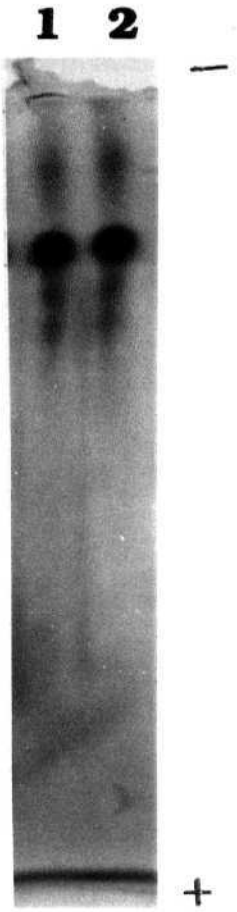
Fig : 19 Electrophoretic profile of leaf peroxidase

Lane 1 - 6 day old normal leaf
2 " " virescent
3 - 8 " normal
4 " " virescent
5 - 10 " normal
6 " " virescent
7 - 6 " normal (dialysed)
8 " " virescent
9 - 8 " normal
10 - 8 " virescent
11 " " "

Fig : 20 Magnified electrophoretic pattern of leaf peroxidase

Lane 1 - 6 day old virescent leaf
2 " normal

18



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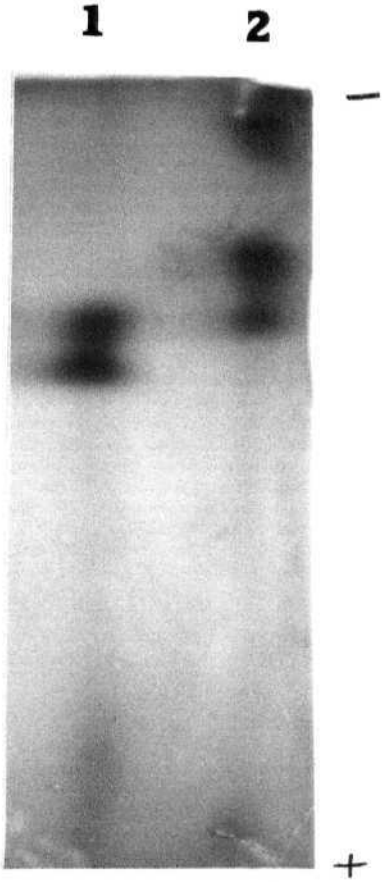

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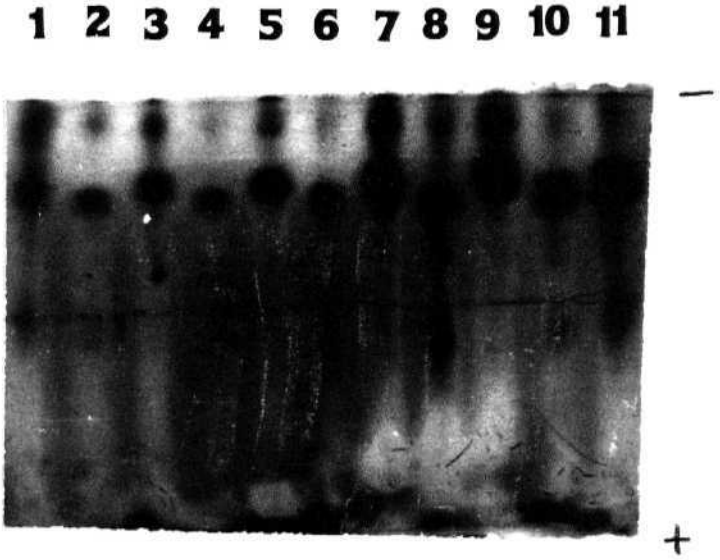


Catalase levels in the germinating normal and mutant kernels of 3, 4 and 5 day old stages are given in table b.3.3. As can be observed, the levels of catalase do not differ significantly between normal and mutant kernels at all the three stages studied. Further, the activity profiles of cataiase are also similar in both normal and mutant kernels. The activity is found to increase from the 3rd to the 4th day and then decrease by the 5th day in both normal and mutant genotypes.

Table b.3.4 gives the levels of catalases in leaves of b, 8 and 1u day old seedlings of normal and mutant genotypes. There are no significant differences in enzyme levels at all the three stages studied. Activity profiles were also found to be similar in normal and mutant genotypes. The enzyme activity is gradually found to decrease from 3rd day to 5th day in both the genotypes.

A schematic diagram of the cataiase isozymes of 3, 4 and 5 day old normal and mutant kernels is shown in Fig.21a. Three isozymes of cataiase activity are noticed in normal and mutant germinating kernels of 3, 4 and 5 day old stages. However, no qualitative difference was noticed in the

Table 6.3.3 : Catalase levels in kernels of $\underline{et}^{+}$ $\underline{et}^{+}$ and $\underline{et}$ $\underline{et}$ genotypes

S.No.	Genotype	Days after germination	Specific activity
1	$\underline{et}^{+}$ $\underline{et}^{+}$	3	348 ± 9
2	$\underline{et}$ $\underline{et}$	3	322 ± 11
3	$\underline{et}^{+}$ $\underline{et}^{+}$	4	456 ± 8
4	$\underline{et}$ $\underline{et}$	4	438 ± 15
5	$\underline{et}^{+}$ $\underline{et}^{+}$	5	356 ± 22
6	$\underline{et}$ $\underline{et}$	5	316 ± 14

Each value is an average of b-b experiments.

Table 6.3.4 : Catalase levels in leaves of normal and mutant seedlings

S.No.	Genotype	Days after germination	Specific activity
1	$\frac{+}{et}\ \frac{+}{et}$	6	242 ± 5
2	et et	6	216 ± 11
3	$\frac{+}{et}\ \frac{+}{et}$	8	212 ± 8
4	et et	8	200 ± 13
5	$\frac{+}{et}\ \frac{+}{et}$	10	208 ± 16
6	et et	10	198 ± 7

Each value is an average of 5-b experiments.

Seedlings grown in bright-light

and at a temperature of *2b* ± $^{\circ}$*2 C*

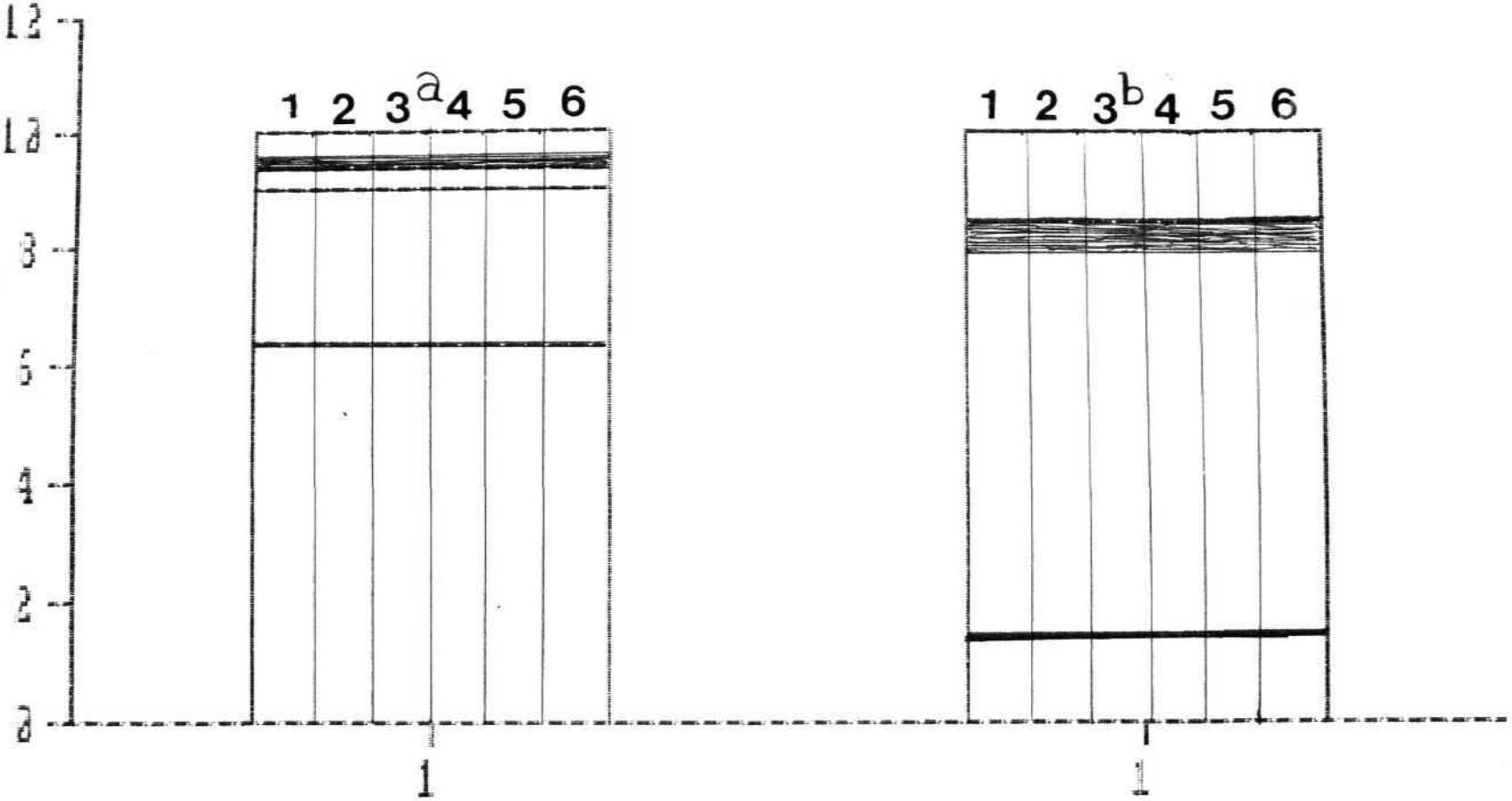
catalase activity zones of normal and mutant kernels.

The zymogram of catalase isozymes of 6, 8 and 10 day old normal and virescent leaves is shown in Fig.21b. The leaf extracts from both normal and mutant seedlings show two clear zones of catalase activity. No detectable allele specific catalase isoenzyme could be seen in the mutant leaves.

The RuBP carboxylase activities in the light-grown normal and virescent seedlings of 6, 8 and 10 day old stages are given in table 6.3.5. Chlorophyll and protein contents in the two genotypes at all the three stages are estimated. The enzyme activity is expressed as μ moles CO_2 fixed /mg. chlorophyll, per mg. protein and also per gram fresh weight of the leaf.

RuBP-carboxylase activity is found to be significantly lower in the virescent leaves of 6 day old stage as compared to normal leaves of the same stage. The activity of the enzyme is found to be lower when expressed in terms of chlorophyll, protein and also fresh weight of the leaf. Normal leaves of 6 day old stage show about three and half times higher activity as compared to 6 day old virescent leaves. The enzyme activity when expressed on the basis of protein content shows about six times higher activity in the

(a) CATALASES OF GERMINATING KERNELS
(b) CATALASES OF LEAF EXTRACTS



(Fig 21)SCHEMATIC ZYMOGRAM OF CATALASES

Table 6.3.5. RuBP - CARBOXYLASE ACTIVITIES IN SEEDLINGS OF NORMAL AND MUTANT GENOTYPES

S.No.	Age in days	Genotype	Chlorophyll (μgms/gm. leaf)	Protein (μgms/gm. leaf)	RuBP-Carboxylase activity		
					μmoles Co_2 fixed/min. mg.chl.	μ moles/min/mg. Co_2 fixed protein	μmoles/min gm. Co_2 fixed fresh wt.
1.	6	et^+et^+	895.56	13820	3.49 ± 0.05	0.226 ± 0.03	3.125 ± 0.17
2.	6	et et	490.66	12682	1.03 ± 0.11	0.039 ± 0.02	0.504 ± 0.03
3.	8	et^+et^+	1723.56	12968	4.61 ± 0.02	0.612 ± 0.10	7.94 ± 0.9
4.	8	et et	1495.42	13310	426 ± 0.04	0.477 ± 0.08	6.368 ± 0.23
5.	10	et^+et^+	1782.40	13912	4.90 ± 0.13	0.627 ± 0.03	8.73 ± 0.11
6.	10	et et	1871.62	13668	4.85 ± 0.08	0.659 ± 0.11	9.07 ± 0.16

Each value is an average of atleast 8 independent experiments.

6 day old normal leaves as compared to mutant leaves. The enzyme activity in terms of fresh weight also shows six times higher activity in the normal leaves at the 6 day old stage. At the 8 day old stage, however, the enzyme activity is slightly higher in the normal leaves as compared to virescent leaves. The higher activity in enzyme levels is noticed with respect to all the three parameters, chlorophyll, protein and fresh weight. The 10 day old normal and mutant leaves do not show significant differences in the RuBP-carboxylase activity when expressed in terms of the chlorophyll content of the leaf. However, when the enzyme activity is expressed in terms of the protein content of the leaf and the fresh weight of the leaf, the virescent leaves of 10 day old stage show slightly higher activities as compared to normal leaves.

The activities of PEP carboxylase in the normal and virescent leaves of 6, 8 and 10 day old seedlings are given in table 6.3.6. The enzyme activities are expressed in terms of protein content, chlorophyll content and also fresh weight of the leaf.

The 6 day old leaves of virescent seedlings show about 25% increase in PEP-carboxylase activity as compared to the

Table 6.3.6. PEP - CARBOXYLASE ACTIVITIES IN SEEDLINGS OF NORMAL AND MUTANT GENOTYPES

S.No.	Age in days	Genotype	Chlorophyll (μgms/gm. leaf)	Protein (μgms/gm. leaf)	PEP - Carboxylase activity		
					μmoles / min/mg.chl. Co_2fixed	μmoles / min / mg. protein Co_2fixed	μmoles / min / gm. fresh W Co_2fixed
1.	6	et^+et^+	738	12210	13.26 ± 0.21	0.795 ± 0.03	9.78 ± 0.20
2.	6	et et	392	11899	16.84 ± 0.16	0.5388 ± 0.08	6.60 ± 0.06
3.	8	et^+et^+	1634	13485	13.91 ± 0.19	1.684 ± 0.10	22.72 ± 0.18
4.	8.	et et	1321	12963	16.91 ± 0.09	1.707 ± 0.05	22.33 ± 0.72
5.	10	et^+et^+	1658	13534	20.85 ± 0.28	2543 ± 0.04	34.56 ± 0.31
6.	10	et et	1600	13521	24.38 ± 0.13	2.873 ± 0.12	38.96 ± 0.26

normal, when the enzyme activity is expressed on the basis of chlorophyll content of the leaf. The enzyme activity when expressed on the basis of protein content of the leaf and fresh weight of the leaf, higher activities were noticed in the normal leaves of 6 day old stage. The 8 day old virescent leaves show slightly higher PEP-carboxylase activity when the activity is expressed in terms of chlorophyll content. Enzyme activity in terms of protein content and fresh weight of the leaf do not differ significantly in the normal and virescent leaves of 8 day old seedlings. The 10 day old leaves of normal seedlings show slightly lower enzyme activity on the basis of chlorophyll content of the leaf. However, in terms of the protein content and fresh weight of the leaf, the enzyme activity is slightly higher in the 10 day old virescent leaves of the etched mutant.

A high degree of correlation is observed between RuBP-carboxylase and the chlorophyll content of the leaf, both in the normal and virescent seedlings. The carboxylating enzyme activity paralleles the synthesis of chlorophyll in virescent leaves. Hence, the difference noticed in the enzyme activity at earlier stages of seedling growth is not maintained at

later stages of greening. This is reflected at the 10 day old stage when the mutant leaves are phenotypically normal and contain chlorophyll and RuBP-carboxylase amount similar to that of the normal leaves of the same stage.

There is a significant difference in the RuBP-carboxylase activity at the 6 day old stage of virescent leaves in terms of all the three parameters (Chlorophyll, protein and fresh weight,). Understandably, in terms of protein content and fresh weight of the leaf, the difference in enzyme activity is more than in terms of the chlorophyll content of the leaf.

A close relation between the synthesis of chlorophyll and ribulose diphosphate carboxylase has been suggested earlier (Trown, 1965). Huffaker et al, have shown that there is a close relationship between carboxylase activity and chlorophyll synthesis (Huffaker et al, 1966).

The activity of the enzyme PEP-carboxylase is found to be higher in the mutant leaves when the activity is expressed in terms of the chlorophyll content. However, the activity is not high enough to be seen when the enzyme activity is expressed in terms of protein and fresh weight. Therefore, the

activity is actually found to be lower in virescent leaves when it is expressed in terms ot protein content or fresh weight of the leaf.

Analysis of the enzyme data reveals that the *et* mutation does not affect the general metabolism of the cell. The levels of the two carboxylating enzymes are reduced in the mutant, during the early stages of seedling growth. It is possible that photosynthesis is limited by the levels of the two enzymes RuBP and PEP carboxylases.

7. BIOCHEMICAL STUDIES ON CHLOROPLAST PREPARATION FROM LEAVES OF *et et* SEEDLINGS

7.1 INTRODUCTION:

In higher plants, greening of leaves involves a series of distinct morphological and biochemical processes leading to chloroplast development and its structural and functional differentiation. it is known that chlorophyll biosynthesis and chloroplast development are spatially and temporally correlated . Grana formation is reported to be directly correlated with the rapid phase of chlorophyll synthesis (Virgin *et al*,1963). 1 ranscri ptional regulation of gene expression plays an important role in the greening process. The general transcriptional activity of the plastid genome is known to increase after illumination and decrease during leaf development (Deng and Gruissem, 1987).

Mature chloroplasts under an electron-microscope abpear as elongated discs enclosed by a double membrane structure called the envelope. Inside the envelope is the amorphous stroma and the highly organized membrane system which consists of thylakoids stacked in structures called grana. Dispersed in the stroma of the ch lorop last are other

structures like plastoglobuli rich in lipids, and starch grains lying close to the thylakoid membranes. Ribosomes occur free in the stroma and also bound to the outer surface of the thylakoid membrane. Chloroplast ribosomes contain four RNA molecules. The 23S (Mol.wt 1,100,000) bs (40,000) and 4.5S are present in the large sub-unit and 1bS (560,000) is located in the small sub-unit of the ribosome. Large sub-unit of Rubisco, elongation factors r ana G and twelve ribosomal proteins are some of the proteins in the stroma region. Chloroplast thylakoid membranes contain five major protein complexes 1.PSII reaction center and 'bound' antenna pigment-protein complex (PS II) 2. PSI reaction center and antenna pigment-protein complex (PSI). 3. Mobile chl-a/b - containing LHCP. 4. CF_0-OF_1 coupling factor ATP synthase complex and 5. Cyt b-f complex containing Cyt b-f and Rieske iron - sulphur protein. PS II and LHcP are present in grana membrane and PSi and CF_0-CF_1 are shown to be present in stroma membrane region. The cyt b - f complex is reported to be present in all regions of the thylakoid membrane (Allred and Staehelin, 1986).

in the thylakoid membrane, all pigments occur as discrete lipo-protein units, the chlorophyll-protein complexes (Thornber, 197b; Thornber et al,1979). Smith

provided the first evidence that chlorophyll was complexed with proteins (Smith and Pickels, 1941). Evidence for the occurence of numerous complexes was given py Chiba (1960). Use of a zwitterionic detergent revealed that all of the chlorophyll in the thylakoid membrane is complexed with proteins (Markwell set al,1979). All the biophysical functions of light harvesting, energy transfer and photochemical energy conversion carried out by the photosynthetic pigments are performed by the pigment-protein complexes. These complexes are generally hydrophobic in nature and since most of them contain the same pigments in similar proportions, their absorption spectra are almost similar.

Two of the complexes CPI and CPIa are derived from photosystem 1. CP is derived from PS11 and contains little if any, chlorophyll b. The three complexes which contain the majority of the chlorophyll a and b are LHCP , LHCP and LHCP . The major thylakoid proteins are α, β, ϵ subunits of CF , CF -1 and CF III, apoprotein of chlorophyll-protein complex I, 32000 dalton protein, Cyt b_{559}, cyt f and cyt b_{563}.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of the stromal fraction of the chloroplasts: Fresh leaves were harvested from seedlings grown under defined conditions and were washed thoroughly with distilled water. Leaves were cut into small pieces and were ground in buffer (2 ml/gm leaf) consisting of 4uo mM sorbitol, 50 mM Tricine-NaoH (pH 7.8), 5 mM sodium ascorbate, 50 µ gms/ml Bovine Serum Albumin.

The homogenate was filtered first through 4 layers and then twelve layers of cheese cloth. The filtrate was then centrifuged at 1uuug for 5 min. and the supernatant was discarded. The resulting chloroplast pellet was then osmotically shocked by resuspending in 25mM Hepes-NaoH, pH 7.5, for 15 min. at 0 °C, and centrifuged at 10,000g for one min. The supernatant is called the soluble fraction.

1.2.2 a Preparation of the membrane fraction of the chloroplast: Weighed leaves were washed in distilled water and then cut into small pieces and homogenized in an ice-cold buffer containing 5umM potassium phosphate pH 7.2, 10mM potassium chloride, 0.3 M sucrose in a prechilled mortar. For 1 gm of leaf material, 10 ml of buffer was used. The homogenate was filtered through 4 layers of cheese cloth and the filtrate was centrifuged at 2000g for 10 min. The pellet was resuspended in 50mM potassium phosphate pH 7.2, 10mM

potassium chloride and centrituged at 4000 g tor 1U min. I he membrane pellet was washed by centritugation and resuspension in cold distilled water at 4000 g for 10 min. I he resultant pellet was resuspended in 1 mM sodium EDTA, pH 8.0 and spun at 10000 g tor 1U min. I he membrane preparation was suspended in 6umM tricine, pH 8.U and spun at 10000g for 1u min. Finally, the washed thylakoid membrane pellet was resuspended in 50mM tricine, pH 8.0 and stored at 4 °C. The entire procedure of thylakoid preparation was carried out under cold and dark conditions (as per Dunkley and Anderson, 1979).

7.2.2b. Estimation of protein and chlorophyll in the thylakoid membrane extract: Protein in the thylakoid membrane was estimated by the Lowry method (Lowry, 1951). optical density was taken at 740nm, since chlorophyll also absorbs at 670 nm. Chlorophyll was estimated using cold 80% acetone. Optical density was recorded at bb2 nm. I he total amount of chlorophyll was expressed as u gms/gm. leaf fresh weight.

7.2.2 c Solubilization of the thylakoid preparation: The thylakoid preparation was washed with the buffer consisting of (2b mM Tricine-KOH, pH 8.0 containing 5mM fci - Mercaptoethanol.). It was then sedimented at 20000 g tor 10 min at 4 °C. The washed pellet was taken up in 1 ml of the

buffer to produce a homogeneous suspension to which 9ml of chilled acetone was added at room temperature with mixing to avoid formation of clumps of protein. The protein was allowed to precipitate by standing on ice in the dark for one hour and then pelleted by centrifugation at 5000 g for 15 min. at. 0 C. The pellet was then washed by the addition of 10 ml acetone and sedimented by centrifugation at 5000 g for 15 min. at 0 C. The white thylakoid pellet was suspended in 100 µl of *b%* SUS at room temperature to solubilise the suspension. The suspension was then placed in a boiling water bath for 2 min. in a sealed tube. After cooling, 1 ml of 0.3 M Tris-HCl (pH 8.0) was added.

7.2.3 Polyacrylamide gel electrophoresis of thylakoid membrane fraction and stroma fraction: SDS-PAGE with a discontinuous buffer system was used . SDS-PAGE was carried out according to the procedure of Laemmli (Laemmli, 1970).

Running gel (10%)

10 ml acrylamide : bis-acrylamide solution
30 '. 0.8 gms w/w

7.5 ml Tris-HCl buffer, pH 9.18

0.6 ml 10% SUS

11.9 ml Double distilled water

25 µl TEMED

1UU µl 10% APS

Stacking gel (5%):

1.7 ml acrylamide: bis-acrylamide solution

30 : 0.8 gms w/w

2.5 ml Tris-H_2SO_4 buffer pH 6.14

0.2 ml 10% SDS

5.63 ml Double distilled water

6.25 µl TEMED

25 µl 10% APS

The gels were polymerised in slabs. The upper reservoir contained Tris-borate buffer, pH 8.64. The lower reservoir contained Tris-HCl buffer, pH y.4.

The stroma fraction and the solubilized thylakoid preparations were loaded onto the slab gel at 4 °C. The appropriate volumes containing known amounts of protein and chlorophyll was loaded. Because the thylakoid preparations are coloured, no tracking dye was used. Electrophoresis was carried out for about 8 hrs. in dark at 4 °C. A constant current of 6mA/slab was used for the first one hour and then tor the next seven hours 12 mA/slab was used.

7.2.4 1MBZ staining of polyacrylamide gels: The cytochromes f and b-5b3 and the Rieske iron -sulphur protein (Cyt-b -f) present in the chloroplast thylakoid protein complex was **stained** by the following procedure. The TMBZ solution (6.3 mM) was freshly prepared in methanol. Just before use, three parts of TMBZ solution was mixed with seven parts of 0.2M sodium acetate, pH 5.0. The gels were immersed in this mixture at room temperature in the dark. After one to two **hours** with occasional mixing hydrogen peroxide was added to a final concentration of 30 mM. The staining was visible within 3 min. and increased in intensity over the next 30 min. The gels were then placed in a solution of isopropanol : 0.25 M sodium acetate (pH 5.0) mixed in a ratio of 3:7. The acetate buffer (with 30% isopropanol) was then replaced once or twice with fresh solution to remove any precipitated TMBZ. TMBZ stain was removed from gels by adding sodium sulphite solution to a final concentration of at least 70 mM. The stain was cleared in 1-3 hrs. and the gel was then washed in 30% isopropanol solution 2-3 times to remove sodium sulphite.

7.2.5 Coomassie staining of polyacrylamide gels:

Staining solution: 0.2% Coomassie blue, 50% methanol and 7% acetic acid

Destaining solution : 40% methanol, 7% acetic acid.

The gel was put in the staining solution and left for 'i hours with occasional shaking. It was then washed twice with the destaining solution and left overnight in the destaining solution.

7 . 'i. 6 b 1 uti on of ch lorophv l l-prote in comp lexes from SDS-PAGE gels and reelectrophoresis:

The green band CP1 and ye l low-green band LHCP , LHCP and LHCP were excised from the gel and frozen immediately in liquid nitrogen. Absorption specra of gel slices was recorded. 1 he gel pieces were then homogenized in Iris-HCl buffer containing 1mM PMSF, β-mercaptoethanol and SDS. I he homogenate was then left at -4 °C overnight. It was then centrifuged at 50000 rpm and the supernatant was lyophilized and aliquots were then loaded onto gel slots in the gel system described earlier. Since protein concentration was extremely low in the e luted gel slices even after lyophilisation, staining was carried out by the silver staining method. Apparent mol.wts were determined by comparison with the following proteins. Glyceraldehyde-3-phosphate dehydrogenase (36,000 mol wt), Bovine albumin (66,000), carbonic anhydrase (29,000) and phosphorylase

(97,400)daltons.

7.2.7 Silver staining of proteins in polyacrylamide gels:

(1) Fixing	50% Methanol 12.5% Acetic Acid 0.5% Formalin (38%)	1 1/2 hours
(2) Wash	50% ethanol	20 x 2 mm.
(3) Pretreat	200 mg Sodium thiosulphate/litre 0.5% formalin	1 min.
(4) Wash	Distilled water	20 x 2 sec.
(5) Impregnation	2 gms silver nitrate 0.75 ml Formalin/litre	20 min.
(6) Wash	Distilled water	20 x 2 sec
(7) Develop	60 gms sodium carbonate, 0.5 ml formalin/litre	10 min
(8) Wash	Distilled water	20 X 2 sec
(9) Wash	50% methanol	20 mins

Distilled water was then added to dilute 50 % methanol to retain the stain on the gel.

7.2.8 Measurement of photosynthetic electron transport:

(a) Measurement of whole chain electron transport H_2O ——— Methyl viologen in isolated chloroplasts was measured as

oxygen uptake.

Donor————————> PSII————————>PSI————————>MV

The reaction mixture in a final volume of 1.5 ml contained 20mM Tris-HCl, pH 7.5, 5mM Magnesium chloride, 10mM sodium chloride, 100mM sorbitol, 1mM methyl viologen, 0.1mM sodium azide, 5mM ammonium chloride and thylakoid membrane equivalent to 20 µ gms chlorophyll/ml.

b) PS1 electron transport:

Donor————————-->PSI————————>MV

PS 1 electron transport from artificial electron donors TMPD to methyl viologen was assayed polarographically as oxygen uptake. The reaction medium in a final volume of 1.5 ml contains 20mM Tris-HCl, pH 7.5; 2mM sodium ascorbate, 0.1mM TMPD,5uM DCMU, 0.1mM sodium azide, 1mM methyl viologen and 10 µ gms chlorophyll / ml.

The electrode vessel was filled with the reaction mixture and after equilibriation at the temperature of 30 °c for 30 seconds atleast, the sample was added in dark. The recorder was then turned on. When a good base line was recorded after about 30 secs, light was turned on and the change in oxygen concentration during 3-5 min. was recorded. To measure oxygen consumption, the pen was set as far away as

possible from the zero line.

Air saturated water contains 0.28 μmoles of oxygen/ml at 20^{o} C, about 0.25 μ moles at 30^{o} C and 1 atm. pressure. When a few crystals of dithionite was added, the oxygen concentration fell to zero. Therefore, the span on the chart of the recorder, between "air-line" and "dithionite-line" will be equal to (0.25 x Volume) of the electrode vessel in ml)/μ moles oxygen/ml.

PSI activity was represented as μ moles of oxygen/hr/mg.chl.

$$= \frac{0.240 \times \text{Volume of the vessel} \times \text{Deflection of pen on chart/min.} \times 1000 \times 60}{\text{chlorophyll } (\mu \text{ gms/ml}) \times \text{Span of chart}}$$

Span of chart = dithionite value

0.240 = standard

c) PSII Electron Transport: The rate of PSII electron transport was determined spectrophotometrically by measuring the bleaching of DCPIP at 600 nms.

$$H_2O \text{ ---------- } > \text{DCPIP}$$

The sample was illuminated for 30 seconds with saturating white light. The final volume of the reactions

mixture was 3 ml and it contained chlorophyll equivalent to 20 gms/ml; 20mM Tris-HCl, pH 7.5; 5mM Potassium chloride, 10mM sodium chloride, 5mM Ammonium chloride and 25u M DCPIP.

PSI1 activity is represented as μ moles DCPIP reduced/hr/mg.chl.

$$= \frac{\text{Absorbance at 600 nms/min} \times 60 \times 1000}{19 \times \text{chlorophyll conc (} \mu \text{ gms/100 } \mu \text{ l)}}$$

7.2.9: Pigment estimations after hormone treatments

Normal and virescent stocks (for 6 day old seedlings) were germinated as described earlier. Leaves were then treated with certain hormone solutions. The cut leaves were incubated in different concentrations of hormone solutions for different time intervals. In addition to hormones, the leaves were incubated in a solution of isotonic sucrose. The leaves were incubated in distilled water for the same period of time for the purpose of control. Extraction of the pigment was then carried out with cold 80% acetone. The homogenate was centrifuged and the pellet was repeatedly washed with cold 80% acetone until no more pigment was extractable from it. The volume of the total supernatant was measured. The optical density of an aliquot was measured at 663, 648, 626 and 480 nm. To avoid any possible degradation

of pigments, the extraction was carried out in cold and dark conditions.

The following equations were used to estimate amounts of chl. a and chl. b. The pigment content was expressed in μ gms/gm of leaf fresh weight.

$$\text{Chl.a} = \frac{(12.67\ A_{663} - 2.65\ A_{645} - 0.29\ A_{626})V}{Y}$$

$$\text{Chl.b} = \frac{(23.6\ A_{645} - 4.23\ A_{663} - 0.33\ A_{626})V}{Y}$$

where A_{663} A_{645} and A_{626} = Absorbance at 663, 645 and 626 nms. V = Volume of the extract in ml and Y = Wt. of the leaf material in gms. The carotenoid content wa calculated using the formula of Jensen and Jenson (1972). It is expressed as μ gms/gm of leaf fresh weight.

$$\text{Carotenoids} = \frac{A_{480}^{\text{carotenoids}} \quad V}{25\ Y}$$

where A_{480}^{carot} = Absorbance of carotenoids at 480nm.

$$= A_{480} - 0.111\ A_{663} - 0.638\ A_{645}$$

A_{480} = Absorbance of leaf extract at 480nm.

V = Volume of the extract in ml.

Y = Weight of the leaf material in grams.

7.3 RESULTS AND DISCUSSION :

The protein content in the stromal and thylakoid fraction of the b,8 and 10 day old normal and virescent leaves are given in table:7.3.1. At the b-day old stage, about 40% reduction in protein content of the thylakoid is noticed in the mutant seedlings as compared to the normal. However, the differences between normal and virescent seedlings in the protein content of the stromal fraction is very much reduced. Only about 22% reduction is noticed at the 6 day old stage, in the stromal proteins of virescent seedlings as compared to 6 day old normal seedlings. At the 8th day old stage, the virescent leaves show about 18% reduction in the thylakoid proteins. In contrast, the virescent leaves show a 10% reduction in stromal proteins. The 10 day old normal and virescent leaves however do not show significant differences in either the stromal or the thylakoid protein content.

Fig.22 shows the electrophoretic separation of the proteins of the stroma fraction of b,8 and 10 day old normal and virescent leaves. Lanes '1' and '~z' represent the stromal peptides of 6 day old normal and mutant seedlings respectively. Atleast 18 bands are clearly seen in each of the lanes. However, no difference is noticed in these two

Table 7.3.1 : Thylakoid and stromal protein content in the seedlings of normal and mutant genotypes

S.No	Days after germination	Genotype	Thylakoid* Protein	Stromal* Protein
1	6	et+ et+	973.84	615.18
2	6	et et	588.11	471.03
3	8	et+ et+	1285.36	839.10
4	8	et et	1051.94	756.94
5	10	et+ et+	1410.90	1092.94
6	10	et et	1389.31	1078.56

* µ gms/gm leaf fresh weight

Each value is an average of atleast 5 experiments

lanes either in the number or in the intensity of bands. Equal amounts of protein are loaded in both the lanes. Lanes 3 and 4 show the separation of proteins in the 8-day old normal and virescent seedlings respectively. No difference is noticeable between the two lanes and further they do not differ from the proteins of 6-day old seedlings. Similarly, lanes 5 and 6 show the stromal proteins of H) day old normal and virescent seedlings and the banding patterns in the gel do not suggest any differences in the stromal proteins. Lanes 7,8,9 and 10 reveal the stromal peptide separation of b and 8 day old normal and virescent leaves. Higher protein concentrations were loaded in these lanes. However, the peptide profiles remain the same in the soluble fraction of the normal and virescent leaves.

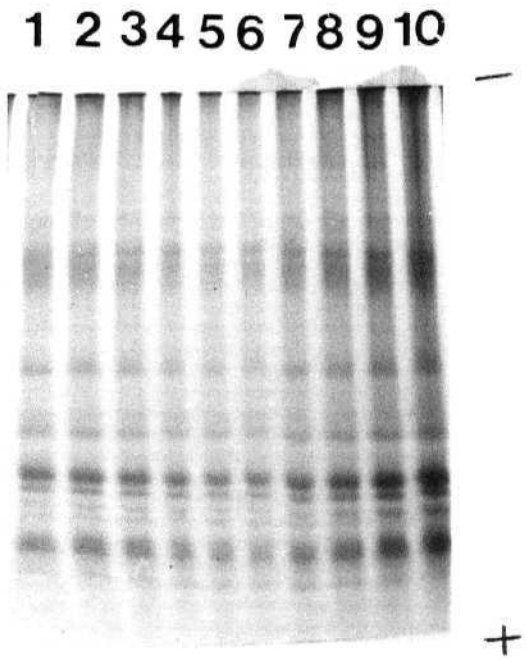
Fractionation of thylakoid extracts of normal and virescent leaves for their chlorophyll-protein complexes revealed the presence of at least four pigment-protein complexes and a free-pigment zone. The complexes identified on the gels are CPI, LHCP , LHCP and LHCP .
1 2 3

Fig.23 shows the electrophoretic separation of the pigment-protein complexes-CP-I, LHCP-I,LHCP-2,LHCP-3 and the free-pigment zone. To determine the polypeptide composition of these complexes, the green bands were excised and the eluted

Fig : *22* Electrophoretic profile of stromal proteins

Lane	1 - 6	day old	normal leaves
	2	"	virescent
	3 - 8	"	normal
	4	"	virescent
	5 - 10	"	normal
	b	"	virescent
	7 - 6	"	normal
	8	"	virescent
	y - 8	"	normal
	10	"	virescent

22



protein was reelectrophoresed. Fig:24 shows the polypeptide composition of the above mentioned complexes of the bday old normal and virescent leaves. Lane 1 and 2 show the polypeptide profiles of the UP complex of normal and mutant seedlings respectively. Lane ^{1} 1 shows seven polypeptides in the molecular-weight range of 94- 20 kD. In lane 2, only five very faint bands are observed.

Two polypeptides in the 43 kD mol.wt.region are present in extremely low quantities, if not absent, in the b-day old virescent seedlings. Polypeptide compositon of the complexes LHCP and LHCP of normal leaves are shown in lane 3. only three of the eight polypeptides observed in Lane 3, are noticed to be contaminants of UP ^{1} ^{2}. Lane 4 gives the polyeptide profiles of LHUP and LHUP ^{1} of virescent leaves of b day old mutant seedlings. Polypeptides in the molecular ^{1} ^{2} weight range of *67* kD are clearly found to be present in very low quantities as observed in lane 4. Further a 20 kD polypeptide is clearly absent in LHUP and LHUP complex of the b day old mutant seedlings. Lanes S and 6 show the ^{1} ^{2} polypeptide composition of LHCP of normal and virescent leaves respectively. Polypeptide composition of LHCP is found to be similar in both mutant and normal seedlings, although they stained less intensely in the mutant. Lanes 7

and 8 show the reelectrophoresis of the tree-pigment zone of normal and virescent leaves. As expected they do not stain for protein in both the lanes.

Fig.25 shows the electrophoretic separation of the cytochrome complex from the thylakoid extracts of normal and virescent 6-day old leaves. Lane 1 shows the control (Cyt.c -mol.wt.12,800). Lane 2 shows the cytochrome complex of the 6 day old normal leaves and lane 3 of the mutant leaves. Normal and mutant seedlings do not show any difference in the intensity of staining of the cytochrome complex.

Table 7.3.2 gives the photosynthetic electron transport (whole chain, PSI and PSII) rates of 6,8 and 10 day old normal and virescent leaves in terms of the chlorophyll content of the leaf. It is observed that the PSI, PSII and the whole chain electron transport rates are unaffected in the mutant at 6,8 and 10 day old stages.

Table 7.3.3 gives the results of the pigment levels in normal and virescent 6 day old leaves after treatment with gibberellic acid. Chlorophyll,a and chlorophyll.b and carotenoid levels were estimated after 4,8 and 24 hours of incubation of the leaves in hormone solution. No significant difference was noticed in the pigment levels after GA

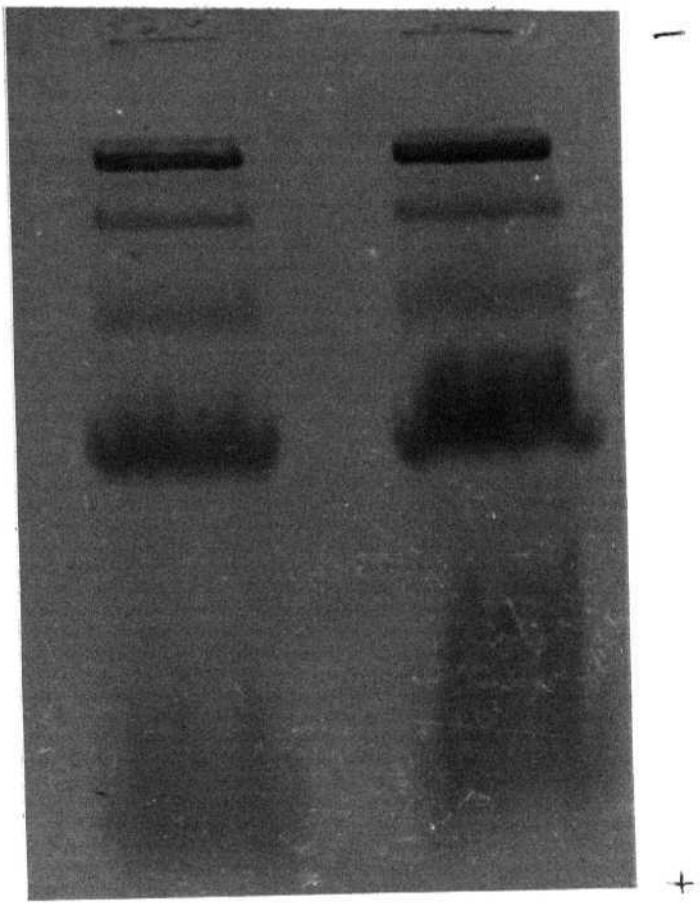
Fig : 23 Electrophoretic profile of thylakoid chlorophyll-protein complexes

Lane 1 - 6 day old normal leaves
2 " virescent "

Fig : 24 Polypeptide profiles of individual complexes

Lane 1 - CPI of 6 day old normal leaves
2 " " virescent "
3 LHCP1,LHCP2 " normal "
4 " " virescent "
5 LHCP3 " normal "
6 " " virescent "
7 free pigment " normal "
8 " " virescent "

23



24

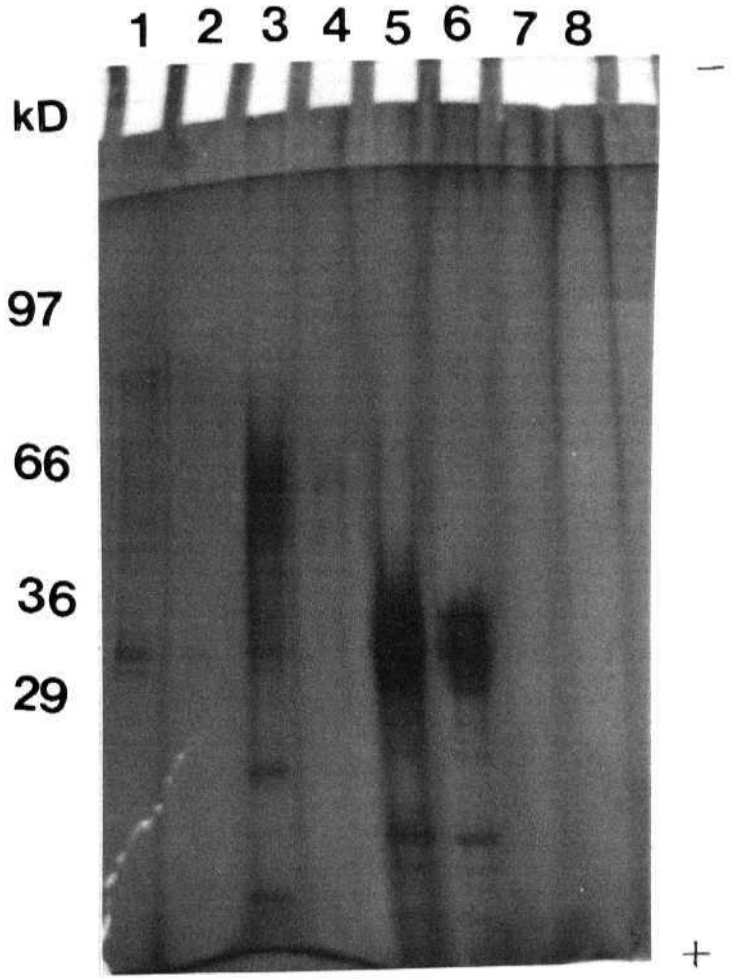


Fig : 25 Electrophoretic profile of cytochrome complex

Lane 1 - control
2 - 6 day old normal leaves
3 " virescent »


25
1 2 3
−
+

Table 7.3.2. PHOTOSYNTHETIC ACTIVITY IN LEAVES OF NORMAL AND MUTANT SEEDLINGS

S.No.	Days after germination	Genotype	Whole chain $H_2O \rightarrow MV$ (μmoles O_2/mgchl/hr) Consumed	PS I TMPD $\rightarrow$ MV (μmoles O_2/mgchl./hr) Consumed	PS II $H_2O \rightarrow$ DCPIP (μ.moles DCPIP/mgchl/hr.) reduced
1.	6	$\underline{et}^{+}\underline{et}^{+}$	128 ± 16	378 ± 14	185 ± 15
2.	6	$\underline{et}\ \underline{et}$	131 ± 10	367 ± 16	173 ± 13
3.	8	$\underline{et}^{+}\underline{et}^{+}$	136 ± 11	412 ± 23	188 ± 10
4.	8	$\underline{et}\ \underline{et}$	134 ± 6	398 ± 11	193 ± 8
5.	10	$\underline{et}^{+}\underline{et}^{+}$	160 ± 13	415 ± 18	198 ± 8
6.	10	$\underline{et}\ \underline{et}$	152 ± 15	423 ± 20	210 ± 17

Table 7.3.3 : Pigment content in 6-day old leaves of normal and mutant genotype after GA treatment

S.No	Genotype	Time of incu-bation (hours)	chl.a (µgms/ gm.leaf	chl.b (µgms/ gm.leaf	chl.a / chl.b	Total chloro (µgms/gm. leaf)	carote-noids (µgms/ gm.leaf)	chloro-phyll / caro-tenoid
1	$\frac{+}{et}\ \frac{+}{et}$	-	340.54	264.44	1.287	604.98	58.36	10.36
2	$\frac{et}{et}$	-	249.62	222.18	1.123	471.80	43.19	10.92
3	$\frac{+}{et}\ \frac{+}{et}$	4	342.63	258.81	1.323	601.44	54.86	10.96
4	$\frac{et}{et}$	4	230.86	201.70	1.144	432.56	43.01	10.05
5	$\frac{+}{et}\ \frac{+}{et}$	-	332.60	270.86	1.227	603.46	60.04	10.05
6	$\frac{et}{et}$	-	240.91	201.08	1.198	441.99	46.11	9.58
7	$\frac{+}{et}\ \frac{+}{et}$	8	321.31	246.84	1.301	568.15	52.67	10.78
8	$\frac{et}{et}$	8	209.81	179.86	1.166	389.67	41.82	9.31
9	$\frac{+}{et}\ \frac{+}{et}$	-	311.21	250.91	1.240	562.12	65.67	8.55
10	$\frac{et}{et}$	-	228.36	199.31	1.145	427.67	40.88	10.46
11	$\frac{+}{et}\ \frac{+}{et}$	24	246.63	165.66	1.488	412.29	47.79	8.62
12	$\frac{et}{et}$	24	185.51	143.74	1.290	329.25	39.32	8.37

treatment. Similarly, table 7.3.4 and 7.3.5 show the pigment levels after incubation of normal and virescent leaves in IAA and sucrose respectively. As is evident from the table, no significant differences were noticed between normal and virescent leaves in response to the treatments given. Table 7.3.6 gives the pigment levels after incubation of leaves in kinetin. After 4 hours of incubation in kinetin solution a slight increase in pigment level was noticed both in the 6 day old normal and virescent leaves. However, no difference was noticed in the response of the normal and virescent leaves to kinetin treatment.

The observations on chloroplast studies suggest that the stromal protein content is reduced in the virescent leaves though the extent of reduction is not as much as that ot the lamellar proteins. However, absolutely no qualitative difference was detectable in the protein profiles ot the stroma of normal and virescent leaves at all the seedling stages studied. Further, significant differences were noticed in the polypeptide profile of the individual chlorophyll-protein complexes of the normal and virescent leaves. Intenity of the staining of the bands and also the number of bands were found to differ between the two genotypes.

Earlier studies on the etched mutant, clearly showed

Table 7.3.4: Pigment content in 6-day old leaves of normal and mutant genotypes after IAA treatment

S.No	Genotype	Time of incu-bation (hours)	chl.a (µgms/ gm.leaf)	chl.b (µgms/ gm.leaf)	chl.a / chl.b	Total chloro (µgms/gm leaf)	carote-noids (µgms/ gm.leaf	chloro-. phyll caro-tenoid
1	$\underline{et}^{+}\ \underline{et}^{+}$	-	340.54	264.44	1.287	604.98	58.36	10.36
2	$\underline{et}\ \underline{et}$	-	249.62	222.18	1.123	471.80	43.19	10.92
3	$\underline{et}^{+}\ \underline{et}^{+}$	4	338.86	258.34	1.311	597.20	53.17	11.23
4	$\underline{et}\ \underline{et}$	4	241.81	216.64	1.116	458.45	41.80	10.96
5	$\underline{et}^{+}\ \underline{et}^{+}$	-	332.60	270.86	1.227	603.46	60.04	10.05
6	$\underline{et}\ \underline{et}$	-	240.91	201.08	1.198	441.99	46.11	9.58
7	$\underline{et}^{+}\ \underline{et}^{+}$	8	326.83	269.70	1.211	596.53	56.64	10.53
8	$\underline{et}\ \underline{et}$	-	239.87	198.39	1.209	438.26	45.89	9.55
9	$\underline{et}^{+}\ \underline{et}^{+}$	-	311.21	250.91	1.240	562.12	65.67	8.55
10	$\underline{et}\ \underline{et}$	-	228.36	199.31	1.145	427.67	40.88	10.46
11	$\underline{et}^{+}\ \underline{et}^{+}$	24	252.38	226.84	1.112	479.22	58.14	8.24
12	$\underline{et}\ \underline{et}$	24	208.68	171.93	1.213	380.61	32.63	11.66

Table 7.3.5 : Pigment levels in 6-day old leaves of normal and mutant genotypes after sucrose treatment

S.No	Genotype	Time of incubation (hours)	chl.a (µgms/gm.leaf)	chl.b (µgms/gm.leaf)	chl.a / chl.b	Total chloro (µgms/gm leaf)	carotenoids (µgms/gm.leaf	chloro-. phyll caro-tenoid
1	$\frac{+}{et}\ \frac{+}{et}$	-	340.54	264.44	1.287	604.98	58.36	10.36
2	$\frac{et}{et}$	-	249.62	222.18	1.123	471.80	43.19	10.92
3	$\frac{+}{et}\ \frac{+}{et}$	4	335.52	270.10	1.242	605.62	64.83	9.34
4	$\frac{et}{et}$	4	252.83	231.92	1.047	484.75	49.15	9.86
5	$\frac{+}{et}\ \frac{+}{et}$	-	332.60	270.86	1.227	603.46	60.04	10.05
6	$\frac{et}{et}$	-	240.91	201.08	1.198	441.99	46.11	9.58
7	$\frac{+}{et}\ \frac{+}{et}$	8	346.83	269.80	1.285	616.63	70.68	8.72
8	$\frac{et}{et}$	8	240.11	220.90	1.086	461.01	51.82	8.89
9	$\frac{+}{et}\ \frac{+}{et}$	-	311.21	250.91	1.240	562.12	65.67	8.55
10	$\frac{et}{et}$	-	228.36	199.31	1.145	427.67	40.88	10.46
11	$\frac{+}{et}\ \frac{+}{et}$	24	352.83	276.98	1.273	629.81	69.13	9.11
12	$\frac{et}{et}$	24	249.32	228.64	1.090	477.96	39.96	11.20

Table 7.3.6 : Pigment content in 6-day old leaves of normal and mutant genotypes after kinetin treatment

S.No	Genotype	Time of incu-bation (hours)	chl.a (µgms/ gm. leaf)	chl.b (µgms/ gm. leaf)	chl.a / chl.b	Total chloro (µgms/gm leaf)	carote-noids (µgms/ gm. leaf	chloro-. phyll caro-tenoid
1	$\frac{+}{et}\ \frac{+}{et}$	-	340.54	264.44	1.287	604.98	58.36	10.36
2	$\frac{et}{et}$	-	249.62	222.18	1.123	471.80	43.19	10.92
3	$\frac{+}{et}\ \frac{+}{et}$	4	395.86	296.28	1.336	692.14	64.21	10.77
4	$\frac{et}{et}$	4	286.29	239.61	1.194	525.90	47.83	10.99
5	$\frac{+}{et}\ \frac{+}{et}$	-	332.60	270.86	1.227	603.46	60.04	10.05
6	$\frac{et}{et}$	-	240.91	201.08	1.198	441.99	46.11	9.58
7	$\frac{+}{et}\ \frac{+}{et}$	8	372.61	282.83	1.317	655.44	63.39	10.33
8	$\frac{et}{et}$	8	262.74	230.64	1.139	493.38	47.64	10.35
9	$\frac{+}{et}\ \frac{+}{et}$	-	311.21	250.91	1.240	562.12	65.67	8.55
10	$\frac{et}{et}$	-	228.36	199.31	1.145	427.67	40.88	10.46
11	$\frac{+}{et}\ \frac{+}{et}$	24	279.56	186.66	1.497	466.22	58.81	7.85
12	$\frac{et}{et}$	24	172.80	130.69	1.322	303.49	37.71	8.04

that the chloroplastogenesis is delayed in virescent seedlings and at the 6-day old stage, the mutant leaves show a very poor membrane development and thylakoid stacking. Since the light reactions of photosynthesis take place in the thylakoid, the major reduction observed in the mutant leaves in thylakoid stacking might affect the photosynthetic electron transport chain. However, the analysis of our data on photosynthetic electron transport rates clearly reveals that the mutant and the normal leaves show similar rates of photosynthetic electron transport in terms of the chlorophyll content of the leaf. Further, the results also show that the hormone treatment on seedlings and cut leaves do not increase the pigment content drastically. Therefore, the etched kernels give rise to seedlings which are virescent even after treatment with the hormones studied.

8. SUMMARY AND CONCLUSIONS

Maize is one of the most amenable systems among higher plants for developmental genetic analysis. A number of well defined morphological and developmental mutants, affecting virtually every stage of its life-cycle have been documented. One such interesting mutant called *etched*, affects both kernel and seedling phenotypes.

This work deals with studies on various aspects of the *etched* mutant. The inheritance and phenotypic expression of the *et* allele has been investigated by a set of appropriate genetic crosses, mainly test-crosses and selfing. scanning electron microscopy was performed to investigate the etching patterns in *et et* kernels of the selfed ears. Starch grains were isolated and observed under the SEM. Starch content in developing as well as in germinating kernels and seedlings have been estimated. Also qualitative and quantitative analysis of certain enzymes like amylases, peroxidases and catalases have been performed. Effect of certain phyto-hormones on the pigment levels of the normal and mutant genotypes has been tested. Chloroplasts have been isolated from *et et* seedlings and their biochemical properties have been investigated. Stromal peptide profiles and polypeptides of certain chlorophyll-protein complexes and cytochrome complex were analysed by standard electrophoretic procedures. The levels of carboxylating enzymes RuBP-carboxylase and PEP-carboxylase were estimated in normal and mutant seedlings at different stages.

The results are summarised below :

1. Data from selfing of the F_1 heterozygotes and test-crossing revealed that the *et* allele is inherited as a Mendelian recessive. Further, *et* allele was found to be 100% penetrant.

2. The extent of etching on *et et* kernels was found to be variable among segregating ears as well as among the *et et* kernels on the same ear. An etched-phenotype scale was prepared based upon the variation exhibited in the extent and depth of cracks on the endosperm. Plants grown from kernels of defined type were selfed and the progeny was classified as per the scale. The results revealed that the extent of etching appears to be highly variable and none of the specific phenotypes are found to breed true tor that particular phenotype. It can be concluded that *et* allele shows variable expressivity.

3. All etched kernels were found to exhibit virescene at seedling level, under defined conditions.

4. The etched endosperms were subjected to scanning electron microscopy tor a detailed analysis of etching. The data revealed gaps which are full of starch-less broken cells. The deep cracks on the endosperm clearly revealed the total absence of cells.

5. Isolated starch grains of normal and mutant kernels show similar grain size and shape as revealed by scannning electron microscopy.

6. Developing mutant kernels of 20 UAH stage show a significant reduction in starch content as compared to normal kernels at the same stage. However, at the 30 and 40 DAP stages, the differences in starch content between normal and mutant kernels are very much reduced.

7. The difference noticed in the starch content at 4u UAP stage is also reflected in mature kernels. However, at the later stages of germination, the kernels of normal and mutant genotypes do not show any differences in starch content.

8. The starch levels during germination of mutant kernels remain constant, whereas, the normal kernels show a significant reduction during the same period.

9. Amylolytic enzyme activities (α and β amylases) in et + et + kernels were found to be higher than that of mutant. The total amylase and fc-amylase trends during germination differed between normal and mutant kernels and seedlings. On the other hand, the <x-amylase profiles were found to be similar in both genotypes. Electrophoretic analysis of amylases of both genotypes during germination and greening did not reveal any qualitative differences in the isoenzyme pattern.

10. Peroxidase activity profiles were found to follow similar patterns in normal and mutant kernels and seedlings. However, peroxidase isozyme profiles of mutant and normal seedlings were found to differ significantly.

11. The *etched* and normal kernels and seedlings did not reveal any differences in catalase activity profiles and isozyme patterns, although slight differences were noted at the quantitative level.

12. Hormone treatment (GA, IAA and Kinetin) of both mutant and normal seedlings during germination did not significantly alter the pigment content.

13. Polyacrylamide gel electrophoresis of stromal fraction did not reveal any difference between normal and mutant seedlings.

14. Elution of chlorophyll-protein complexes and their reelectrophoresis revealed that certain poiypeptides of CP1 and LHCP complex are not detectable in the virescent seedlings.

15. TMBZ staining of the thylakoid fraction did not show any differences in the cytochrome complex of normal and mutant genotypes indicating that the cyt. complex is unaltered in the mutant.

16. Measurement of photosynthetic electron transport activities of thylakoid preparations of normal and mutant genotypes show similar photosynthetic rates in terms of chlorophyll content indicating that the photosynthetic electron transport chain is not affected by the *etched* mutation.

17. Level of RuBP carboxylase is found to be lower in virescent leaves and definite correlation was observed in the rate of synthesis of RuBP carboxylase and chlorophyll.

18. PEP carboxylase levels were found to be slightly lower in the virescent "leaves of the etched mutant indicating differences in the carboxylating enzyme levels between the normal and virescent seedlings.

In summary, the results establish the influence of the etched mutation on the endosperm and seedling phenotypes. Further, the genetic inseparability of virescence and etched traits is substantiated. It is suggested that the et mutation belongs to a unique class of developmental virescent mutants, whose phenotypic expression is reversible and influenced by light and temperature or an interaction between the two.

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The effect of the etched (et) mutation on the amylolytic enzyme activities in germinating kernels and seedlings of *Zea mays*

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Summary. The etched *(et)* mutation in maize causes distinct depressions and structural gaps in the endosperm and also gives rise to virescent seedlings. a- and β-Amylase activities were observed to be higher in et^+ et^+ kernels and seedlings as compared to that of the *et et* mutant. The total amylase and β-amylase trends during germination also differed between normal and mutant kernels and seedlings (it increases in the wildtype and decreases in *et et).* On the contrary, the overall α-amylase trend was found to be similar in both genotypes (slight decrease during germination). The native gel electrophoresis of crude enzyme extracts did not reveal any qualitative differences in a and β amylases during germination. The germinating *et et* kernels initially showed lower levels of starch compared with the wild type kernels, whereas no such difference was found at later stages of germination. It is concluded that *et* gene associated endosperm lesions lead to an impairment of starch degradation in germinating kernels resulting in virescent seedlings.

Key words: Etched – Vircsccnce – Starch – Amylolytic enzymes

Introduction

In maize, of the many known endosperm mutants, the etched *(et)* mutant (Stadler 1940) is unique for its distinct kernel and seedling phenotypes. Kernels homozygous recessive for *et* allclc (3L; 153) exhibit the so-called etched phenotype, i.e., cracks or depressions in the endosperm, whereas the seedlings express vircsccnce during early stages of growth. (Neuffer et al. 1968; Coe and Neuffer 1977; Coe et al. 1983). The characteristic virescence phenotype of *etet* seedlings has been recorded both under field and laboratory conditions. Under certain defined conditions of light and temperature, *et et* seedlings begin their growth with leaves devoid of chlorophyll and gradually turn normal green in about 10 days after germination (Ramesh 1983). At this stage, both normal (et^+ et^+) and mutant *(et et)* seedlings are phenotypically indistinguishable. Standard in vitro procedures as well as photoacoustic spectral analysis revealed significant quantitative differences in chlorophyll and carotenoids during the greening process under light (Ramesh et al. 1984; Sangeetha 1985). It was observed that *et et* seedlings accumulate reduced levels of chlorophyll and carotenoids up to the 8th day. Transmission electron microscopy of chloroplast preparations revealed that chloroplastogenesis is delayed in mutant seedlings. Further, SDS-PAGE analysis of solubilized thylakoid membrane extracts of virescent leaves showed reduced levels of chlorophyll-protein complexes (Sangeetha 1985; Sangeetha et al. 1986).

The association of *et* mutation with both kernel as well as the seedling phenotypes is interesting. The inseparability of these phenotypes by genetic crossing-over led to the conclusion that these two traits are controlled by the *et* gene itself. Although an absolute correlation between endosperm defect and seedling virescence in *et* mutant has been unequivocally established, the underlying biochemical and physiological basis is not yet understood.

The present study attempts to look into the nature of the relationship between kernel and seedling phenotypes of the *et et* mutant. We report here on starch-degrading enzyme activities in *et et* kernels during germination as well as greening of seedlings.

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Materials and methods

The isogenic maize lines of homozygous normal (et^+ et^+) and mutant *(et et)* genotypes arc in the background of line R168. Mature kernels were soaked in water for 8-10 h, surface sterilized with sodium hypochlorite and germinated in vermiculite. The seedlings were maintained at 25±2"C under continuous illumination with cool fluorescent lights (4-5 watts/m^2).

Determination of starch content in germinating kernels and seedlings

Germinating kernels of both genotypes were ground in 75% elhatiol. The suspension was heated for 30 min in a boiling water bath and centrifuged at 10,000 x g for 15 min. The starch precipitate was suspended in 0.2NKOH and boiled for 30 min and neutralized to pH 5.5 with glacial acetic acid. The suspension was incubated with amyloglucosidase (E.C. 3.2.1.3, Sigma) at 37 "C for 18 h. After centrifugation of the suspension, the supernatant was used for glucose estimation (Nelson 1944). Starch content in leaf tissue was estimated according to the procedure of Mac Rac (1971).

Estimation of total amylase, α-amylase and β-amylase in germinating kernels and leaves

The amylolytic enzyme activities were estimated in normal and mutant kernels as well as leaves. The day of soaking the seeds for germination is considered day 1 and the age of seedlings is referred to as days after germination (DAG). Kernel or leaf material was homogenized in 2 ml of chilled extraction buffer (20 mmol sodium citrate), pH 6.1. The homogenate was diluted to 7 ml with extraction buffer and then spun at 30,000 x g for 30 min. All steps were performed at 4"C. Total amylase activity was assayed in a reaction mixture (4 ml) containing 240 µmol of sodium-citrate buffer pH 6.1, 8 mg soluble starch, 6 µmol sodium flouride and 500 µl of enzyme extract. Aliquots of 500 µl were withdrawn at 0, 30 and 60 min and the reaction was stopped by adding 500 µl of 3,5 dinitrosalicylic acid reagent (Bernfield 1955) and boiling for 5 min in a water bath. After cooling, the mixture was diluted with distilled water to a final volume of 6 ml. Reducing sugars were determined colorimetrically at 540 nm using maltose as a standard. α-Amylase was assayed under the above mentioned reaction conditions, except β-limit dextrin was the substrate instead of soluble starch.

For estimation of β-amylase, the enzyme extract was preincubated with 5 m*M* EDTA for 48 h and then assayed as described. Micromoles of maltose liberated/second was taken as the unit of enzyme activity. Enzyme activity was expressed as nkat/mg seed and nkat/gm leaf. Protein estimations were according to the procedure of Lowry et al. (1951).

Native gel electrophoresis of amylases in crude enzyme extracts of kernels and leaves was performed at 4"C according to Davis (1964). Amylases were visualized on gels by the negative staining method of Work and Work (1972).

Results

Total a- and ft-amylase activities in germinating kernels

The total amylase and specific activity profiles of mutant and normal kernels (3, 4 and 5 DAG) are shown in Fig. 1 a. The total amylase levels and specific activity of

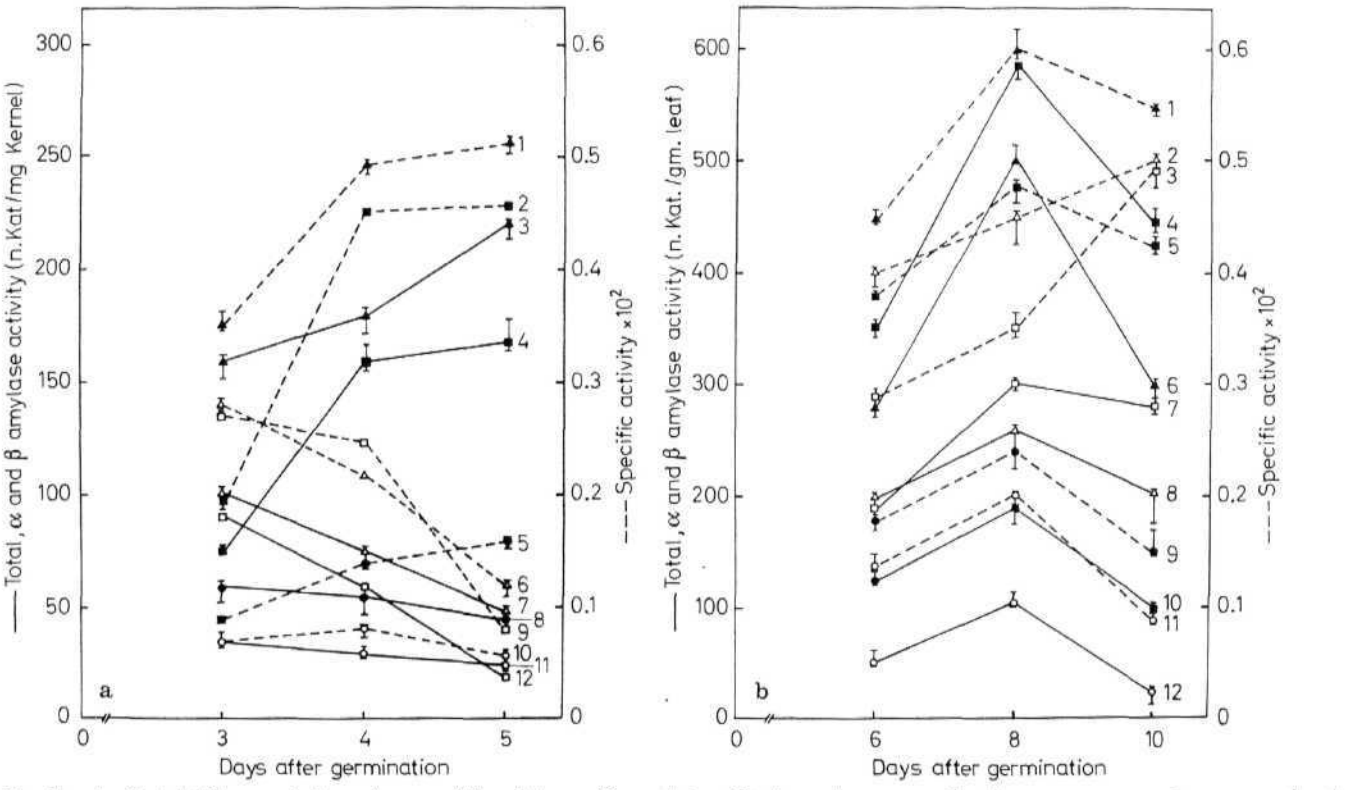

Fig. 1 a–b. Total (T), α and β amylase profiles. SA-specific activity. Total amylase: ▲ = et^+ et^+ △ = *et et*; α amylase: ● = et^+ et^+ ○ = *et et*; β amylase: ■ = et^+ et^+ □ = *et et*. (**a**) Germinating kernels: *1* = T, SA; *2* = β, SA; *3* = T; *4* = β; *5* = α, SA; *6* = T, SA; *7* = T; *8* = α; *9* = β, SA; *10* = α, SA; *11* = α; *12* = β. (**b**) Developing seedlings: *1* = T, SA; *2* = T, SA; *3* = β, SA; *4* = β; *5* = β, SA; *6* = T; *7* = β; *8* = T; *9* = α, SA; *10* = α; *11* = α, SA; *12* = α. Each data point represents an average of 5–6 experiments

amylase in $et^+ et^+$ kernels increased between the 3rd and 5th day after soaking, while in the *et et* kernels they decreased during the same period. The total amylase content was always significantly higher in the $et^+ et^+$ kernels as compared with the *et et* kernels.

α-Amylase activity profiles are shown in Fig. 1 a. The α-amylase level of $et^+ et^+$ kernels was observed to be significantly higher than that of *et et* kernels at all three stages studied. However, both in the mutant and normal kernels α-amylase related data follow the same trends, i.e., they decreased slightly during germination.

The β-amylase activity profiles are also shown in Fig. 1 a. The β-amylase levels increased from the 3rd to the 5th day after germination in $et^+ et^+$ kernels, whereas its levels actually decreased in the *et et* kernels. Further, β-amylase levels were observed to be significantly higher in $et^+ et^+$ kernels of 4 and 5 DAG compared with that of *et et.*

Total a- *and fi-amylase activities in leaves*

Total amylase profiles of seedlings (6, 8 and 10 DAG) are shown in Fig. 1 b. It was observed that amylase levels were significantly higher in normal ($et^+ et^+$) leaves than that of virescent *(et et)* leaves. Total amylase levels in leaves of both genotypes peaked at 8 DAG. Similarly, the specific activity of total amylase peaked on the 8th day in $et^+ et^+$ seedlings and then decreased. On the contrary, specific activity of total amylase in *et et* seedlings increased from the 6th to the 10th DAG.

The a-amylasc profiles of leaves harvested from seedlings of 6, 8 and 10 DAG are shown in Fig. 1 b. The a-amylase level was significantly higher in normal leaves as compared with the virescent leaves at 6, 8 and 10 days after germination. The α-amylolytic profiles and specific activity profiles peaked on the 8th day in both $et^+ et^+$ and *et et* leaves.

The β-amylase profiles in seedlings (6, 8 and 10 DAG) are shown in Fig. 1 b. β-Amylasc activity was significantly higher in normal leaves as compared with virescent leaves. β-Amylase activiy peaked on the 8th day in $et^+ et^+$ leaves, whereas it increased up to the 10th day in *et et* leaves.

Electrophoretic analysis

Electrophorctic analysis of amylases in $et^+ et^+$ and *et et* kernels during germination and in the seedlings during greening did not reveal any qualitative differences in the isozyme pattern (data not shown). It was concluded that the *et* mutation affects only the level but not the isozymic pattern of amylases.

Correlation between amylase levels and starch content

The mature *et et* kernels accumulated significantly lower levels of starch (42.84% fresh weight) compared with that of $et^+ et^+$ kernels (68.31% fresh weight). Similarly, germinating kernels of the 3 DAG stage also showed the same trend ($et^+ et^+$ −56.39%; *et et* −40.22%). On the contrary, there was no difference between *et et* (4 DAG -39.86%; 5 DAG -41.86%) and $et^+ et^+$ kernels (4 DAG.....43.11%; 5 DAG -42.30%) at 4 and 5 DAG stages. Mutant kernels consistently showed the same amount of starch during germination (1-5 DAG), whereas normal kernels indicated a decrease. Leaves of both *et et* and $et^+ et^+$ seedlings at 6, 8 and 10 DAG exhibited similar levels of starch. Further, the starch profiles of growing seedlings were the same for both genotypes.

Discussion

The present data on starch hydrolyzing enzymes during germination of kernels suggest that the total amylase levels are significantly reduced by *et* mutation. This could be due to an impaired movement of amylases or of their reaction products caused by structural gaps in the endosperm, as revealed by Scanning Electron Microscopic analysis (data not shown). Similarly, β-amylase levels also show such a reduction during germination. In *et et* kernels, a-amylase also shows significantly reduced activity. Taken together, these data suggest that during germination of *et et* kernels, the starch-hydrolyzing enzymes are somehow affected by the structural discontinuity in the endosperm, which might in turn lead to an early virescence of seedlings. It is unlikely that the *et* gene has any direct effect on the expression of genes encoding amylases.

The amylolytic enzyme activity profiles in the growing seedlings under light did not show any significant deviations from that of the germinating kernels, indicating that the *et* effect continues up to 10 DAG. These observations lead to the suggestion that the virescence of *et et* seedlings has its origin in the endosperm lesions caused by *et* allele during kernel development. This conclusion is supported by the following observations: (1) the mutant kernels consistently show the same amount of starch during germination (1–5 DAG), whereas normal kernels show a decrease; (2) leaves of both $et^+ et^+$ and *et et* seedlings were found to have similar levels of starch at all three stages studied; (3) the leaves of young virescent seedlings (6-8 DAG), when grown under light, accumulate greatly reduced amounts of chlorophyll pigments as shown by in vivo and in vitro procedures (Ramesh 1983; Ramesh et al. 1984); and (4) chloroplastogenesis is delayed in virescent seedlings as shown by transmission electron microscopy and this transient delay is reversed by 10 DAG (Sangeetha et al. 1986).

In summary, we show here that there is a positive correlation between the impairment of starch degrada-

tion during germination of *et et* kernels and virescence of seedlings. However, there could be several other factors leading to virescence, besides the *et* mediated lesions in the endosperm. It has been reported that the virescent mutant of maize *v16* has a deficiency of chloroplastic 16s and 23s rRNA (Hopkins and Elfman 1984). Interestingly, the kernels of *v16* are phenotypically normal as in the case of other virescent mutants known in maize. The availability of a number of non-allelic virescent mutants simplifies the task of elucidating the causes leading to virescence. A comparative analysis of several such non-allelic virescent mutants that do not show endosperm lesions is in progress.

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