INDUCTION OF LEUCINE AUXOTROPHS IN *Neurospora crassa* AND THEIR GENETICAL & BIOCHEMICAL INVESTIGATIONS

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Dedicated to my mother

and

to the memory of my father

INDUCTION OF LEUCINE AUXOTROPHS IN *Neurospora crassa* AND THEIR GENETICAL & BIOCHEMICAL INVESTIGATIONS

A dissertation submitted for the degree of Doctor of philosophy to the Department of Botany under the faculty of Biological Sciences of the University of Dhaka

By

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May 2006

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DECLARATION

I hereby declare that this thesis entitled 'Induction of *leucine* auxotrophs in *Neurospora crassa* and their genetical &biochemical investigations" has been composed by myself and all the works presented herein are my own. I further declare that this work has not been submitted any where for any academic degree.

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CERTIFICATE

This is to certify that this thesis contains the results of research on "Induction of *leucine* auxotrophs in *Neurospora crassa* and their genetical & biochemical investigations" and has been carried out by Tasmin Akter under my supervision. It is further certified that the work presented here is original and suitable for submission as a Ph.D. thesis.

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List of Abbreviation

%	=	Percentage
ade-1	=	*adenine*-1
arg-5	=	*arginine*-5
arg-10	=	*arginine*-10
αβIC	=	α hydroxy-βcarboxy isocaproate
αKIC	=	α-keto isocaporoate
αKIV	=	α- keto isovalerate
ββIC	=	βcarboxy β-hychoxy isocaproate
b	=	Buffer
c	=	conidia formed
cg	=	conidia germinating
cm	=	Centimeter
CM	=	Centimorgan
Co-n	=	comb- needle
Conc	=	Concentration
Em'a'	=	Emerson stock Mating type 'a'
Em'A'	=	Emerson stock Mating type 'A'
g	=	Gram
his-2	=	*histidine*-2
hrs	=	Hours
i.e.	=	idest (that is)
leu-1	=	*leucine*-1
leu-2	=	*leucine*-2
leu-3	=	*leucine*-3
leu-4	=	*leucine*-4
leu-5	=	*leucine*-5
lys	=	*Lysine*
M	=	Mole
met-2	=	*methionine*-2

mg = milligram

Min = minute

ml = milliliter

mm = millimeter

mM = milli mole

nm = nano meter

nt = *nicotinic acid*

PEG = poly Ethylene Glycol

pyr-1 = *pyrimidine*-1

rpm = rotation per minute

sec = second

SM = Sorbose minimal medium

trp-1 = *tryptophan*-1

trp-5 = *tryptophan*-5

UV = Ultra Violet

VM = Vogel's minimal medium

WCM = Westergard`s Crossing medium

ABSTRACT

Seventy nine biochemical mutants were UV-induced and isolated by following the modified technique of Filtration Enrichment Method from *Neurospora crassa* Em'a'(5297) using 3-4 days old conidia. They were classified into five nutritional groups: tryptophan 26, leucine 28, lysine 10, histidine 9 and adenine 6 in numbers. Only the leucine mutants were studied.

The 28 leucine mutants were tested for heterocaryon test in all possible pair-wise combination in solid, liquid and hanging drop methods. All the mutants were also tested for complementation with the standard 5 leucine markers [*leu*-1(33757)IIIa, *leu*-2(37501)IVa, *leu*-3(R156)Ia, *leu*-4(R359)Ia and *leu*-5(45208(t)Va]. Six leucine mutants (*leu* M2a, leu M6a, *leu* M8a, leu M9a, *leu* M13a and *leu* M52a) belong to locus *leu* 1(33757)IIIa, one leucine mutant (*leu* M51a) to locus *leu*-2(37501)IVa, three (*leu* M7a, *leu* M59a and *leu* M62a) to locus *leu*-3(R156)Ia and three (*leu* M5a, *leu* M42a and *leu* M138a) to locus *leu* 4(R359)Ia, but all the leucine mutants are positively complemented with *leu*-5(45208(t))Va. The rest 15 showed positive complementation with all the 5 leucine standard markers. A complementation map is prepared from the complementation matrix with Roman numerals I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII and XIV represent the 14 groups of mutants. The map has 11 complons designated by A, B, C, D, E, F, G, H, I, J and K. The complementation map of 15 leucine mutants is linear and complex but only continuous defects were found in all of the mutants.

The effect of different concentrations of both the gastric and intestinal extracts on release of protoplasts from the three leucine mutants (*leu* M9a, *leu* M30a and *leu* M51a) were investigated. Fusion of protoplast was carried out by complementing *leu* M9a + *leu* M30a and *leu* M9a + *leu* M51a and *leu* M30a + *leu* M51a and all gave positive results. Gastric extract gave the best result than that of the intestinal extract with a ratio of 1:4 compared to the other ratios (3:2 & 2:3).

The fertility and mating type of the 28 leucine mutants were tested by crossing them with Em'A'(5296) and Em'a'(5297) respectively, all were fertile with Em'A', showing no changes of mating type during the mutation. When the segregation data was analyzed, it was observed that the wild type and mutant progenies were segregating in 1:1 ratio. This confirms the mutation in the nuclear gene and not in the cytoplasm or due to any suppression.

The linkages of the fertile crosses were studied and the distance of the mutant loci were calculated. Eight leucine mutants (*leu* M11a, *leu* M23a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a and *leu* M202a) were found to be linked with *trp*-1(10575)A of the linkage group III; four (*leu* M1a, *leu* M3a, *leu* M9a and *leu* M25a) were found to be linked with pyr-1(H25a) A of linkage group IV; two (*leu* M4a and *leu* M8a) were found to be linked with his-2(T51M152(t))A of linkage group I and only one (*leu* M262a) was found to be linked with *trp*-5(A420)A of linkage group V. Genetic map shows that the mutant *leu* M63a is the nearest (I9.444 CM) and *leu* M23a is the furthest (38.613 CM) from *trp*-1(10575)A. On the other side, *leu* M3a is the nearest (19.047 CM) and *leu* M25a is the furthest (28.301 CM) from *pyr*-1(H263)A.

The gene order and distances between the above mentioned 15 leucine mutants with four leucine standard markers [*leu*-1(33757)IIIA, *leu*-2(37501)IVA, *leu*-3(R156)IA and *leu*-5(45208(t))VA] were found out. From the data, mutant *leu* M39a is the nearest (0.101 CM) and *leu* M146a is the furthest (0.239 CM) from *leu*-1(33757)IIIA; *leu* M9a is the nearest (0.144 CM) and *leu* M3a is the furthest (0.281 CM) from *leu*-2(37501)IVA.

CHAPTER I

INTRODUCTION

CHAPTER I
INTRODUCTION

Genetics is the study of heredity and variation. It makes important contributions to the progress of biological sciences. Critical experiments in 1952 and the discovery of the structure of DNA in 1954 by Watson and Crick changed all these. Genetics entered the DNA age and over the last decade we came to understand the chemical nature of genes and how genetic information is stored, released to a cell and transmitted from one generation to the next. Currently it is providing new tools of great economic importance and value in agriculture, medicine, microbial fermentations and genetic transfer from one to unrelated organism. This branch of genetics is known as genetic engineering.

Two recent events in the genetics arena are attracting large amounts of attention, firstly the launching of the "Human genome project" and secondly the imminent green light for the first human somatic cell "Gene therapy" experiment. Criminal law is another arena in which the spot light now focuses on genetics. "Human DNA prints" or "DNA fingerprints" are known to possess greater specificity by several orders of magnitude than "human finger prints".

Benjamin Lewin (2000) mentions, "gene (cistron) is the segment of DNA involved in producing a polypeptide chain, it includes coding regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons)."

A chromosme consists of an uninterrupted length of duplex DNA that contains many genes. A gene may have multiple alleles. Each chromosome consists of a linear array of genes. The genes are composed of DNA a molecule that lies within the nucleus of a eukaryotic cell, cytoplasmic organelles, the nucleiod of prokaryotes and in the cytoplasm (Plasmid). Genes play important roles in essentially all aspects of our

lives. They not only control what look physically, but also influence our behavior, personalities, susceptibility to diseases (either they inherited or infectious), aging processes and indeed extending the longevity. Chromosome is the bearer of gene which controls the metabolic process of life.

The experimental material, *Neurospora crassa* is a well known pink bread mold, which grows in tropical and subtropical regions. It is an excellent tool for genetical study. Thus we may study *Neurospora crassa* and can apply the knowledge and principles what we learn, to wheat, rice, humans, any other plant or animal. It is the filamentous fungus that belongs to the class Ascomycetes, Subclass Pyrenomycitadae, Order Xylariales, Family Sordariaceae. The vegetative phase consists of mycelia of branching hyphae. It is heterothallic and obviously possess two opposite mating types called 'A' and 'a'. It has distinct male and female sexual structure. *Neurospora crassa* can be easily grown on a suitable mixture of mineral salts in water plus a carbon source (sucrose) and the vitamin biotin (Beadle and Tatum (1941, 1945), Anonymous (1966). It has a simple life history in which the zygote is only diploid. Dominance is not a problem and even the recessive genes are expressed. As a consequence the unique advantages of *Neurospora crassa* for research the simple nutritional requirements, wide diversity of mutant genotypes, rapid growth of mycelia, ease of cloning from conidia, rapidity and ease of handling of the sexual cycle, haploid in nature, non-pathogenic, linear arrangement of ascospores, it is convenient to work with the organism using ordinary microbiological techniques. It has been intensively studied by geneticists, biochemists and microbiologists, *Neurospora crassa* has contributed to the understanding of diverse biological phenomena. The mutant characters have evolved due to biochemical deficiencies or morphological differences in which mutant phenotypes are distinguishable in haploid strains. It has produced two types of conidia which are macroconidia and microconidia. They are rich in carotenoids and in mass results in most of the bright orange color observed in the culture. *Neurospora crassa* can be used for any classical as well as molecular investigations and its genetics have been studied fairly intensively during the recent years (Akins and Lambowitz 1985; Yassin and Wheals 1992; Bruchez *et.al.* 1993; Perkins and Kinsey 1993; Aranson *et.al.* 1994; Maloisel *et.al* 1994; Atkinson *et.al.*

1995; & McDonald *et.al.* 1995). The ability of the fungus to produce large number of identical predominantly haploid uninucleate conidia enables such units to be used for the investigation of spontaneous and natural mutations. (Ahmad *et al* 1977; Stadller *et al* 1993; & Watters *et al* 1995).

Inheritance is based on genes that are transmitted from parents to offspring during reproduction and that the genes store genetic information encoded in the sequence of nucleotide pairs in DNA or nucleotides in RNA. The term 'mutation' refers to the change in the genetic material resulting in the subsequent development. An organism that exhibits a novel phenotype resulting from a mutation is called a mutant. Mutation is the ultimate source of all genetic variation and it provides the raw material for evolution. Mutations provide decisive evidence that DNA is the genetic material. When a change in the sequence of DNA causes an alteration in the sequence of a protein, we may conclude that the DNA codes for that protein. Furthermore a change in the phenotype of the organism may allow us to identify the function of the protein. The existence of many mutations in a gene may allow many variant forms of a protein to be compared and a detailed analysis can be used to identify regions of the protein responsible for individual enzymatic functions. Without mutation all genes would exist in only one form. Some level of mutation is essential to provide new genetic variability and allow organism to adapt to new environments (Snustad *et al* 1997).

A point mutation changes only the amino acid represented by the codon in which the mutation occurs. Point mutations may be reverted by back mutation of the original mutation. Mutation may be disadvantageous and are called deleterious. When disastrous to the individual they are called 'lethal'. Most mutations will lie at different sites but some will lie at the same position. Two independently isolated mutations at the same change in DNA or they may constitute different changes. The occurrence of mutations can be increased by treatment with certain compounds. These are called mutagens and the changes they cause are referred to as induced mutations. Most mutagens act directly by virtue of an ability either to modify a particular base of DNA or to become incorporated into the nucleic acid. The effectiveness of a mutagen is

judged by the degree to which it increases the rate of mutation above background. By using mutagens it becomes possible to induce many changes in any gene. Mutagens used by the geneticists may be of two kinds. These are physical and chemical. Physical mutagens include X-ray and UV ray. Ultra violet (UV) radiation does not possess sufficient energy to induce ionizations. It is readily absorbed by many organic molecules such as the purines and pyrimidines in DNA, which then enter a more reactive or excited state. UV rays penetrate tissues only slightly. Thus in multicellular organisms only the epidermal layer of cells usually is exposed to the effects of UV. The maximum absorption of UV by DNA is at a wavelength of 254 nm. The effect of an ultraviolet photon is very localized it is limited to one atom. Two major products of UV absorption by pyrimidines are pyrimidine hydrates and pyrimidine dimmers. Thymine dimerization is probably the major mutagenic effect of UV.

UV radiation of 254nm which was used for induction of mutation in *Neurospora crassa*. The original *Neurospora* mutants were isolated by treating conidia with physical mutagens (UV ray) and crossing the treated conidia with the opposite mating type, establishing cultures from random ascospore isolates and testing the genotype (Beadle and Tatum 1945). From over 80,000 individual ascospore isolates a wide variety of auxotrophic and morphological strains were obtained. The study on suitable mutagens for this organism and the study on molecular mechanism of mutation have been the subject of the investigation during the past years (Flavel and Fincham 1968; Drake 1970; Schroeder 1970; De Serres *et al* 1971; Ahmed *et al* 1977; Santosa and Kuwana 1992; Stephens *et al* 1993; & Vellanji *et al* 1994). Catcheside (1954) induced mutant of *Neurospora crassa* by UV irradiation using filtration enrichment method.

The *leucine* is an important amino acid. The *leucine* auxotrophs can be obtained by physical mutagens like UV. Proust isolated *leucine* from cheese in an impure form in 1819. In 1820, Braconnot isolated the crystalline amino acid hydrolyzates of muscle and wood, then gave it the name *leucine*. The *leucine* is generally more prevalent than valine or isoleucine in proteins. It is also a branched chain amino acid and is the next higher homologue of valine. As such, it has much in common with valine from the

viewpoint of reactivity and function. It is also one of the few amino acids that are sparingly soluble in water. The synthesis of amino acids by such organisms as *E.coli* and *N. crassa* has been the subject of a number of detailed investigations.

The details of *leucine* biosynthesis are known only in a few microorganisms. Biosynthesis of *leucine* takes place in the following manner:

α-ketoisovalerate + acetyl co=enzyme		
β-isopropylmalate synthetase	↓	*leu*-4
β-hydroxy β-carboxy isocaproate(α-isopropylmalate)		
α-isopropylmalate isomerase	↓	*leu*-2, *leu*-3
Dimethylcitraconate		
β-isopropylmalate dehydrogenase	↓	*leu*-2, *leu*-3
α-hydroxy β-carboxy isocaproate (β-isopropylmalate)		
β-isopropylmalate dehydrogenase	↓	*leu*-1
α-keto isocaproate		
leucine transasaminase	↓	
leucine		

[The *leucine* precursors alpha and beta-isopropylmalate were formerly called beta-OH-beta carboxyisocaproate and alpha-OH-beta carboxyisocaproate, respectively.]

Intermediates, genes and enzymes in *leucine* biosynthesis of *Neurospora crassa* is shown diagrammatically in Fig. 1-1.

The biosynthesis of *leucine* involves the following reaction sequence:

⇨ The condensation of acetyl Co-A and α-ketoisovalerate in the formation of α-isopropylmalate (α-IPM) catalyzed by α-IPM synthetase (the synthetase) (Gross and Webster 1963; Webster *et al* 1965).

⇨ The reversible isomerization of α and β-IPM catalyzed by IPM isomerase (the isomerase) (Gross *et al* 1963).

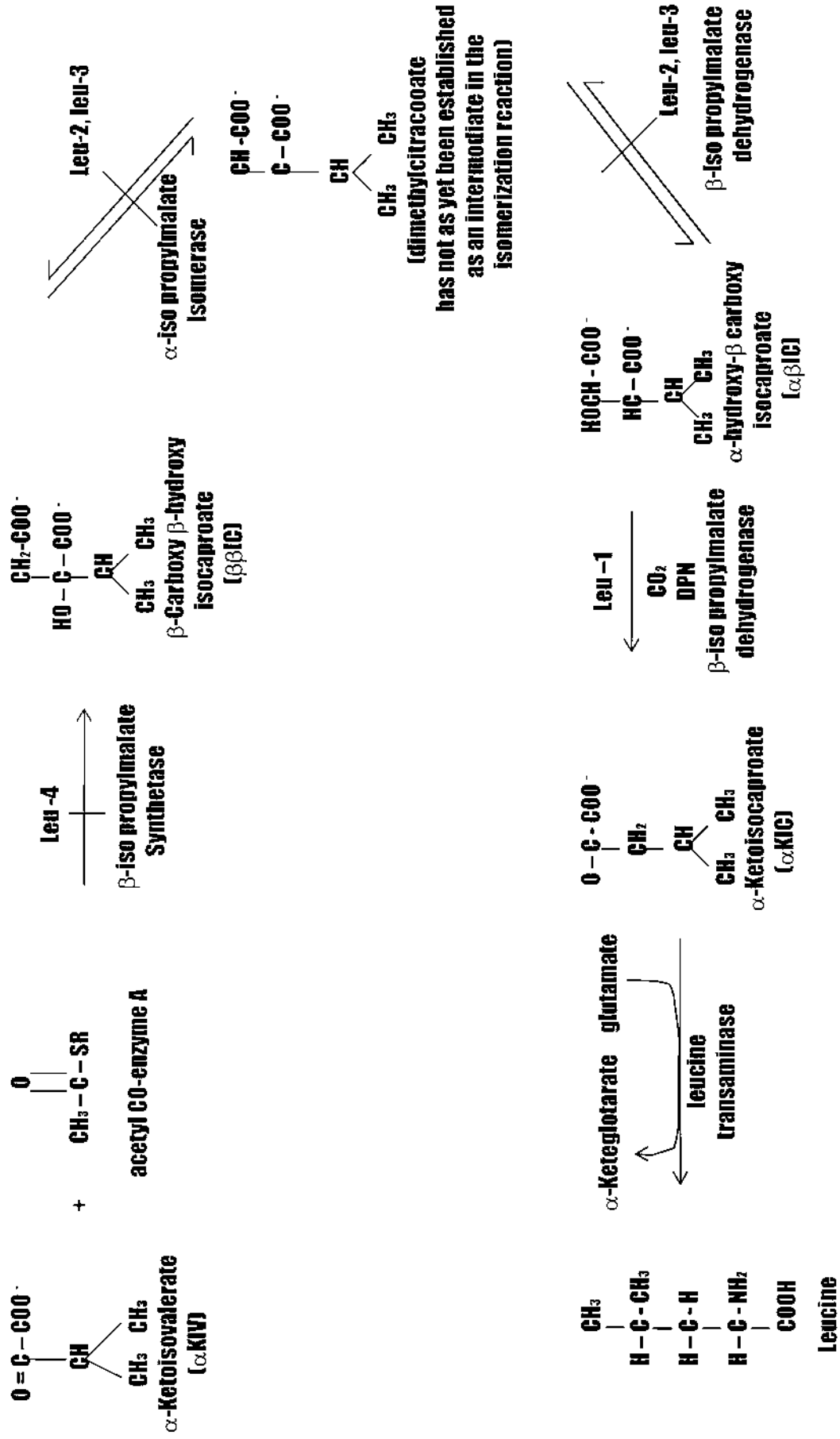


Fig. 1-1. The biosynthetic pathway to leucine.

⇨ NAD-linked dehydrogenation and decarboxylation of β IPM yielding α-ketoisocaproate the ketoacid analogue of *leucine* catalyzed by β-IPM dehydrogenase (the dehydrogenase) (Burns et al 1963). Synthesis begins by utilizing α-ketoisovaleric acid which is also the immediate precursor of valine.

The biosynthesis of *leucine* in both *Neurospora* and *Salmonella* has been elucidated (Jungwirth *et al* 1961; Gross *et al* 1962). The *leucine* auxotrophs have been used extensively for studies of regulation (S.R. Gross 1969; Polacco *et al* 1973). This mutants acquire suppressors when grown on Difco-agar sorbose medium. The suppressors are leaky auxotrophs blocked at various steps in sulfur metabolism, apparently these blocks allow more efficient use of the traces of *leucine* in the agar. Most aliphatic and aromatic amino acids can inhibit growth of *leucine* mutants at appropriate concentrations probably because of competition for a common uptake system (S.R. Gross 1969).

The biosynthesis of *leucine* is controlled by six loci in *Neurospora crassa*. These are *leu*-1, *leu*-2, *leu*-3, *leu*-4 (S.R Gross 1965) and *leu*-5, *leu*-6 (Chow *et al* 1989). The *leu*-1 lie in linkage group IIIR, *leu*-2 lie in linkage group IVR, *leu*-3 and *leu*-4 lies in linkage group IL, *leu*-5 lie in linkage group VR and *leu*-6 lie in linkage group IIR.

The *leu*-1 locus of *Neurospora* has been shown to control the synthesis of the enzyme or one of the enzymes involved in the formation of α-ketoisocaproate the keto analogue of *leucine* by oxidative decarboxylation of β-isopropylmalate (α-hydroxy β-carboxy isocaproate)(S.R. Gross 1962). The *leu*-1 cistron involved in the determination of the structure of the dehydrogenase (S.R. Gross 1965). The *leu*-1 cistron accumulates α-isopropylmalate and β-isopropylmalate. Synthesis of the enzyme also requires the function of regulatory gene *leu-3*$^{+}$ and the presence of α-isopropylmalate which acts as inducer (S.R. Gross 1969). Resistant to aminotriazole (Ryan *et al* 1946; Perkins *et al* 1963; Crawford 1967; Tan 1972; & Kuwana *et al* 1979).

The *leu*-2 locus of *Neurospora crassa* controls the synthesis of isomerase enzyme. The *leu*-2 mutants are deficient in isomerase enzyme (S.R. Gross1965). All *leu*-2 mutants accumulate α-isopropylmalate (β-hydroxy β-carboxy isocaproate) in their growth medium. Synthesis of the enzyme also requires the function of regulatory gene *leu-3*$^{+}$ and the presence of α-isopropylmalate which acts as an inducer (S.R. Gross 1969). Resistant to aminotriazole. Complementation between *leu*-2 mutants involves the production of an enzyme (isomerase) whose total synthesis is controlled by two separate unlinked genes (*leu*-2 in linkage group IV while *leu*-3 in linkage group-I).

Allele 37501 is heat sensitive (30^{0}C versus 20C) and is leaky at 25^{0}C (Regnery 1944 & 1947; Maling 1959; Smith 1962; & Kuwana *et al* 1969).

The *leu*-3 cistron is involved in isomerase and dehydrogenase synthesis. The *leu*-3 mutants are also deficient in the isomerase and accumulate α-isopropylmalate (β-hydroxy β-carboxy isocaproate). Regulatory mutation prevents synthesis of α-isopropylmalate isomerase and β-isopropylmalate dehydrogenase and prevents full derepression of α-isopropylmalate synthetase. Also involved in regulation of isoleucine and valine synthesis (S.R. Gross 1969; Olshan *et al* 1974; & Polacco *et al* 1973). The original allele 47313 is leaky, but some other alleles e.g. R156 are not leaky (Barry *et al* 1982).

The *leu*-4 locus of *Neurospora* controls the synthesis of the enzyme that catalyzes the synthesis of α-isopropylmalate ((β-hydroxy β-carboxy isocaproate) by condensation of α- Ketoisovalerate and acetyl coenzyme-A. The *leu*-4 cistron specifies the structure of the synthetase (S.R. Gross 1962 & 1965).

The *leu*-5 is the structural genes for mitochondrial leucyl-tRNA synthetase. Strains carrying the only auxotrophic allele 45208t, have a partial *leucine* requirement at low temperatures and a tighter *leucine* requirement at 34^{0}C and stop growth at 37^{0}to 39^{0}C. Apparently a gene complex consisting of structural genes for cytoplsmic leucyl-

tRNA synthetase and a separate mitochondrial leucyl-tRNA synthetase. Mutations mapping in the *leu*-5 region can affect either enzyme separately both simultaneously. Assembly of glycerolkinase and glycerol-3- phosphate dehydrogenase into inner mitochondrial membrane is not impaired in *leu*-5 strains. Allele 45208t is somewhat unstable (S.R. Gross *et al* 1968; Courtright 1976; Beauchamp *et al* 1977; Hopper *et al* 1982; Perkins *et al* 1982; Metzenberg *et al* 1984 & 1985; Kunugi 1986; Orbach *et al* 1986; Benarous *et al* 1988; & Chow *et al* 1989).

The *leu*-6 gene is linked to *trp*-3 on linkage group II. This is the structural gene for cytoplasmic leucyl-tRNA synthetase. The nuclear genes for the cytoplasmic and mitochondrial leucyl-tRNA synthetase (leuRS) of *Neurospora crassa* are distinct in their encoded proteins, codon usage, mRNA levels and regulation. The *leu*-5 and *leu*-6 both genes are described here. Although cytoplasmic LeuRS shares with mitochondrial LeuRS some general features common to most aminoacyltRNA syntheses, there is little amino acid sequence similarity between them. mRNA levels for cytoplasmic LeuRS were much higher than those for mitochondrial LeuRS. The genes for cytoplasmic and mitocondrial LeuRS are regulated independently. The cytoplasmic LeuRS gene is regulated by the cross-pathwaty control system in *Neurospora crassa.* The cytoplasmic LeuRS mRNA levels are induced by amino acid starvation resulting from the addition of aminotriazole. In contrast, the mitochondrial LeuRS gene is not induced by amino acid limitation (Breetenberger *et al* 1985; Giles *et al* 1985; Akins *et al* 1987; Koerner *et al* 1987; Chatton *et al* 1988; Kuiper *et al* 1988; Paluh *et al* 1988; Roberts *et al* 1988; Chow *et al* 1989; Kreader et al 1989; & Sachs *et al* 1989).

Complementation is a process in which two defective mutants mutually overcome their defects. In *Neurospora crassa* heterokaryon between two biochemical mutants of the same mating type is formed when the defective region of the genes are not overlapped. The complementation test allows geneticists to determine whether mutations that produce the same or similar phenotype are in the same gene or in different genes. This phenomenon is one of the three basic tools of genetics and the other two genetic tools are recombination and mutation. The results of complementation tests indicate whether mutations are allelic, whereas the results of

recombination analysis indicate whether mutations are linked and if so provide estimates of how far apart they are on a chromosome. Complementation should occur in every trans-heterozygote containing mutations in two different genes or sites of the same gene.

Complementation refers to the ability of independent (non-allelic) genes to provide diffusible products that produce wild phenotype when two mutants are tested in trans configuration in a heterozygote (Lewin 2000)

In a cell containing two mutant alleles however their products can mix to form multimeric proteins that contain both types of subunit. Sometimes the two mutations compensate, so that the mixed-subunit protein is active, even though the proteins consisting solely of either type of mutant subunit are inactive. This effect is called interallelic (intragenic) complementation.

In common with many fungi *Neurospora crassa* also possesses a growth phase in which genetically different haploid nuclei can exist within the same cytoplasm in a hyphae. This phenomenon known as hetercaryosis, is equivalent in many ways to the diploid but differs since more than one nucleus take part in the determination of the phenotype (Davis 1966). Heterocaryons can originate by direct fusion between two hyphae as they touch which is called anastomosis. The subsequent flowing of nuclei from one hyphae to the other or by mutation of a nucleus in a heterocaryotic culture (Srb and Infanger 1964). Heterocaryon stability is controlled by a number of factors, including mating type (Sansome 1946; Whitehouse 1949; & S.R. Gross 1952) and several incompatibility genes (Garnjobst and Wilson 1956; Wilson and Garnjobst 1966). Stable heterocaryons are readily obtainable in *Neurospora crassa* and provide a powerful tool for certain types of genetic analysis. Nuclear ratios in heterocaryons and their effect on the phenotype have been investigated fairly extensively (Beadle and Coonradt 1944; Barratt and Garnjobst 1949; Pittenger and Atwood 1956; Pittenger 1964; & Davis 1966).

The heterocaryon incompatibility due to *het* genes is strictly vegetative, it does not reduce fertility. If two strains carry different alleles at one or more *het* loci, they are unable to form stable hcterocaryons. Ten *het* loci have been identified in *Neurospora crassa* and various others are inferred to exist. *het-c, het-d, het-e* and *het-I* were first defined by heterocaryon tests. The remainder *het-5, het-6, het-7, het-8, het-9* and *het-10* were detected by using duplications. The mating type alleles *A* and *a* also act as *het* genes in *Neurospora crassa*, although some slow heterocaryotic growth may occur. *het-c* and *het-d* lie in linkage group II, *het-e* lies in linkage group VII, *het-i* lies in linkage group I or II, *het-5* lies in linkage group I, *het-6* and *het-7* lie in linkage group 11, *het-8* and *het-9* lie in linkage group VI and finally *het-10* lies in linkage group VII.

Now-a-days hybrid formation by the technology of protoplast fusion is flourishing. Scientists have succeeded in production of intergeneric hybrid with desired characters (Vasquez *et al* 1997). A further application of protoplast technology is protoplast fusion which allows combi nation of desirable plant traits from two species to one plant which would not be possible by sexual crossing (Krasnyanski and Menezel 1995). Recently Mozmader and Hadiuzzaman (1998) separated protoplast from *Neurospora crassa* mutant using snail gut enzyme and novozyme-234 to study complementation among themselves through protoplast fusion. For fusion thus isolation of protoplasts is a prerequisite. Fusion of two nuclei of Yeast protoplast is a promising technique for transfer of genes from genetically dissimilar organisms to different species of Yeast (Vasquez *et al* 1977). Regeneration from protoplast is possible for a large number of species, many of important agronomic value (Roset and Gilisson 1993), which makes protoplast cultures into an interesting alternative for genetic engineering and plant improvement.

The term 'linkage' was first coined by Bateson and Punnett in 1909. Linkage may be complete when the genes of one linkage group segregating together and may be incomplete when gene paris in most linkage group assort at least partially independently of each other (Morgan 1944). Linkage describes the tendency of genes to be inherited together as a result of their location on the same chromosome measured

by percent recombination between loci (Lewin 2000). Genes on the same chromosome show genetic linkage because they are present on the same molecule of DNA. Linkage is revealed in the progeny of a genetic cross when the proportion of recombinant genotypes is less than the number of parental genotypes.

Recombination results from a physical exchange of chromosomal material. This is visible in the form of the crossing over that occurs during meiosis. The visible result of a crossing over event is called a chiasma. Genetic analysis allows the construction of a linkage map that connects all the genes carried by one chromosome. The genetic map of a linkage group corresponds to the physical existence of the chromosome. Recombination mechanisms rearrange genetic variability into new combinations and natural or artificial selection preserves the combinations best adapted to the existing environmental conditions or desired by the plant breeder. On the genetic maps of higher organisms established during the first half of this century. The genes are arranged like beads on a string. They occur in a fixed order and genetic recombination involves transfer of corresponding portions of the string between homologous chromosomes. A microbial system in which a very large number of progeny can be obtained from each genetic cross, it become possible to demonstrate that recombination occurs within genes. A linkage map can be expanded by this reasoning to include all the known genes in a chromosome, these genes constitute a linkage map. The number of linkage groups is the same as the haploid chromosome number of the species.

Seven linkage groups have been identified in *Neurospora crassa* in which haploid chromosome number is seven. The haploid chromosome number is seven of *Neurospora crassa* are identified by Arabic numerals 1-7 in the order of decreasing length (B-Mc Clintock 1945) and the seven chromosome constitute the seven linkage groups are identified by Roman numerals I-VII in the chronological order in which they were discovered (Barratt *et al* 1954). Over 350 loci controlling morphology, nutrition, drug resistance etc. have been described and mapped (Barratt and Redford 1969. & 1970; Redford 1972). Many loci have been subjected to fine structure analysis. Linkage was first demonstrated in *Neurospora crassa* by Lindegren. He

published linkage maps of linkage group I and II (Lindegren and Lindegren 1936). The seven chromosomes in *Neurospora crassa* contain hundreds of genes. Each chromosome corresponds to a linkage group (Verma and Wann 1983). Therefore a plan was made to study linkage of the obtained mutants and to find out the linkage group of the new mutants by crossing it with the markers of seven linkage groups.

The aim of the present investigation was based on the following heads:-

- ⇨ Induction of mutants including *leucine* mutants by UV irradiation.
- ⇨ Characterization of the induced *leucine* mutants on the basis of fertility, mating types and segration ratio.
- ⇨ Complementation study of the induced *leucine* mutants and with the help of conventional, new and protoplast fusion techniques.
- ⇨ Determination of Linkage of the *leucine* mutants in their specific linkage groups.
- ⇨ Preparation of the genetic map of some selected *leucine* auxotrophs.

CHAPTER II

MATERIALS

CHAPTER II
MATERIALS

The main material of this experiment was the Ascomycetes, *Neurospora crassa*, the preferred species in genetics. Dodge and Cornelius shear (1927) opened up a new era in the study of inheritance by discovering the Ascomycetes. *Neurospora crassa* is the most favorite and widely used experimental material for researchers of molecular genetics and cytogenetics due to its advantages.

The most important cultures Emerson 'A' (EmA5296), Emerson 'a' (Ema 5297) and some markers were used for investigation. Em'a' was used for treating conidia with mutagens (UV). Both Em'a' and Em'A' of *Neurospora crassa* were used for checking mating types of mutants and the markers for the study of complementation test and linkage study. The morphological feature of wild types of *Neurospora crassa* Em'A'(5296) and Em'a'(5297) is shown in Fig. 2-1.

The following wild types and markers were received from "Fungal Genetics Stock Center", Department of Microbiology, University of Kansas Medical School, Kansas, U.S.A.

The wild types and markers of *Neurospora crassa* were kindly supplied by Prof. T.I.M.A. Mozmader from genetic stock Microbial Genetics Laboratory, University of Dhaka.

Another important material in the experiment was land snail (*Helix sp.* L) that was collected from the Botanical garden at Curzon Hall, University of Dhaka. The wild types of *Neurospora crassa* and biochemical mutants were used for treating conidia with gastrointestinal extracts of *Helix* for isolation and fusion of protoplasts.

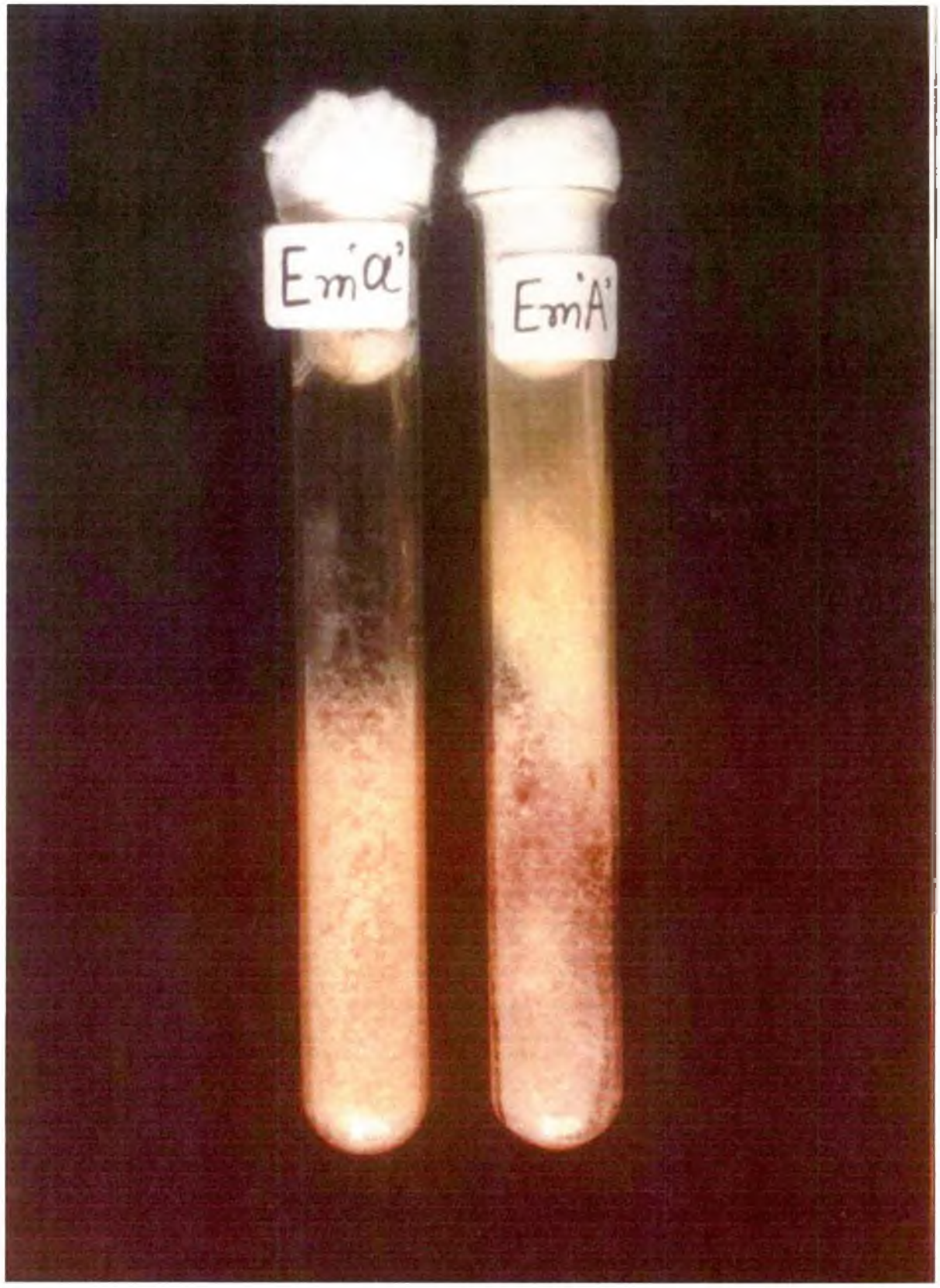


Fig. 2-1. Morphological feature of wild types of *Neurospora crassa* Em'a'(5297) and Em'A'(5296).

2.1. PHYSICAL MUTAGEN

UV irradiation (UV-rays of 254 nm wave length)

2.2. STANDARD MARKERS

The following markers tabulated in Table 2-1 were used for the complementation and linkage study.

Table 2-1. Standard markers.

Fungal Genetic Stock Centre number	Markers with their allele number	Mating type	Position of the gene	linkage group
4002	*leu*-3 (R 156)	A	IL	I
4003	*leu*-3 (R 156)	a	IL	I
4233	*leu*- (R 359)	A	IL	I
4234	*leu*-4 (R 359)	a	IL	I
2278	*his*-3 (C140)	A	IR	I
4624	*his*-2 [T 51M152(t)]	A	IR	I
4034	*arg*-5 (27947)	A	IIR	II
73	*trp*-3 (C83ext)	A	IIR	II
73	*trp*-3 (C83)	a	IIR	II
4047	*leu*-1 (33757)	A	IIIR	III
4048	*leu*-1 (33757)	a	IIIR	III
26	*ad*-2 (ST12)	A	IIIR	III
726	*his*-7 (K577)	a	IIIR	III
3735	*trp*-1 (10575)	A	IIIR	III
4816	*leu*-2 (37501)	A	IIIR	IV
4817	*leu*-2 (37501)	a	IVR	IV
3865	*trp*-4 (Y2198)	A	IVR	IV
72	*pyr*-1 (H263)	A	IVR	IV

939	*leu*-5 [45208(t)]	A	VR	V
340	*leu*-5 [45208(t)]	a	VR	V
7168	*cyh*-2 *leu*-5 (KH5345208)	a	VR	V
2332	*trp*-5 (420)	A	VR	V
2333	*trp*-5 (420)	a	VR	V
4069	*lys*-1 (33933)	A	VL	V
3955	*ilv*-1 (3955)	A	VR	V
4107	*trp*-2 (75001)	A	VIR	V
4108	*trp*-2 (75001)	a	VIR	VI
2281	*ad*-1 (3254)	A	VIL	VI
4091	*arg*-10 (B 317)	A	VIIR	VII
4089	*nt* (65001)	A	VIIR	VII

2.3. THE WILD TYPES

a) Emerson 'A' EmA(5296)

b) Emerson 'a' Ema(5297)

2.4. MUTANTS

The following 28 *leucine* mutants given in Table 2-2 were induced with UV-rays (254 nm wavelength) using filtration enrichment method.

Table 2-2. List of leucine mutants.

Serial Number	Mutant number	Name of the mutants
1	M-1	*leucine*
2	M-2	*leucine*
3	M-3	*leucine*
4	M-4	*leucine*
5	M-5	*leucine*

6	M-5	*leucine*
7	M-6	*leucine*
8	M-6	*leucine*
9	M-7	*leucine*
10	M-8	*leucine*
11	M-9	*leucine*
12	M-11	*leucine*
13	M-23	*leucine*
14	M-25	*leucine*
15	M-30	*leucine*
16	M-31	*leucine*
17	M-39	*leucine*
18	M-42	*leucine*
19	M-47	*leucine*
20	M-51	*leucine*
21	M-52	*leucine*
22	M-59	*leucine*
23	M-62	*leucine*
24	M-63	*leucine*
25	M-138	*leucine*
26	M-146	*leucine*
27	M-202	*leucine*
28	M-262	*leucine*

2.5. APPARATUS

The following apparatus listed below were used during the study:

1. Air pump
2. Autoclave (Hirayame Manufacturing Corporation, Japan)
3. Centrifuge (Temp. 20°-0°C and 10^4-2^4x10^4 rpm)
4. Deep freeze

5. Dissecting tray and box
6. Electric balance
7. Electric shaker (Lab line orbit shaker No.-3591)
8. Filter membrane (Pore size 0.22 μ)
9. Fluorescence microscope
10. Homogenizer (ULTRA-TURBEX)
11. Incubator
12. Inoculation Chamber
13. Laminar flow
14. Millipore filtration device
15. Nikon Camera (FX-35WA)
16. P^H meter
17. Refrigerator
18. Shaker Gy Rotary
19. Stand with clamp
20. Sonicator (The Virsonic Inc. NY.)
21. Thermometer
22. Thermostirrer
23. UV Lamp (254 nm weave length)
24. Water bath with circotherm

2.6. GLASS WARES

The following glass wares used in the experiment are listed in Table 2-3 along with their purposes of use.

Table 2-3. List of glass wares.

No.	Name	Made	Size	Purpose
1.	Test tubes	Pyrex	0.5″ × 2″	Isolating
2.	Test tubes	Pyrex	0.5″ × 3″	Subculturing

3.	Test tubes	Pyrex	5/8" × 5"	Stock
4.	Test tubes	Pyrex	1" × 6"	Crossing
5.	Test tubes	Pyrex	1.5" × 9"	Radiation
6.	Test tubes	Pyrex	0.5"× 15"	Growth (race)
7.	Petridishes	Pyrex	1.5"	Irradiation
8.	Petridishes	Pyrex	3"	Plating
9.	Flasks	Erlenmeyer	100 ml	Media and Water
10	Flasks	Erlenmeyer	250 ml	Media and Water
11.	Flasks	Erlenmeyer	500 ml	Media and Water
12.	Flasks	Erlenmeyer	1000 ml	Media and Water
13.	Pipette	Erlenmeyer		Dropping solution, Water

2.7. CHEMICALS USED FOR THE RESEARCH WORK

The following chemicals classified in two groups, general and special, were used in the study:

General

1. Absolute alcohol
2. Acetic acid
3. Agar (Bacto)
4. Ammonium nitrate (NH_4NO_3)
5. Ammonium Sulphate $[NH_4)_2SO_4]$
6. Biotin ($C_{10}H_{16}N_2O_3S$)
7. Boric acid anhydrous (H_3BO_4)
8. Calcium chloride ($CaCl_2,6H_2O$)
9. Chloroform ($CHCl_3$)
10. Citric acid $[C(OH)(COOH)CH_2COOH)H_2O]$
11. Copper sulfate ($CuSO_4,7H_2O$)
12. Di-Sodium Mono-hydro-phosphate (Na_2HPO_4)

13. Ferrous sulfate ($FeSO_4,7H_2O$)
14. Glucose ($C_6H_{12}O_6$)
15. Hydrochloric acid (HCl)
16. Magnesium Chloride (MgCl)
17. Magnesium sulfate ($MgSO_4$, $7H_2O$)
18. Manganese sulfate ($MnSO_4$, $7H_2O$)
19. Potassium Di-hydrogen phosphate ((KH_2PO_4)
20. Potassium nitrate (MNO_3)
21. Potassium Chloride (KCl)
22. Potassium sulfate (K_2SO_4)
23. Sodium Chloride (NaCl)
24. Sodium Citrate ($NaH_2C_6H_5O_7,2H_2O$)
25. Sodium Carbonate (Na_2CO_3)
26. Sodium molybdate ($NaMoO_4,2H_2O$)
27. Sodium hydroxide
28. Sorbose [$OCH_2(CHOH)_3$]
29. Sucrose ($C_{12}H_{22}O_{11}$)
30. Sodium bi-phosphate ($NaHPO_4$)
31. Zinc sulfate ($ZnSO_4$, $5H_2O$)

Special

1. Sorbitol
2. PEG (Poly-Ethylene Glycol)

2.8. AMINO ACIDS AND THE SUPPLEMENTS

The following amino acids and the supplements used during the study are listed below:

1. *Adenine*
2. Anthranilic acid

3. *Arginine*
4. *Histidine*
5. Indole
6. Isoleucine and Valine
7. *Leucine*
8. *Lysine*
9. Nicotinic acid
10. *Pyrimidine*
11. *Tryptophan*
12. Uracil

2.9. BUFFERS

Phosphate buffer P^H 7.0

CHAPTER III

MEDIA

CHAPTER III
MEDIA

Neurospora crassa grows on a suitable mixture of mineral salts in water plus a carbon source (sucrose) and the vitamin biotin. The nutrition of *Neurospora crassa* is very simple (Beadle and Tatum 1945, Anonymous 1966). The wild type cultures were maintained on VM (Vogel 1956). For the maintenance of auxotrophs VM was supplemented with necessary supplements. For plating 15ml of the medium was taken in each time. Sorbose minimal medium (SM) was used for single colonial isolation and single spore isolation. SM was supplemented with necessary supplement for plating irradiated conidia. Westergaard's crossing medium (Westergaard *et al* 1947) was used to determine the mating types of the mutants and extracts and for the study of linkage. Incase of sterile crosses, Suyama's (1958) crossing medium was used. All media were sterilized in the autoclave at 120°C under 15 lb pressure for 20 minutes.

3.1. STOCK SOLUTIONS NEEDED FOR DIFFERENT MEDIA

For culturing *Neurospora crassa* various stock solutions were needed for the preparation of media. To prepare a stock solution, the chemicals were added one after another and the addition of the next one was followed by the complete dissolution of the previous one. Thousand ml conical flasks were used to keep the stock solutions. All the stock solutions were stored in the refrigerator.

3.1.1. Composition of Biotin Solution per 100 ml

1. Distilled water 100 ml
2. Biotin 10 mg

Biotin was used for the preparation of Vogel's stock solution and for Westergaard's stock solution.

3.1.2. Composition of Vogel's Stock Solution

For preparing Vogel's minimal (VM) medium and sorbose minimal medium Vogel's stock solution is essential. Forty ml of Vogel's stock solution was required for 1000 ml of medium.

1.	Distilled water	1540 ml
2.	Sodium Citrate [$NaH_2C_6H_5O_7$, $2H_2O$]	127 g
3.	Calcium chloride [$CaCl_2$, $6H_2O$]	7.5 g
4.	Ammonium nitrate [NH_4NO_3]	100 g
5.	Magnesium sulphate ($MgSO_4$, $7H_2O$)	10 g
6.	Potassium Di-hydrogen phosphate [KH_2PO_4]	250 g
7.	Trace element solution (as detailed below)	5 ml
8.	Biotin solution [$C_{10}H_{16}N_2O_3S$]	2.4 ml
9.	Chloroform [$CHCl_3$]	4 ml

Instead of 770 ml of distilled water as recommended by Vogel, 1540 ml of distilled water was used.

3.1.3. Composition of Trace Element Solution per 100 ml

1.	Distilled water	95 ml
2.	Citric acid [$C(OH)(COOH)(CH_2COOH)_2$ H_2O]	5 g
3.	Zinc sulfate [$ZnSO_4$, $7H_2O$]	5 g
4.	Ferrous sulfate [$FeSO_4$, $7H_2O$]	1.0 g
5.	Copper sulphate [$CuSO_4$, $5H_2O$]	0.25 g
6.	Manganese sulphate [$MnSO_4H_2O$]	0.65 g
7.	Boric acid anhydrous [H_3BO_4]	0.05 g
8.	Sodium molybdate [Na2MoO_4,$2H_2O$]	0.05 g
9.	Chloroform [$CHCl_3$]	1 ml

3.1.4. Composition of Westergaard's Stock Solution

1.	Distilled water	1000 ml
2.	Potassium nitrate [KNO_3]	4 g
3.	Potassium di-hydrogen phosphate [KH_2PO_4]	4 g
4.	Magnesium sulphate [MgSO, $7H_2O$]	4.1 g
5.	Calcium chloride [$CaCl_2$, $6H_2O$]	0.8 g
6.	Sodium Chloride [Nacl]	0.4 g
7.	Biotin solution [$C_{10}H_{16}N_2O_3S$]	15 drops
8.	Chloroform [$CHCl_3$]	2 ml

For the preparation of Westergaard's crossing medium, Westergaard's stock solution was needed.

3.2. AMINO ACID SUPPLEMENTS

For the preparation of supplemented medium, various amino acids were added with VM medium. Hundred mg of amino acid was dissolved in 100 ml of sterilized distilled water and 300 ml of amino acid solution was used for 1000 ml medium.

Composition of amino acid solution:

1. Distilled water 500 ml
2. Amino acid 500 mg

The stock solution was named after the amino acid used (For instance, *leucine* solution, *tryptophan* solution, *arginine* solution, *histidine* solution, etc.).

Medium for culturing and maintaining stock

Various medium were used for the growth and reproduction of *Neurospora crassa*. Vogel's minimal or VM medium was used for the stock maintenance of

cultures and study of linear growth. VM liquid medium was used for the preparation of conidial suspension and to study of mycelial growth. Sorbose minimal and supplemented medium were used the isolation of single spore and its colony. Westergaard's crossing medium was used for crossing to find met the mating types of mutants.

3.3. VOGEL'S MINIMAL MEDIUM

Vogel's minimal medium usually used for culturing the organism in the test tube. It also used for growing and counting spores in the petriplate. Amino-acid supplements were used in culturing medium to the specific need of different mutants especially for biochemical mutants and the markers. The supplemented media were named after the amino acid used, e.g. *leucine* medium, *tryptophan* medium, *lysine* medium, *arinine* medium etc. 2-4 ml of medium was taken in a small test tube (3″) for maintaining cultures of mutant. 8-10 ml of medium was taken in a large test tubes (6″) for plating and isolating growing spores. Biochemical mutant and wild types were easily recognized by testing them on non-supplemented Vogel's minimal medium plates. Growing one's was wild but the bio-chemical mutants did not grow in this medium until and unless a particular supplement or supplements were added for particular mutants.

3.3.1. Composition of Vogel's Minimal Medium (VM)

1. Vogel's stock solution	40 ml
2. Distilled water	960 ml
3. Sucrose	20 g
4. Agar	15 g

Hundred mg of required amino acid was used for supplemented VM per liter. For subculturing and heterocaryon tests VM with 1.5 % agar was used. The medium is also used for counting spores but in this case the percentage of agar used was 2. For linear growth in test tubes, 4% agar was used.

3.3.2. Composition of Vogel's Minimal Liquid Medium

1. Distilled water	960 ml
2. Vogel's stock solution	40 ml
3. Sucrose	20 g

The medium was supplemented with some selected amino acids when required. It was used for heterokaryon tests.

3.4. SORBOSE MINIMAL MEDIUM (SM)

SM medium, supplemented or not, used for single colony isolation. This medium was used plating as it had the quality to restrict the growth of the colonies. The huge growth rate of *Neurospora crassa* often produce a serious problem which was overcome by adding a ketohexose, sorbose, to the culture media and a restricted colonial growth habit results (Tatum *et al* 1949). The importance of the ratio between other carbon sources and sorbose has been investigated (Tatum et al 1949, Brockman and De Serres 1964, Pithenger 1964).

Composition of SM Medium per liter:

1. Distilled water	960 ml
2. Vogel's stock solution	40 ml
3. Sucrose	1 g
4. Agar	20 g
5. Sorbose	5 g

During the preparation of sorbose medium, all-the constituents except sorbose, were added and then boiled. Sorbose was added after boiling to prevent caramelization. 10 ml of media was used for each plate in plating. To find out wild types or biochemical mutants sorbose supplemented or non-supplemented media were

used. In non supplemented media the wild type ascospores could grow easily, but the ascospores of bio-chemical mutants could not grow but only germinate.

3.5. WESTERGAARD'S CROSSING MEDIUM

This medium was used for crossing, it restricts the vegetative growth but enhances sexual reproduction. It is also used to determine the mating types of the mutants and extracts and for the study of linkage. A rectangular piece of filter paper measuring 1.5" ×2.5" were folded thrice to make the shape of 'W'. The 'W' shape filter paper was introduced into a 6" test tubes and 8-10 ml of the crossing medium was poured into each of the crossing tubes. The medium remained in the furrows of the folded paper when the tubes were slanted.

Composition of Westergaard's Crossing Medium:

1. Westergaard's stock solution	250 ml
2. Distilled water	750 ml
3. Sucrose	20 g
4. Agar	20 g

3.6. SUYAMAS'S CROSSING MEDIUM

In case of infertile crosses, sucrose was used 0.2 g per 100 ml and the percentage of the supplement was increased to 50 mg/100 ml (Suyama *et al* 1958)

Composition of Suyamas's Crossing Medium:

1. Westergaard's stock solution	250 ml
2. Distilled water	750 ml
3. Sucrose	2 g
4. Agar	20 g

In the above two cases, amino acid was added where necessary. For example, *leucine* mutants, 20 mg of *leucine* per 100 ml was generally used.

3.7. REAGENTS USED FOR PROTOPLAS FUSION

Composition of Sorbitol Solution per 100 ml:

1. Distilled Water	81.8 ml
2. Sorbitol	18.2 g

This solution was sterilized with the help of autoclave.

Composition of fusion mixture:

1. Poly Ethylene Glycol (PEG)	1 g
2. Distilled Water	99 ml

Composition of 0.2 M phosphate buffer solution per 200 ml:

1. Distilled Water	200 ml
2. $Na_2HPO_4,12H_2O$ (Molecular weight 357.97)	14.31 g
3. $NaH2PO_4$; $2H_2O$ (Molecular weight 155.97)	6.24 g

This solution was adjusted to $P^{H}7.0$ and stored at 4^0C.

3.8. STERILIZATION

All the media and required instruments were sterilized in the autoclave at 120°C under 15 lb pressure for 20 minutes. The inoculation chamber inoculation needle and the working space sterilized with rectified spirit and the needle was burnt over the flame of a spirit lamp.

CHAPTER IV

METHODS

CHAPTER IV
METHODS

4.1. FUNGAL STOCK MAINTENANCE

Usually 5″ × 5/8″ tubes with 1-2 ml of sterilized media were used for stock maintaining. The stock of *Neurospora crassa* cultures i.e. wild types (Em 'A' & Em 'a'), biochemical mutants, and markers are maintained on it, At 25°C within 4 to 5 days of inoculation the culture then stored in a refrigerator at ± 4°C temperature.

4.2. SUBCULTURING

The availability of fresh culture is a must to initiate a new experiment. That is why subculturing is required. Small tubes containing 1-2 ml of sterilized VM medium, generally used for sub-culturing the parent stock. Necessary supplement was added to the medium at the time of its preparation. It may be mentioned that the wild types and morphological mutants subcultured in VM medium whereas biochemical mutants and markers are subcultured in supplemented VM. Subculturing is a process of transferring few conidia from the old stock to the new culture medium. The process is done in an inoculation chamber.

Subculturing was done is a sterilized inoculation chamber with utmost care to avoid any kind of infection. The chamber, inoculation needle were sterilized with rectified spirit. The inoculation needle was sterilized by heating over the flame of a lamp kept in the chamber and then cooled into the new medium before each time of transferred from old stock to new medium. The culture tube then incubated at 25°C temperature for 4-5 days for the growth and formation of conidia.

4.3. ARTIFICIAL INDUCTION OF MUTATION IN *Neurospora crassa* BY UV IRRADIATION USING FILTRATION ENRICHMENT METHOD

Ultra-violet light seems to induce true gene mutations as well as losses and reversion to the wild type gene and perhaps also mutation to other alleles. Ultra-violet light, unlike X-rays, does not induce gross structural changes in the chromosomes (Catcheside 1949).

The maximum absorption of UV by DNA is at a wave length of 254 nm. Maximum mutagenecity also occurs at 254 nm. UV radiation of 254 nm was used for induction of mutation. Figure 4-1 shows the apparatus of filtration enrichment technique and the technique is described in the following sections.

4.3.1. Obtaining of fresh culture

To obtain fresh culture Ema (5297) was cultured 4 times at an interval of 4 days and 4-5 days old culture was used for treating conidia.

4.3.2. Sterilization

All the media, essential elements, instruments, radiation tubes, filter, pipettes and petridishes were sterilized at 120°C under 15 lb pressure for 20 minutes. The inoculation chamber and needles sterilized with rectified spirit.

4.3.3 Preparation of conidial suspension

Conidial suspension from 4-5 days old culture (Em'a') were prepared by transferring 2-3 loopful of conidia into a tube containing 10-15 ml sterilized distilled water. The tube was shaken gently for homogeneous conidial suspension. The suspension was filtered through a sterilized absorbing cotton filter to eliminate the mycelial debris and conidial lumps.

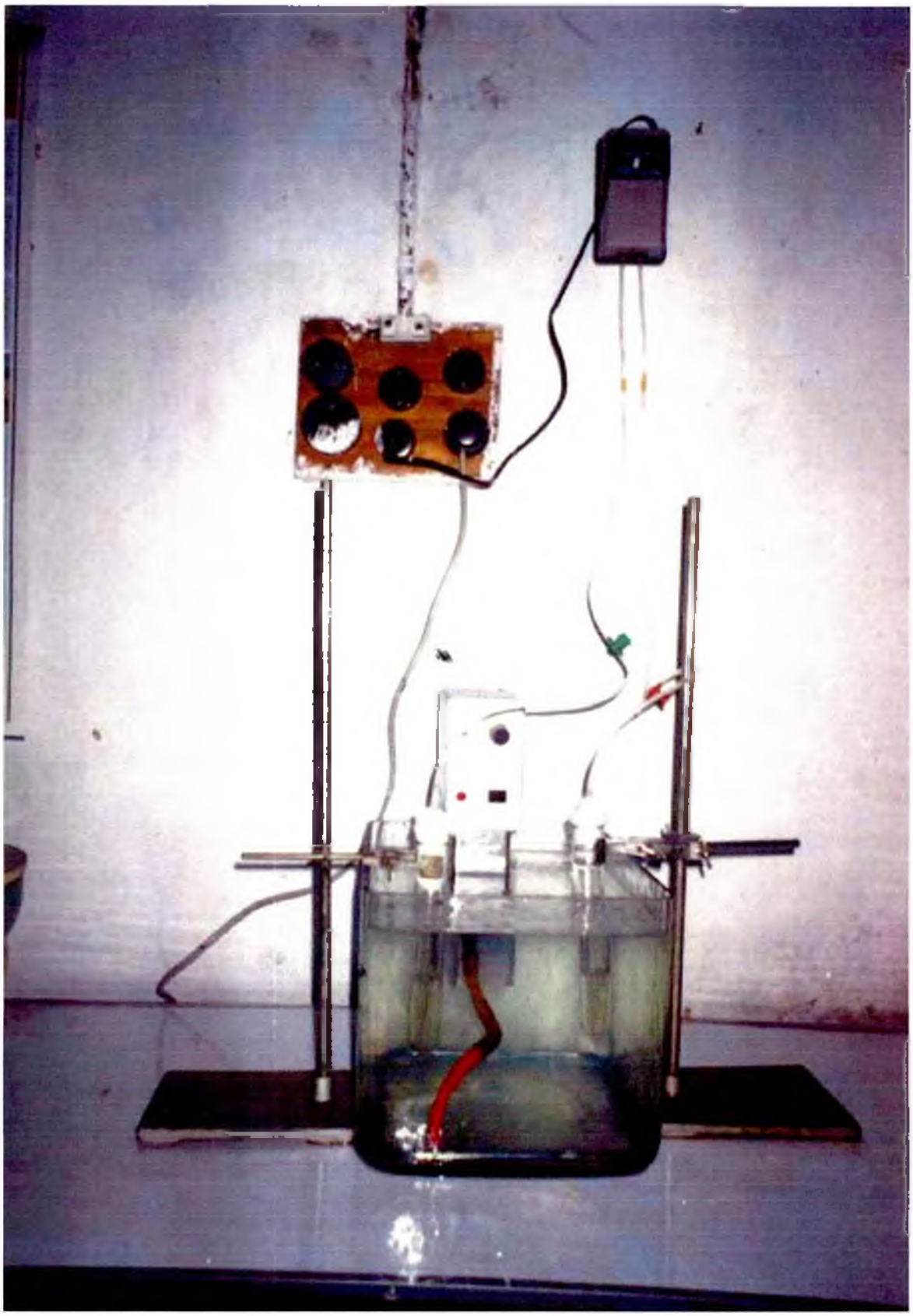


Fig. 4-1. Apparatus of filtration enrichment technique.

4.3.4. Irradiation

Four ml of the filtered conidial suspension was transferred into each of the two small sterilized petridish marking I and II, irradiated with UV light having wave length of 254 nm for 55 seconds and 2 minutes respectively. Radiation was given at a distance of 15 cm. The suspension was continuously rotated during irradiation with a wooden handle to ensure equal dose for all the conidia. After completion of irradiation the suspension was stored at room temperature in the dark for 2 hours to prevent photo reactivation.

4.3.5. Repeated filtration

The irradiated conidial suspensions were taken in 4 separate large radiation tubes (2.5″ × 7.5″) fitted with pipette. The radiation tubes were marked previously. Each radiation tube contained 25 ml VM liquid medium. The inoculated tubes were placed in a water tank maintained at 25^0C. The plastic tubings of air pump were fitted with Pasteur pipettes of the radiation tubes and the air pump was started. Then filtered air was pumped through a pasteurpipette inserted into each radiation tube. To secure continued suspension of the conidial and to keep the germinating conidia separated from each other, it was found necessary to have the orifice of the Pasteur pipette very close to the bottom of the tube. After every six hours, the irradiated conidia were filtered either through a cotton filter or filter made with nylon or canabric cloth of these filters, the nylon filters, gave the best result. After each filtration some fresh liquid VM medium was added into the radiation tube. It was then put back into the controlled temperature tank. This procedure was continued for 2-3 days.

4.3.6. Plating of the irradiated and filtered conidia

After the final filtration, the filtered conidial suspension was taken into sterilized petridishes, 60 petridishes were taken for each suspension and 1-2 drops of suspension were given in each petridish. 10 ml of molten SM medial supplemented

with 30 mg adenine, histidine, leucine, lysine and tryptophan per 100 ml medium was taken for each petridish.

4.3.7. Incubation

The plates were incubated at 25^0C and checked for the growth of colonies up to 1-3 days.

4.3.8. Isolation

When the growth was visible on the media, only the distinctly separated colonies were isolated. A part of the well separated colony was cut with agar with the help of an isolation needle and was inoculated into VM tubes supplemented with *adenine, argenine, histidine, leucine, dysine* and *tryptohan.* The tubes were incubated at 25°C for 4-6 days.

4.3.9. Observation and classification

After 5 days the isolates were observed. Many of the isolates showed morphological variations with Em'a' or among the isolated. The isolated were then classified according to their characters.

4.3.10. Classification of mutants

The induced mutants of *Neurospora crassa* (Em'a') were sub-cultured several times to make the culture refresh. The mutants were then tested for growth on SM and SM supplemented plates separately with *adenine, argenine, histidine, leucine, lysine* and *tryptophan* to classify them into *adenine, arginine, histidine, leucine, lysine* and *tryptophan* auxotroph.

4.3.11. Further test of the *leucine* auxotrophs

All the *leucine* auxotrophs were tested on SM and SM supplemented with *leucine* for finding their growth requirements. Those cultures which did not grow on SM but grow on SM supplemented with *leucine* are *leucine* mutants.

4.4. TEST FOR HETEROCARYON COMPATIBILITY

This test was done in order to classify the mutants basing on their positive (complementation) or negative (non-complementation). The test were done among the mutants of same mating types and for these test always fresh cultures of 4-6 days and fresh medium were used as Suggested by reverend guide for better accuracy.

Test for heterocaryon compatibility were made on Vogel's minimal medium both in solid and liquid medium. This test was first made in tubes ($1.5'' \times 3''$) freshly prepared VM solidified with agar. Conidia from 4-5 days old cultures were used in the test. To prevent infection, inoculation was done inside an inoculation chamber. Those tubes were done in duplication for a check. Controls for the mutants were maintained in VM in duplicate.

In heterocaryon tests in solid VM medium, few conidia from a mutant were put in the tubes in two places and then conidia from another mutant were placed exactly at the same place as the conidia from the first mutant were placed. In heterocaryon tests in liquid VM medium conidia from two mutants were simply put in the test tubes one after another.

After this test the tubes were kept in incubator at 25°C. Observation of both the control and heterocaryon sets were taken after 1, 3, 7, 12, 15, 18, 21 and 30 days.

4.5. TEST FOR HETEROCARYOSIS USING NEW METHOD

If any of the results was doubtful, tests were repeated in new method of complementation. For this method hanging drop culture is used. Ordinary slides, glass rings and cover slips are required. The most convenient size of slide is 1.5 cm and this was firmly fixed over the ordinary slide ring coated with Vaseline. One to two droplets of distilled water was placed in side the ring just to make it a moist chamber.

For heterocaryon test, liquid VM was used and drop of the medium was placed upon the cover slip. Few conidia from a mutant were given first then conidia from other mutant, whose heterocaryon-compatibility are to be determined were placed on that drop of the cover slip and then inverted and pressed firmly against the Vaseline coated margin of the glass ring.

4.6. CROSSING

Neurospora crassa strains of two mating types 'A' and 'a'. Sexual fruiting bodies called perithecia are produced only when two mating types are brought together on a suitable media (Sher and Dodge 1927; Westergards crossing medium was usually used for crossing. In some cases, when the crosses were sterile i.e. perithecia did not form, Suyama's crossing medium was used (Suyama et al 1958). The crossing media was supplemented with the amino acid according to the requirement.

Crossing was done in large test tubes (6") containing 'W' shaped crossing paper in 5-7 ml of Westergard crossing medium. One strain was put on one through of the 'W' shaped crossing paper pleat and the other was put on other through of the pleat. The crosses were then incubated at 25°C which is the optimum temperature for sexual reproduction.

Fertile mutants formed perithecia with in 2-3 weeks. It was observed that the more delay in the formation of perithecia, the less is the number of perthecia formed in

a cross. Further the greater the concentration of amino acid supplement the earlier was the formation of perithecia and also the higher number of perithecia. The perithecia is generally liberated spores with in 20-25 days.

The mutants were crossed with wild type Em'a' and Em'A' for checking the mating types and they were also crossed with biochemical markers of seven linkage groups for the determination of linkage group.

4.7. DETERMINATION OF MATING TYPE

For obtaining fresh culture Em'a', Em'A' and mutants were cultured on VM on VM supplemented (with necessary amino acid) 3-4 times with an interval 4 days. 4-5 days old culture was used for study of mating type. The selected mutant were chosen and their mating types were determined by crossing them with both the wild types (Em'A' & Em'a') separately. Observation was taken for 5-30 days. If perithecia are formed with Em'a' the mating type of the mutant is 'A' and if perithecia are formed with Em'A' it is 'a'.

4.8. IMPROVEMENT OF FERTILITY OF THE MUTANTS

a) Mutants were subcultured 4 times after 4 days interval to improve their fertility.
b) Fresh mutants were crossed in Westergard's medium containing 30 mg amino acid/100 ml media with wild types on the markers.
c) The wild types or the markers as the case might be were sub-cultured 4 times before crosses were made.
d) For good conidia formation, the concentration of amino acid was used 40-50 mg per 100 ml media.
e) The crosses were kept at 25^0C bath.

The fertility of the mutant might be improved by adding mycelial extracts over the surface of the medium before crossing.

4.9. STUDY OF SEGREGATION

When the cross between the mutant and wild formed perthecia and the spores were shed, the segregation was studied. For the study of segregation the following steps were followed.

4.9.1. Spreading of spore

A spreading needle made of glass rod bend twice at right angles was boiled in water old culture was used for and kept ready for spreading. A drop of sterilized water was put on the medium of a petridish. A wire loop needle was burnt red hot and then cooled in sterilized distilled water plate. With the help of this needle a loop of ascospores were taken from the desired cross and were transferred to SM supplemented media plate in which a drop of a sterilized water was put. The spreading needle was then cooled in sterilized water and the spores were uniformly spread with the help of it. The loop needle was again and the glass needle was boiled for sterilization. The name of the cross was written on the upper lid of the petridish. The prepared plates was given a heat shock at 58^0C for 50 minutes and was then incubated at 25°C for 12-16 hours as necessary.

4.9.2. Isolation of single spore

Usually after 12-16 hours spreaded spores were ready for isolation and a good number of well grown single spores colonies were isolated with the help of an arrow shapes isolating needle and inoculated in small culturing test tubes containing culture media (VM supplemented). Precautions were taken so that only the distinctly separated germinated single spores were isolated.

4.9.3. Incubation

The tubes with inoculum were then incubated at 25°C for 4-5 days. The isolates were then analyzed whether they showed similarity with the characteristics of wild

type or mutants. The ratio between the mutant isolates and wild type like isolates were calculated out. For the *leucine* auxotrphs all the isolates were tested on SM and SM+ *leucine* medium. Those culture which did not grow on SM, but grew on SM+*leucine* medium, they were the *leucine* mutants and which did grow son SM, they are wild type. In most cases it was found to be about 1:1.

4.10. DETERMINATION OF LINKAGE GROUP

The method of determination of linkage groups has been described followingly.

4.10.1. Subculturing of induced mutants and markers

The mutants and markers were subcuttured 4- times after 4 days interval to improve their fertile.

4.10.2. Crossing

Mutants were crossed with seven markers and incubated at 25°C.

4.10.3. Observation of the cross

The fertile cultures formed perithecia in 10-19 days and shaded spores with in 15-30 days.

4.10.4. Spreading of spores

About 10 ml sterilized medium preserved in 20 ml w as taken in the clean dried and sterilized petridish. The medium was supplemented with the amino acid according to the requirement. A spreading needle made of glass rod bent twice at right angle was boiled in water and kept ready for spreading. One drop of sterilized water was taken on the medium of the Petri dish. A loop needle was burnt red hot and then cooled in sterilized distilled water. A loop of spore was taken from the desired cross with the

help of this needle and were transferred to water drop on the SM supplemented plate. The spreading needle was then cooled in sterilized distilled water plate and the spores were uniformly spread with it. The needle was again boiled for sterilization. The name of the cross was written on the upper lid of the petridish. The prepared petridish was given a heat shock at 58^0C for minutes and was then incubated at 25°C for 12-16 hours.

4.10.5. Isolation of single spore

The plate was opened by taking the upper lid and the growing spores were isolated with the help of an isolating needle in the VM supplemented tubes. The tubes were incubated at 25°C for growth.

4.10.6. Checking the isolates

After 4 days isolates were analyzed. The ratio between the mutant isolates and wild isolates was calculated.

4.10.7. Plate tests of the extracts of mutants

For linkage study the isolates were tested on SM and supplemented SM plates with necessary amino acid (according to cross). Four types of progenies were obtained after plate test, that is (i) Mutant (ii) Marker (iii) Mutant + Marker and (iv) Wild. The linkage was studied by analyzing the percentage of recombinates and parental types.

4.10.8. Checking the mating type of the extracts

The selected extracts were chosen and their mating type was determined by crossing them with both wild type Em'a' the mating type of the extracts would be 'A' and it perithecia formed with Em'A' it would be 'a'.

4.11. PROTOPLAST ISOLATION & FUSION OF *leucine* AUXOTROPH

4.11.1. Preparation of phosphate buffer

Two types of phosphate were used, viz.,

i) NaH_2PO_4, $2H_2O$ (Molecular weight = 155.97 g)
ii) Na_2HPO_4, $12H_2O$ (Molecular weight = 357.97 g)

Preparation of 200 mM Stock buffer:

When 155.97 g of NaH_2PO_4. $2H_2O$ is dissolved in 1000 ml water, its molality is 1M. So to prepare 200 ml 200 mM (0.2 M) buffer solution 6.24 g NaH_2PO_4. $2H_2O$ was dissolved in 200 ml water. The solution was termed 'A'.

Again 357.97 g of $Na_2HPO_4.12H_2O$ was dissolved in 1000 ml water to produce 1M solution. So to prepare 200 ml 200 mM (0.2 M) buffer 14.31 g of $Na_2HPO_4.12H_2O$ was dissolve in 200 ml of distilled water. The solution was termed as 'B'.

The formula for the preparation of 200 ml 200 mM (0.2M) phosphate buffer is X ml of 'A' + Y ml of 'B' was diluted to a total of 200 ml.

So, 39 ml of 'A'+ 61 of 'B'+ 100 ml of sterilized distilled water produced a phosphate buffer of 200 mM (0.2 M). It was then stored at 4°C.

Preparation of 40 mM Phosphate buffer:

40 ml of stock phosphate buffer (200 mM) was mixed with 160 ml of sterilized distilled water. The nascent buffer has then a molality of 40 mM. Its P^H was fixed at 7.0. This 40 mM buffer was used in the experiment.

4.12. ISOLATION AND FUSION OF PROTOPLAST

Protoplasts were isolated by following the method of Browne, Peberdy and Gibson (1983) with a modification outlined below.

4.12.1. Preparation of Conidical suspension

5-6 days old culture of selected biochemical mutants *leu* M9a, *leu* M30a and *leu* M51a were taken. 2-3 lopes of fresh conidia (10^6) from the culture were taken in another tube with 5 ml of liquid VM supplemented medium. The tubes were then shaken to make a homogeneous suspension of conidia.

4.12.2. Production of *Neurospora crassa* colony on cellophane paper

Vogel's minimal medium supplemented with leucine for the growth of *leucine* mutants. 10 ml of supplemented VM medium was taken in each of the several sterilized cellophane paper was then spread over the medium in each petridish. The conidial suspension was then placed at each pore of the cellophane paper with the help of loop needle. The cellophane papers were pored so that the conidia placed over it can get nourishment. After inoculation the plates were inoculated for about 16 hours at 29°C for the formation of distinct colony with mycelia on cellophane paper. Which was photographed and shown in

Different concentrations of intestinal and gastric extracts with sorbitol solution were taken in different sterilized petridishes. The colonies of one mutant with cellophane paper were removed from the first petriplates by the sterilized forceps and were placed over the gastric extracts and the colonies of the same mutant with cellophane paper from the other Petri plate were removed and placed over the intestinal extract having upside down so that the snail extract can come in contact with the mycelia. So for treating mycelia in both gastric and intestinal extracts, one type of mutant must be cultured twice. By this we can compare the enzymatic activity of

gastric and intestinal extracts. The plates were then set in a shaken and observed after a regular interval of one hour.

4.12.3. Isolation of protoplast

After about 4-5 hours in most of the plates, the cell wall of the elongated mycelia were digested off and masses of spherical on round protoplast were observed. The concentrations and efficiency of gastric and intestinal extracts on biochemical.

4.12.4. Fusion of protoplast

For fusion of protoplasts 200 ml of sorbose minimal medium was prepared. 1% polyethylene glycol (PEG) solution was used as fusogenic agent for successful fusion. PEG appears to act as a molecular bridge between the surfaces of adjacent protoplast either directly or indirectly. Protoplasts obtained from the enzymatic treatment were used for fusion experiments. The isolated protoplast suspensions of different mutants were taken in sterilized test tubes. For fusion of protoplasts between two different mutant strains, several drops of protoplast suspension of both the mutants were taken in a petridish that contained 1 ml PEG (1%) solution. Then 10 ml of VM medium without any supplement was poured in the petridish and shaken gently so that the mixed homogenously. For control, the protoplasts of two mutants also separately inoculated in the Petri dish containing VM medium 1% PEG solution. Thus 3 Petri dishes were prepared for protoplast fusion. Observations were taken for fusion experiment after 24, 48 and 72 hours.

CHAPTER V

RESULTS

CHAPTER V
RESULTS

5.1. INDUCTION OF MUTATION WITH UV IRRADIATION

UV radiation of 254 nm (Mercury source) wave length was used for treating conidia and the treatment was done in the dark to avoid photo reactivation. The conidia were treated for 45 sec, 50 sec and 1 minute at a distance of 6 inches. SM media containing *leucine*, *lysine*, *tryptophan*, *adenine*, *histidine*, etc. were used for plating the irradiated conidia and isolating conidial cultures, respectively.

The isolates in small tubes containing supplemented VM were incubated at 25°C. After 5 days, they were observed and classified according to their nutritional requirements.

5.1.1. Classification of biochemical mutants induced with UV

All the isolates were tested in the non-supplemented SM plates. The cultures those did not grow in the non-supplemented SM medium were selected for further test of biochemical mutants and the results of the plate tests are given in Table 5-1.

Table 5-1. Observation of the growth of the biochemical *leucine*, *lysine*, *tryptophan*, *histidine* and *adenine*.

Serial No.	Culture No.	SM + *leucine*	SM + *lysine*	SM + *tryptophan*	SM + *histidine*	SM + *adenine*	Inference of mutants
1	1	●	○	○	○	○	*leucine*
2	2	●	○	○	○	○	*leucine*
3	3	●	○	○	○	○	*leucine*
4	4	●	○	○	○	○	*leucine*
5	5	●	○	○	○	○	*leucine*
6	6		○	○	○	○	*leucine*

7	7		○	○	○	○	*leucine*
8	8		○	○	○	○	*leucine*
9	9	●	○	○	○	○	*leucine*
10	10	●	○	○	○	○	*leucine*
11	11	●	○	○	○	○	*leucine*
12	12	●	○	○	○	○	*leucine*
13	22	○	○	○	○	○	*tryptophan*
14	23	●	○	○	○	○	*leucine*
15	24	○	○	●	○	○	*tryptophan*
16	25	●	○	○	○	○	*leucine*
17	27	○	●	○	○	○	*lysine*
18	28	○	●	○	○	○	*lysine*
19	29	○	●	○	○	○	*lysine*
20	30	●	○	○	○	○	*leucine*
21	31	●	○	○	○	○	*leucine*
22	35	○	○	●	○	○	*tryptophan*
23	37	○	○	○	●	○	*histidine*
24	39	●	○	○	○	○	*leucine*
25	40	○	○	○	●	○	*histidine*
26	41	○	○	○	●	○	*histidine*
27	42	●	○	○	○	○	*leucine*
28	44	○	○	○	●	○	*histidine*
29	45	○	○	○	●	○	*histidine*
30	46	○	○	○	●	○	*histidine*
31	47	●	○	○	○	○	*leucine*
32	50	○	○	○	○	○	*histidine*
33	51	●	○	○	●	○	*leucine*
34	52	●	○	○	○	○	*leucine*
35	53	○	○	○	●	○	*histidine*
36	55	○	○	○	○	●	*adenine*
37	58	○	○	○	●	○	*histidine*
38	59	●	○	○	○	○	*leucine*
39	60	○	○	○	○	●	*adenine*
40	62	●	○	○	○	○	*leucine*
41	63	●	○	○	○	○	*leucine*
42	68	○	○	○	○	●	*adenine*
43		○	○		○	○	*tryptophan*

44		○	○		○	○	*tryptophan*
45		○		○	○	○	*lysine*
46		○	○	○	○	●	*adenine*
47		○	○	○	○	●	*adenine*
48		○	●	○	○	○	*lysine*
49		○	●	○	○	○	*lysine*
50		○	○	○	○	○	*adenine*
51		○	○	●	○	●	*tryptophan*
52		○	○	●	○	○	*tryptophan*
53		○	○	●	○	○	*tryptophan*
54		○	○	●	○	○	*tryptophan*
55		○	●	○	○	○	*lysine*
56		○	●	○	○	○	*lysine*
57		○	●	○	○	○	*lysine*
58		○	●	○	○	○	*lysine*
59		○	○	●	○	○	*tryptophan*
60		○	○	●	○	○	*tryptophan*
61		○	○	●	○	○	*tryptophan*
62		○	○	●	○	○	*tryptophan*
63		○	○	●	○	○	*tryptophan*
64		○	○	●	○	○	*tryptophan*
65		○	○	●	○	○	*tryptophan*
66		○	○	●	○	○	*tryptophan*
67		○	○	●	○	○	*tryptophan*
68		○	○	●	○	○	*tryptophan*
69		○	○	●	○	○	*tryptophan*
70	138	●	○	○	○	○	*leucine*
71	146	●	○	○	○	○	*leucine*
72	160	○	○	●	○	○	*tryptophan*
73	167	○	○	●	○	○	*tryptophan*
74	170	○	○	●	○	○	*tryptophan*
75	177	○	○	●	○	○	*tryptophan*
76	180	○	○	●	○	○	*tryptophan*
77	202	●	○	○	○	○	*leucine*
78	252	○	○	●	○	○	*tryptophan*
79	262	●	○	○	○	○	*leucine*

Note: ⇒ Indicates growth; ○ ⇒ Indicates no growth

Classification of UV induced mutants were performed by testing them on plates containing supplements as *leucine, lysine, tryptophan, histidine* and *adenine*. Classification of UV induced biochemical mutants on the basis of their nutritional requirements is given in Table 5-2.

Table 5-2. Biochemical mutants on the basis of their nutritional requirements.

No. of mutants which grow in the medium	Designation of the classes of mutants
26	*tryptophan*
28	*leucine*
10	*lysine*
9	*histidine*
6	*adenine*

5.1.2. Testing of the *leucine* mutants on SM and SM supplemented media

Out of a total of 79 biochemical mutants, only 26 were tryptophan, 28 were leucine, 10 were *lysine*, 9 were *histidin*e and 6 were *adenine* mutants. The result is summarized in Table 5-3. The 28 *leucine* mutants were used for further study and the rest were stored in the refrigerator at 4°C±1. The characteristics, classification and nomenclature of UV induced mutants are tabulated in Table 5-4.

Table 5-3. The result of plate test of *leucine* mutants.

Serial No.	Culture No.	Plate – A (SM)	Plate – B (VM)	Plate – C (SM+*leucine*)	Interference of mutant types
1	1	○	○	●	*leucine*
2	2	○	○	●	*leucine*
3	3	○	○	●	*leucine*
4	4	○	○	●	*leucine*
5	5	○	○	●	*leucine*
6	6	○	○		*leucine*

7	7	○	○		*leucine*
8	8	○	○		*leucine*
9	9	○	○	●	*leucine*
10	10	○	○	●	*leucine*
11	11	○	○	●	*leucine*
12	12	○	○	●	*leucine*
13	23	○	○	●	*leucine*
14	25	○	○	●	*leucine*
15	30	○	○	●	*leucine*
16	31	○	○	●	*leucine*
17	39	○	○	●	*leucine*
18	42	○	○	●	*leucine*
19	47	○	○	●	*leucine*
20	51	○	○	●	*leucine*
21	52	○	○	●	*leucine*
22	59	○	○	●	*leucine*
23	62	○	○	●	*leucine*
24	63	○	○	●	*leucine*
25	138	○	○	●	*leucine*
26	146	○	○	●	*leucine*
27	202	○	○	●	*leucine*
28	262	○	○	●	*leucine*

Note: ● => Indicates growth
○ => Indicates no growth

Table 5-4. Characteristics, classification and nomenclature of UV induced mutants.

Group	Characteristics	Time of treatment	No. of mutants obtained	Name of the mutants
A	Mutants only grow on SM + *leucine* medium and not in others	45″, 50″ & 1’	28	*leucine* (auxotroph)
B	Mutants only grow on SM + *tryptophan* medium and not in others	45″, 50″ & 1’	26	*tryptophan* (auxotroph)
C	Mutants only grow on SM + *lysine* medium and not in others	45″, 50″ & 1’	10	*lysine* (auxotroph)

D	Mutants only grow on SM + *histidine* medium and not in others	45″, 50″ & 1'	9	*histidine* (auxotroph)
E	Mutants only grow on SM + *adenine* medium and not in others	45″, 50″ & 1'	6	*adenine* (auxotroph)

5.2. TEST FOR HETEROCARYON COMPATIBILITY

Tests for heterocaryon compatibility were made on Vogel minimal medium in small tubes. Heterocaryon tests were carried out between two mutants of the same mating type. Quite a few conidia were taken fresh culture of ~ 4-6 days old as one mutant and were put inside the tube. Similarly a few conidia from the other mutants were taken and put on the top of the conidia of the former mutant. They were also put separately in two different tubes as controls. These tests were carried out in duplicate and triplicate. The tubes after inoculation were incubated at 25°C for 96 hours. Inoculation was performed inside the inoculating chamber to prevent infection. The result of plate test of *leucine* mutants of different concentrations in supplemented SM medium is tabulated in Table 5-5.

The 28 *leucine* mutants were tested for heterocaryon formation in both solid and liquid VM media method and hanging drop method. The tests were observed on 2^{nd}, 4^{th}, 7^{th}, 14^{th} and 21^{st} day. The complementation matrix of 5 *leucine* markers used in the test is shown in Fig. 5-1 and the complementation matrix of 28 *leucine* mutants in solid and liquid VM medium.is shown in Fig. 5-2. The complementation of 28 new *leucine* auxotrophs along with the *leu*-1, *leu*-2, *leu*-3, *leu*-4 and *leu*-5 standard markers is shown in Fig. 5-3. Similarly, the complementation matrices of *leu*-1(33757)aIII, *leu*-3(R156)aI and *leu*-4(R359)aI mutants are shown in Figs. 5-3 to 5-6. Finally, the complementation matrix of newly induced 15 *leucine* auxotrophs used in solid and liquid VM medium method and hanging drop method are shown in Fig. 5-7. The *leucine* mutants and their complementation groups are tabulated in Table 5-6. The complementation map of the *leucine* auxotrophs mutants is shown in Fig. 5-8.

Table 5-5. The result of plate test of *leucine* mutants in supplemented SM medium.

Serial No.	Name of the *leucine* mutants	Plate-A (SM+5mg *leucine*)	Plate-B (SM+10mg leucine)	Plate-C (SM+20mg leucine)	Plate-D (SM+30mg leucine)	Plate-E (SM+50mg leucine)	Inference on mutant type
1	*leu* M-1	•	•	•	●	●	*leucine*
2	*leu* M-2	●	●	•	•	●	*leucine*
3	*leu* M-3	•	•	●	●	●	*leucine*
4	*leu* M-4	●	●	•	●	●	*leucine*
5	*leu* M-5	•	•	•	●	●	*leucine*
6	*leu* M-5	●	●	●	●	●	*leucine*
7	*leu* M-6	●	●	●	●	●	*leucine*
8	*leu* M-6	●	●	•	●	●	*leucine*
9	*leu* M-7	•	•	•	•	●	*leucine*
10	*leu* M-8	●	●	•	•	●	*leucine*
11	*leu* M-9	●	•	●	●	●	*leucine*
12	*leu* M-11	●	●	●	●	●	*leucine*
13	*leu* M-23	●	●	●	●	●	*leucine*
14	*leu* M-25	•	•	•	•	●	*leucine*
15	*leu* M-30	●	•	•	●	●	*leucine*
16	*leu* M-31	●	•	●	●	●	*leucine*
17	*leu* M-39	●	●	●	•	●	*leucine*
18	*leu* M-42	•	•	•	•	●	*leucine*
19	*leu* M-47	●	●	•	•	•	*leucine*
20	*leu* M-51	●	●	•	•	•	*leucine*
21	*leu* M-52	•	●	•	•	●	*leucine*
22	*leu* M-59	•	●	●	●	●	*leucine*
23	*leu* M-62	•	•	●	•	●	*leucine*
24	*leu* M-63	•	•	•	●	●	*leucine*
25	*leu* M-138	•	●	●	●	●	*leucine*
26	*leu* M-146	•	●	●	•	●	*leucine*
27	*leu* M-202	•	●	●	●	●	*leucine*
28	*leu* M-262	•	•	●	●	●	*leucine*

Note: ⇒ Large growth; ⇒ Medium growth; • ⇒ Small growth

5.2.1. Complementation matrix of 5 *leucine* markers

Name of mutants	*leu*-1 (33757)a	*leu*-2 (37501)a	*leu*-3 (R 156)a	*leu*-4 (R 359)a	*leu*-5 (45208[t])
leu-1 (33757)a	○	●	●	●	
leu-2 (37501)a	○	●	●		
leu-2 (37501)a	○	●			
leu-3 (R 156)a	○				
leu-4 (R 359)a	○				

Fig. 5-1. Complementation matrix of 5 leucine markers.

5.2.2. Complementation matrix of 28 *leucine* auxotrophs

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M23	M25	M30	M31	M39	M42	M47	M51	M52	M59	M62	M63	M138	M146	M202	M262
M1	●	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●	●	●	●
M2	●	●	●	●	●	●	○	○	○	●	●	●	●	●	●	○	○	○	●	●	●	●	●	●	○	●	●	●
M3	●	●	○	○	○	●	○	●	●	●	●	○	○	●	●	●	●	○	○	○	○	○	○	○	○	○	○	●
M4	●	●	●	●	●	○	○	○	○	●	●	●	●	●	●	○	○	●	●	○	○	●	●	●	●	○	○	●
M5	○	○	○	●	●	●	●	●	○	○	●	●	●	●	○	○	○	○	○	○	●	●	●	●	●	●	●	●
M6	●	●	○	○	○	●	●	○	○	○	○	○	○	○	○	●	●	○	○	○	●	●	○	○	○	○	○	●
M7	●	●	○	○	○	○	○	●	●	●	●	●	●	●	●	○	○	○	○	○	●	●	●	●	●	●	●	●
M8	●	○	●	●	●	●	○	●	○	○	●	○	○	○	○	●	○	○	○	○	○	○	○	○	○	●	○	●
M9	○	○	○	○	●	●	○	○	●	●	●	○	○	○	○	●	○	○	●	●	●	●	●	●	●	○	○	●
M10	●	●	●	●	●	○	○	○	●	●	●	○	○	○	○	●	○	○	○	○	○	○	○	○	○	●	○	●
M11	○	○	○	●	●	○	○	○	●	●	●	●	●	○	○	●	○	○	○	○	●	●	●	●	●	●	○	●
M12	○	○	○	●	●	○	○	○	●	●	●	●	●	●	○	●	○	○	○	○	●	●	●	●	○	●	●	●
M23	●	●	●	○	○	○	○	○	○	○	○	○	●	●	●	○	○	○	●	●	●	○	○	○	○	●	●	○
M25	○	○	○	●	●	○	○	○	○	○	○	○	●	●	●	●	○	○	○	○	○	○	○	○	●	●	○	○
M30	●	●	●	○	○	○	○	○	○	○	○	○	●	●	●	○	○	○	●	●	●	●	●	●	●	●	●	●
M31	●	●	○	○	○	○	○	○	○	○	○	○	●	●	●	●	○	○	●	●	●	●	●	●	●	●	●	●
M39	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	●	○	○	○	○	○	○	○	●	●	●	●
M42	●	●	●	●	○	○	○	○	○	○	○	○	●	○	○	○	○	●	○	○	○	○	○	○	●	●	●	●
M47	○	●	●	●	○	○	○	○	○	○	○	○	●	○	○	○	○	○	●	○	○	○	○	○	●	●	●	●
M51	●	●	●	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	●	○	○	○	○	●	●	●	●
M52	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	○	○	○	●	●	●	●
M59	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	●
M62	○	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	●	●	○	●
M63	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M138	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M146	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
M146	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Fig. 5-2. Complementation matrix of 28 *leucine* mutants in solid and liquid VM medium. Heterokaryon positive (○), Heterokaryon negative (●).

5.2.3. Complementation matrix of 28 *leucine* auxotrophs with 5 *leucine* standard markers

Name of the *leucine* markers	M2	M6	M8	M9	M31	M52	M51	M7	M59	M62	M5	M42	M138	M1	M3	M4	M8	M9	M11	M23	M25	M30	M39	M47	M63	M146	M202	M262
leu-1 (33757) IIIa	○	○	○	○	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
leu-2 (37501) IVa	●	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
leu-3 (R156)Ia	●	●	●	●	●	●	●	○	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
leu-4 (R359)Ia	●	●	●	●	●	●	●	●	●	●	○	○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
leu-5 (45208{t})Va	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Fig. 5-3. Complementation of 28 new *leucine* auxotrophs along with the *leu*-1, *leu*-2, *leu*-3, *leu*-4 and *leu*-5 standard markers.

	M2	**M6**	**M8**	**M9**	**M31**	**M52**
M2		○	○	○	○	○
M6			○	○	○	○
M8				○	○	○
M9					○	○
M31						○
M51						

Fig. 5-4. Complementation matrix of *leu*-1(33757)aIII mutants.

	M7	**M59**	**M62**
M7		○	○
M59			○
M62			

Fig. 5-5. Complementation matrix of *leu*-3(R156)aI mutants.

	M5	**M42**	**M138**
M5		○	○
M42			○
M138			

Fig. 5-6. Complementation matrix of *leu*-4(R359)aI mutants.

5.2.4. Complementation matrix of newly induced 15 *leucine* auxotrophs

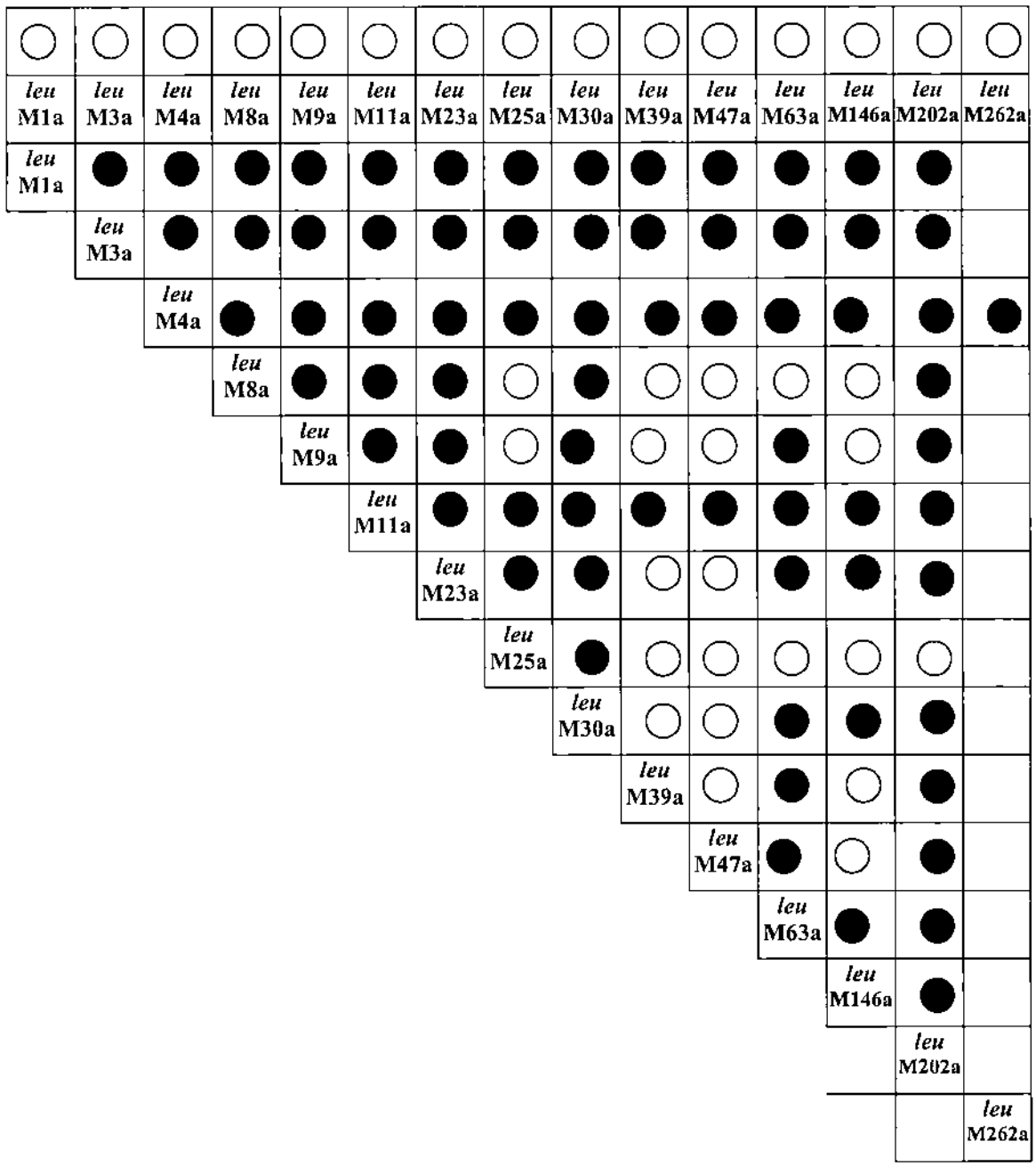


Fig. 5-7. Complementation matrix of newly induced 15 *leucine* auxotrophs used in solid and liquid VM medium method and hanging drop method. Heterokaryon positive (◯), Heterokaryon negative (●).

Table 5-6. *leucine* mutants and their complementation groups.

Nutritional requirements	Complementation group	Mutants in each group	Heterocaryon reactions
leucine	I	*leu* M1a	Positive with *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a & *leu* M262a and negative with none
	II	*leu* M3a	Positive with *leu* M1a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a & *leu* M262a and negative with none
	III	*leu* M4a	Positive with *leu* M1a, *leu* M3a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a & *leu* M262a and negative with none
	IV	*leu* M11a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a & *leu* M262a and negative with none
	V	*leu* M262a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a & *leu* M202a and negative with none
	VI	*leu* M202a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a & *leu* M262a and negative with *leu* M25a only
	VII	*leu* M63a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M146a, *leu* M202a & *leu* M262a and negative with *leu* M8a & *leu* M25a
	VIII	*leu* M9a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M11a, *leu* M23a, *leu* M30a, *leu* M63a, *leu* M202a & *leu* M262a and negative with *leu* M25a, *leu* M39a, *leu* M47a & *leu* M146a
	IX	*leu* M23a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M25a, *leu* M30a, *leu* M63a, *leu* M146a, *leu* M202a & *leu* M262a and negative with *leu* M39a & *leu* M47a
	X	*leu* M30a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M63a, *leu* M202a & *leu* M262a and negative with *leu* M39a & *leu* M47a
	XI	*leu* M8a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M30a, *leu* M202a & *leu* M262a and negative with *leu* M25a, *leu* M39a, *leu* M47a, *leu* M63a & *leu* M146a
	XII	*leu* M146a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M11a, *leu* M23a, *leu* M30a, *leu* M63a, *leu* M202a & *leu* M262a and negative with *leu* M8a, *leu* M9a, *leu* M25a, *leu* M39a & *leu* M47a
	XIII	*leu* M25a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M11a, *leu* M23a, *leu* M30a & *leu* M262a and negative with *leu* M8a, *leu* M9a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a & *leu* M202a
	XIV	*leu* M39a & 47a	Positive with *leu* M1a, *leu* M3a, *leu* M4a, *leu* M11a, *leu* M63a, *leu* M202a & *leu* M262a and negative with *leu* M8a, *leu* M9a, *leu* M23a, *leu* M25a, *leu* M30a & *leu* M146a

5.2.5. Complementation map of newly induced 15 *leucine* auxotrophs

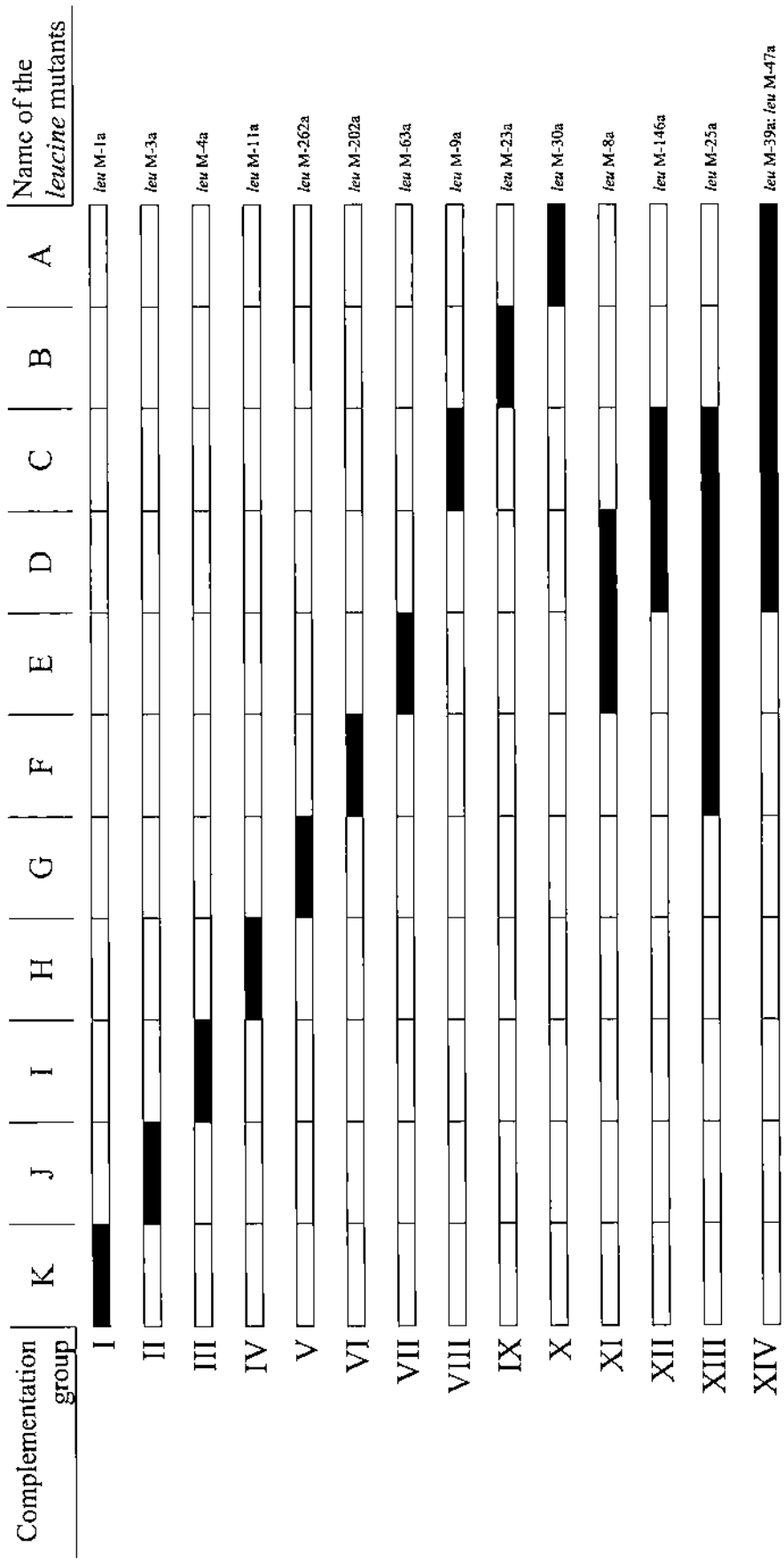


Fig. 5-8. Complementation map of newly induced 15 *leucine* auxotrophs.

- ⇨ ▬ = Defective region; ▭ = Functional region
- ⇨ A, B, C, D, E, F, G, H, I, J, K = denotes complons; I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV = complementation groups
- ⇨ *leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a, *leu* M262a = 15 *leucine* auxotrophs
- ⇨ Complementation groups are shown at the left side and mutants are at the right side
- ⇨ Hollow bar indicates functionally active regions and defective region is indicated by solid bar

5.3. ISOLATION AND FUSION OF *leucine* AUXOTROPH IN *Neurospora crassa* FOR DETECTION OF COMPLEMENTATION

Protoplasts were isolated enzymatically from the *leucine* auxotrophs in *Neurospora crassa* with the help of enzymic digestion using snail gastric and intestinal extracts. Complementation studies by the fusion of their protoplasts were shown in Tables 5-7 to 5-10.

Table 5-7. Efficiency of different concentrations both gastric and intestinal extracts on three *leucine* mutant strains.

Name of the mutant strains	Ratio of sorbitol and gastric extracts	Percentages (%) of protoplasts observed (approx.)	Ratio of sorbitol and intestinal extracts	Percentages (%) of protoplasts observed (approx.)
leu M9a	3:2	25	3:2	15
	2:3	38	2:3	20
	1:4	60	1:4	30
leu M30a	3:2	28	3:2	18
	2:3	35	2:3	22
	1:4	65	1:4	35
leu M51a	3:2	30	3:2	22
	2:3	40	2:3	28
	1:4	70	1:4	30

Fig. 5-9. Morphology of garden snail.

Fig. 5-10. Production of *Neurospora crassa* colony on cellophane paper

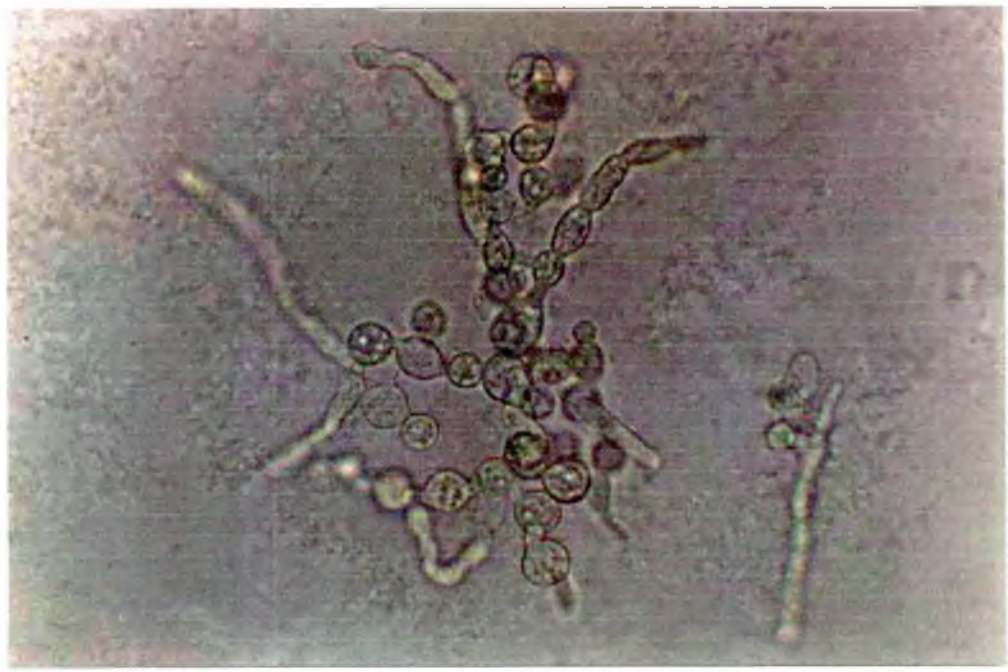


Fig. 5-11. Mycelial cell wall were being digested.

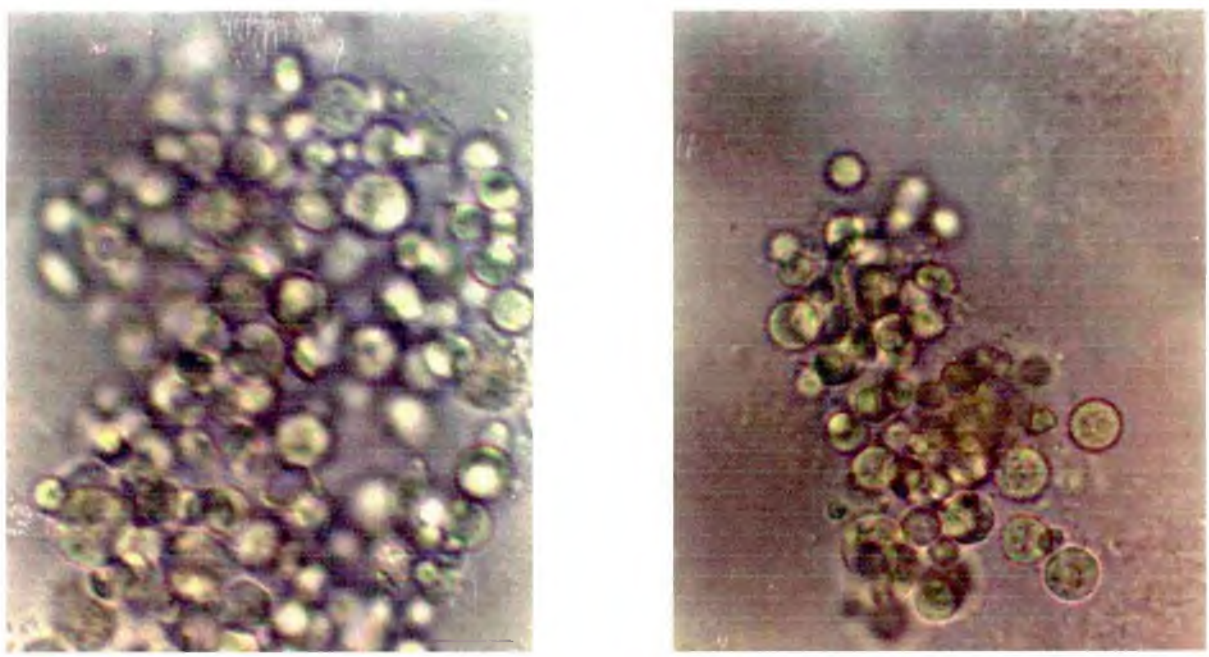


Fig. 5-12. Micrographs of protoplasts of *Neurospora crassa*
a) Cell wall was digested with gastric extracts of *Helix sp*,
b) Cell wall was digested with intestinal extracts of *Helix sp*.

Table 5-8. Protoplast fusion & growth of colonies on SM plates.

No. of plates	Name of the mutants that were taken for protoplast fusion	Colony formed after the following hours		
		24	48	72
1st plate	*leu* M9a + *leu* M30a	Few colonies	Several colonies	Several colonies
2nd plate	*leu* M9a + *leu* M51a	Few colonies	Several colonies	Several colonies
3rd plate	*leu* M30a + *leu* M51a	Few colonies	Several colonies	Several colonies

Table 5-9. The growth and complementation of protoplasts between three *leucine* mutants on different SM medium.

Name of the mutant strains	Growth of protoplast on SM media	Growth of protoplast on PEG unsupplemented SM media	Interference on complementation
leu M9a	No growth	No growth	No growth due to non-complementation
leu M30a	No growth	No growth	No growth due to non-complementation
leu M51a	No growth	No growth	No growth due to non-complementation
leu M9a + *leu* M30a	Growth	Growth	Growth due to complementation
leu M9a + *leu* M51a	Growth	Growth	Growth due to complementation
leu M30a + *leu* M51a	Growth	Growth	Growth due to complementation

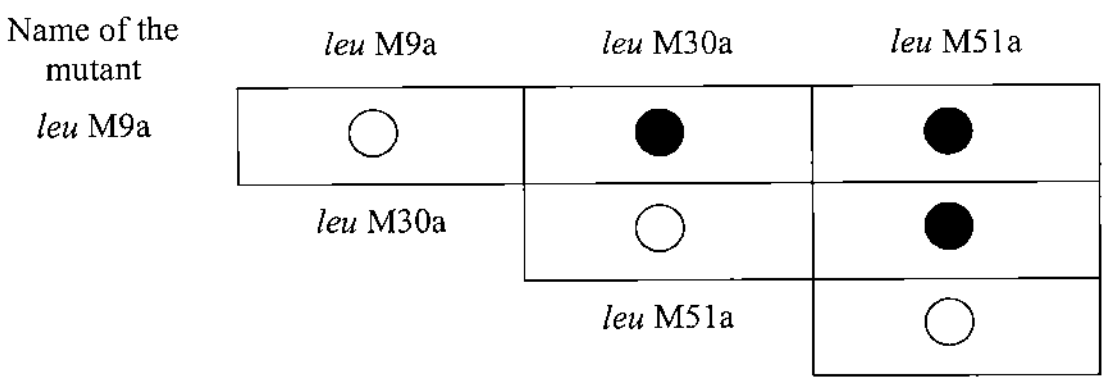


Fig. 5-13. Complementation matrix of *leucine* mutants based on protoplast.

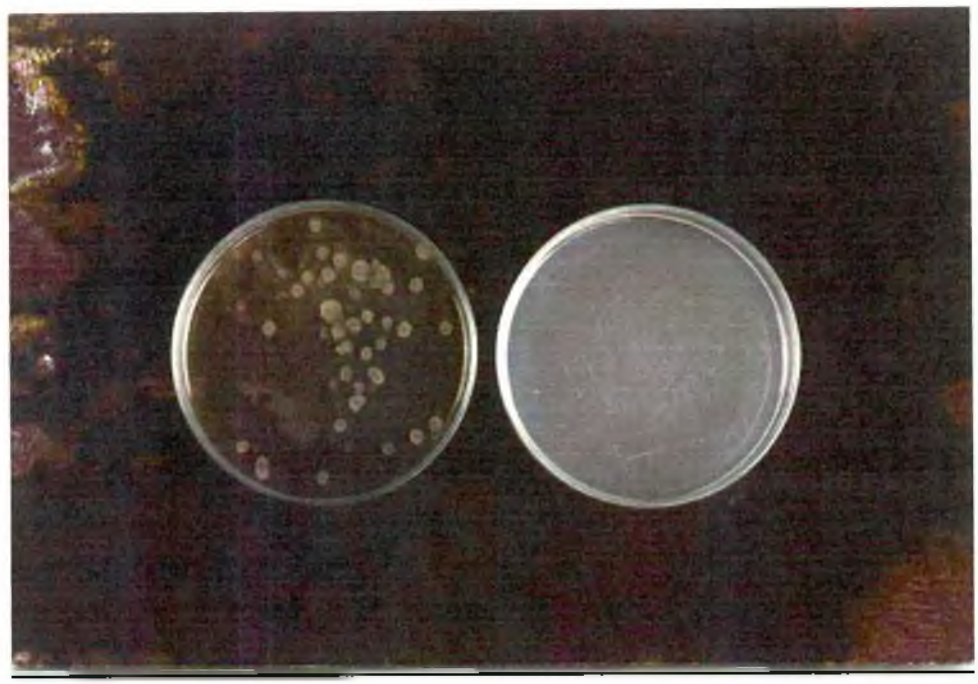


Fig. 5-14. Protoplast fusion of *leu* M9a with *leu* M30a.

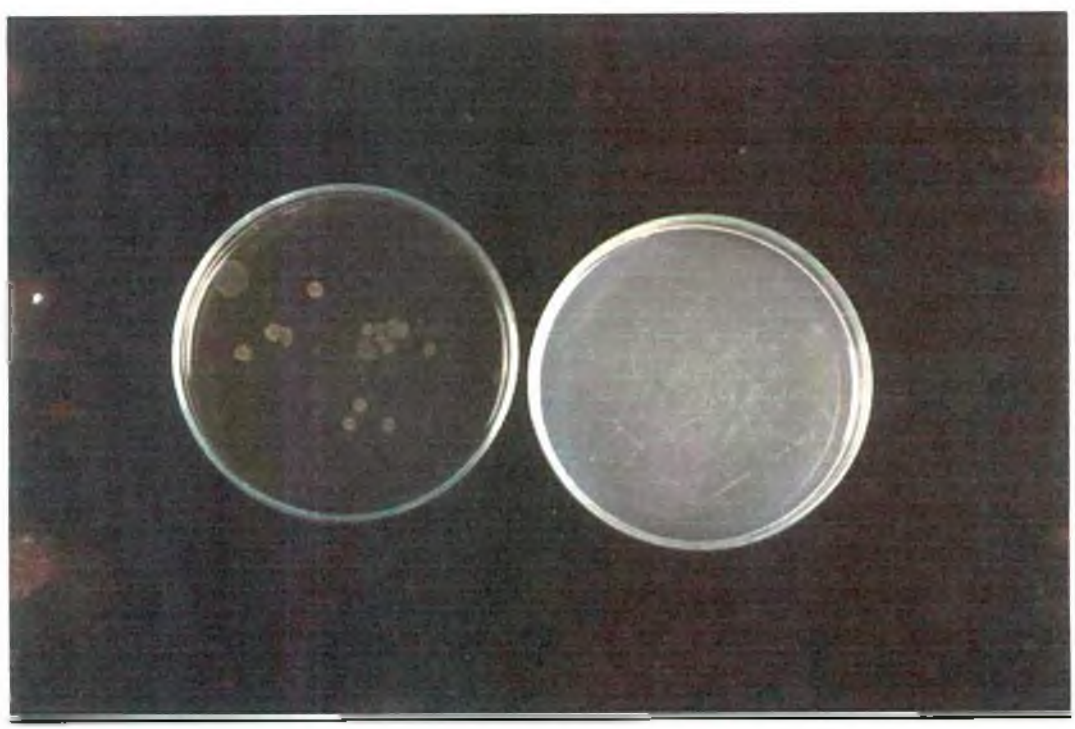


Fig. 5-15. Protoplast fusion of *leu* M30a with *leu* M51a.

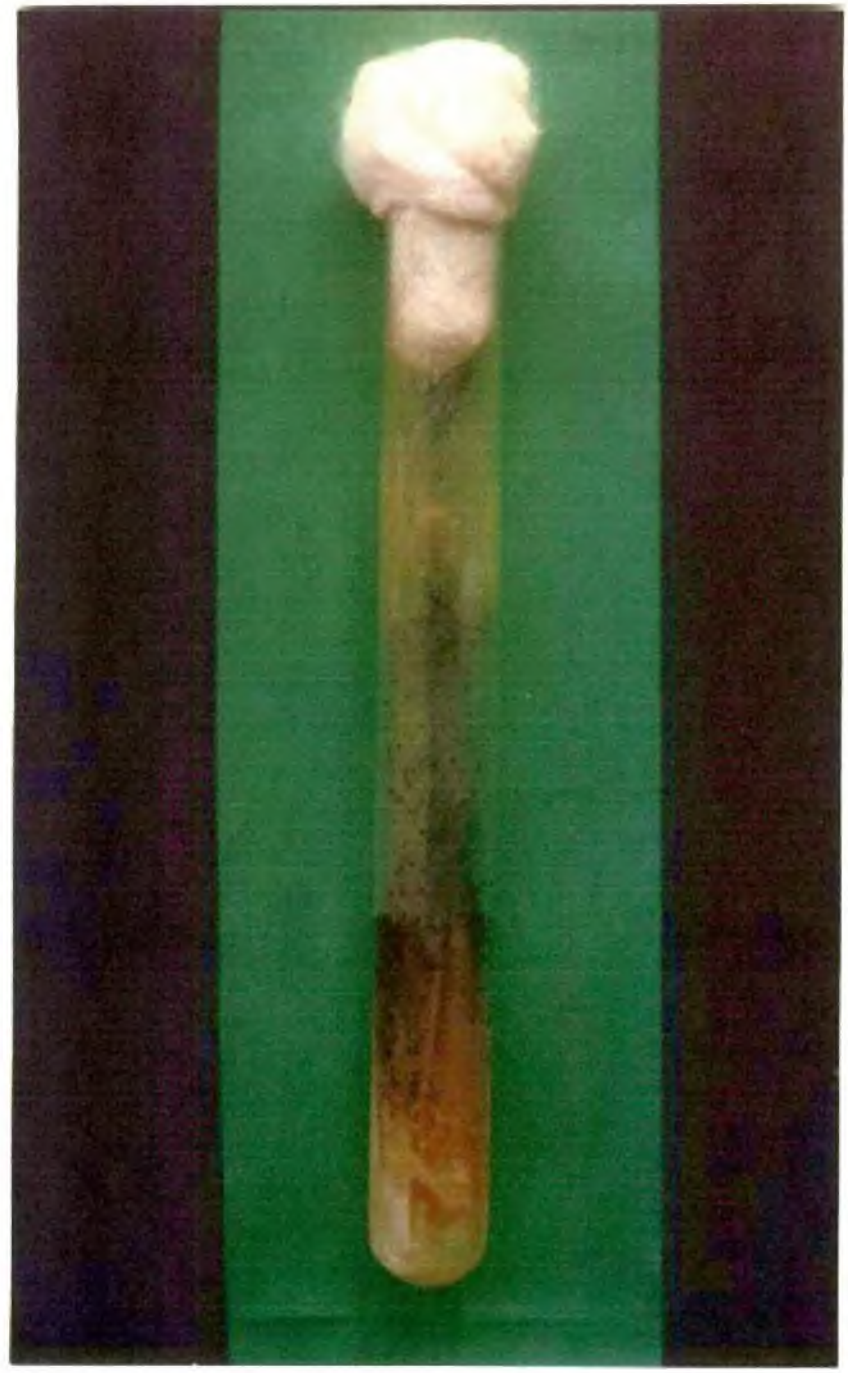


Fig. 5-16. A fertile cross of *Neurospora crassa*.

5.4. MATING TYPE AND FERTILITY TEST OF *leucine* AUXOTROPHS WITH Em'A' AND Em'a' OF *Neurospora crassa*

The experiment was performed in Westergaard's crossing medium. In the experiments, 2% and 4% phosphate supplements per 100 ml and 4-6 days old culture were used. The results are tabulated in Table 5-10.

Table 5-10. Mating type and fertility of leucine auxotrophs with wild types.

Serial No.	Name of the cross	Perithecia formed (Yes/No)	Days taken for the formation of Perithecia	Frequency of Perithecia	Size of Perithecia	Spore Sheding days	Fertility	Mating type
1	*leu*M1xEmA	Yes	7	Many	Large	15	Fertile	a
	leu M1xEma	No	-	-	-	-	Sterile	-
2	*leu*M2xEmA	Yes	7	Many	Medium	15	Fertile	a
	*leu*M2xEma	No	-	-	-	-	Sterile	-
3	*leu*M3xEmA	Yes	7	Many	Medium	15	Fertile	a
	leu M3xEma	No	-	-	-	-	Sterile	-
4	*leu*M4xEmA	Yes	7	Many	Large	17	Fertile	a
	*leu*M4xEma	No	-	-	-	-	Sterile	-
5	*leu*M5xEmA	Yes	7	Many	Large	15	Fertile	a
	leu M5xEma	No	-	-	-	-	Sterile	-
6	*leu*M5xEmA	Yes	7	Many	Large	16	Fertile	a
	*leu*M5xEma	No	-	-	-	-	Sterile	-
7	*leu*M6xEmA	Yes	7	Many	Medium	20	Fertile	a
	leu M6xEma	No	-	-	-	-	Sterile	-
8	*leu*M6xEmA	Yes	7	Many	Medium	20	Fertile	a
	*leu*M6xEma	No	-	-	-	-	Sterile	-
9	*leu*M7xEmA	Yes	7	Many	Medium	15	Fertile	a
	leu M7xEma	No	-	-	-	-	Sterile	-
10	*leu*M8xEmA	Yes	7	Many	Medium	15	Fertile	a
	*leu*M8xEma	No	-	-	-	-	Sterile	-
11	*leu*M9xEmA	Yes	8	Many	Medium	22	Fertile	a
	leu M9xEma	No	-	-	-	-	Sterile	-
12	*leu*M11xEmA	Yes	7	Many	Large	19	Fertile	a
	leuM11xEma	No	-	-	-	-	Sterile	-
13	*leu*M23xEmA	Yes	7	Few	Large	20	Fertile	a
	leu M23xEma	No	-	-	-	-	Sterile	-

14	*leu*M25xEmA	Yes	9	Many	Large	20	Fertile	a
	*leu*M25xEma	No	-	-	-	-	Sterile	-
15	*leu*M30xEmA	Yes	9	Many	Large	15	Fertile	a
	leu M30xEma	No	-	-	-	-	Sterile	-
16	*leu*M31xEmA	Yes	9	Few	Small	25	Fertile	a
	*leu*M31xEma	No	-	-	-	-	Sterile	-
17	*leu*M39xEmA	Yes	9	Many	Large	20	Fertile	a
	leu M39xEma	No	-	-	-	-	Sterile	-
18	*leu*M42xEmA	Yes	7	Few	Large	17	Fertile	a
	*leu*M42xEma	No	-	-	-	-	Sterile	-
19	*leu*M47xEmA	Yes	7	Many	Large	17	Fertile	a
	leu M47xEma	No	-	-	-	-	Sterile	-
20	*leu*M51xEmA	Yes	10	Many	Large	18	Fertile	a
	*leu*M51xEma	No	-	-	-	-	Sterile	-
21	*leu*M52xEmA	Yes	10	Many	small	23	Fertile	a
	leu M52xEma	No	-	-	-	-	Sterile	-
22	*leu*M59xEmA	Yes	10	Many	Small	23	Fertile	a
	*leu*M59xEma	No	-	-	-	-	Sterile	-
23	*leu*M62xEmA	Yes	7	Many	Medium	18	Fertile	a
	leu M62xEma	No	-	-	-	-	Sterile	-
24	*leu*M63xEmA	Yes	10	Many	Large	20	Fertile	a
	*leu*M63xEma	No	-	-	-	-	Sterile	-
25	*leu*M138xEmA	Yes	8	Few	Small	25	Fertile	a
	leu M138xEma	No	-	-	-	-	Sterile	-
26	*leu*M146xEmA	Yes	8	Many	Large	18	Fertile	a
	*leu*M146xEma	No	-	-	-	-	Sterile	-
27	*leu*M202xEmA	Yes	8	Many	Medium	18	Fertile	a
	leu M202xEma	No	-	-	-	-	Sterile	-
28	*leu*M262xEmA	Yes	78	Many	Large	17	Fertile	a
	*leu*M262xEma	No	-	-	-	-	Sterile	-

Conclusion: If perithecia formed with Em'a' the mating type will be 'A' and if perithecia formed with Em'A' it will be 'a'.

5.5. SEGREGATION RATIO OF *leucine* MUTANTS INDUCED WITH UV

Spores of the crosses were spread on SM plate and were counted, isolated, analyzed and recorded and are given in Table 5-11.

Table 5-11. Segregation ratio of *leucine* mutants induced with UV ray.

Serial No.	Name of the cross	Total No. of Spore isolated	No. of wild types isolated	No. of mutant isolated	Segregation ratio of Mutant: Wild
1	*leu* M1a x EmA	106	57	49	1:1.632
2	*leu* M3a x EmA	105	53	52	1:1.019
3	*leu* M4a x EmA	110	61	49	1:1.244
4	*leu* M8a x EmA	103	55	48	1:1.145
5	*leu* M9a x EmA	101	52	49	1:1.016
6	*leu* M11a x EmA	100	51	49	1:1.04
7	*leu* M23a x EmA	115	62	53	1:1.698
8	*leu* M25a x EmA	108	56	52	1:1.076
9	*leu* M30a x EmA	120	61	59	1:1.033
10	*leu* M39a x EmA	104	54	50	1:1.06
11	*leu* M47a x EmA	108	57	51	1:1.117
12	*leu* M63a x EmA	107	59	48	1:1.229
13	*leu* M146a x EmA	109	58	51	1:1.137
14	*leu* M202a x EmA	102	52	50	1:1.04
15	*leu* M262a x EmA	112	62	52	1:1.192

Conclusion: The ratio between mutant isolates and wild type isolates was calculated. In most cases it was found to be ~ 1:1.

5.6. FERTILITY OF *leucine* MUTANTS WITH MARKERS

15 *leucine* mutants (*leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a, *leu* M262a) were crossed with markers of 7 linkage groups. The crosses were performed in Westergaard's crossing medium with required supplements and are shown in Tables 5-12 to 5-26.

Table 5-12. Fertility of *leu* M1a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of initiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M1a X *his*-2(T51M152(t))A	I	Yes	10	Many Medium	Yes	20	Fertile
*leu*M1a X *arg*-5(27947)A	II	Yes	15	Many Medium	Yes	20	Fertile
*leu*M1a X *trp*-1(10575)A	III	Yes	15	Many Large	Yes	22	Good Fertile
*leu*M1a X *pyr*-1(H263)A	IV	Yes	17	Many Large	Yes	25	Good Fertile
*leu*M1a X *trp*-5(A420)A	V	Yes	17	Few Small	Yes	20	Poor Fertile
*leu*M1a X *ad*-1(3254)A	VI	Yes	12	Moderate Large	Yes	25	Fertile
*leu*M1a X *arg*-10(B317)A	VII	Yes	12	Many Medium	Yes	25	Fertile
*leu*M1a X *nt*(65001)A	VII	Yes	12	Few Small	Yes	24	Poor Fertile

Table 5-13. Fertility of *leu* M3a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of initiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M3a X *his*-2(T51M152(t))A	I	Yes	18	Many Medium	Yes	22	Fertile
*leu*M3a X *arg*-5(27947)A	II	Yes	18	Few Small	Yes	20	Poor Fertile
*leu*M3a X *trp*-1(10575)A	III	Yes	16	Few Small	Yes	23	Poor Fertile
*leu*M3a X *pyr*-1(H263)A	IV	Yes	17	Many Large	Yes	23	Good Fertile
*leu*M3a X *trp*-5(A420)A	V	Yes	15	Few Small	Yes	24	Poor Fertile
*leu*M1a X *ad*-1(3254)A	VI	Yes	18	Many Medium	Yes	21	Fertile
*leu*M3a X *arg*-10(B317)A	VII	Yes	18	Many Medium	Yes	24	Fertile
*leu*M3a X *nt*(65001)A	VII	Yes	17	Few Small	Yes	25	Poor Fertile

Table 5-14. Fertility of *leu* M4a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Ycs/No)	Sheding days	Fertility
*leu*M4a X *his*-2(T51M152(t))A	I	Yes	14	Few Small	Yes	25	Poor Fertile
*leu*M4a X *arg*-5(27947)A	II	Yes	14	Few Small	Yes	25	Poor Fertile
*leu*M4a X *trp*-1(10575)A	III	Yes	15	Many Large	Yes	20	Good Fertile
*leu*M4a X *pyr*-1(H263)A	IV	Yes	15	Many Large	Yes	20	Good Fertile
*leu*M4a X *trp*-5(A420)A	V	Yes	15	Many Large	Yes	21	Good Fertile
*leu*M4a X *ad*-1(3254)A	VI	Yes	13	Many Medium	Yes	23	Fertile
*leu*M4a X *arg*-10(B317)A	VII	Yes	14	Many Medium	Yes	23	Fertile
*leu*M4a X *nt*(65001)A	VII	Yes	14	Few Small	Yes	25	Poor Fertile

Table 5-15. Fertility of *leu* M8a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of initiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M3a X *his*-2(T51M152(t))A	I	Yes	18	Many Medium	Yes	22	Fertile
*leu*M3a X *arg*-5(27947)A	II	Yes	18	Few Small	Yes	20	Poor Fertile
*leu*M3a X *trp*-1(10575)A	III	Yes	16	Few Small	Yes	23	Poor Fertile
*leu*M3a X *pyr*-1(H263)A	IV	Yes	17	Many Large	Yes	23	Good Fertile
*leu*M3a X *trp*-5(A420)A	V	Yes	15	Few Small	Yes	24	Poor Fertile
*leu*M1a X *ad*-1(3254)A	VI	Yes	18	Many Medium	Yes	21	Fertile
*leu*M3a X *arg*-10(B317)A	VII	Yes	18	Many Medium	Yes	24	Fertile
*leu*M3a X *nt*(65001)A	VII	Yes	17	Few Small	Yes	25	Poor Fertile

Table 5-16. Fertility of *leu* M9a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M9a X *his*-2(T51M152(t))A	I	Yes	13	Few Small	Yes	22	Poor Fertile
*leu*M9a X *arg*-5(27947)A	II	Yes	11	Few Small	Yes	23	Poor Fertile
*leu*M9a X *trp*-1(10575)A	III	Yes	12	Many Large	Yes	23	Good Fertile
*leu*M9a X *pyr*-1(H263)A	IV	Yes	12	Many Large	Yes	22	Good Fertile
*leu*M9a X *trp*-5(A420)A	V	Yes	13	Many Large	Yes	23	Good Fertile
*leu*M9a X *ad*-1(3254)A	VI	Yes	14	Many Medium	Yes	24	Fertile
*leu*M8a X *arg*-10(B317)A	VII	Yes	14	Few Small	Yes	24	Poor Fertile
*leu*M9a X *nt*(65001)A	VII	Yes	14	Few Small	Yes	24	Poor Fertile

Table 5-17. Fertility of *leu* M11a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M11a X *his*-2(T51M152(t))A	I	Yes	11	Many Medium	Yes	22	Fertile
*leu*M11a X *arg*-5(27947)A	II	Yes	10	Few Small	Yes	24	Poor Fertile
*leu*M11a X *trp*-1(10575)A	III	Yes	11	Many Large	Yes	23	Good Fertile
*leu*M11a X *pyr*-1(H263)A	IV	Yes	12	Many Large	Yes	23	Good Fertile
*leu*M11a X *trp*-5(A420)A	V	Yes	12	Many Large	Yes	24	Good Fertile
*leu*M11a X *ad*-1(3254)A	VI	Yes	13	Many Medium	Yes	24	Fertile
*leu*M11a X *arg*-10(B317)A	VII	Yes	15	Few Small	Yes	20	Poor Fertile
*leu*M11a X *nt*(65001)A	VII	Yes	15	Few Small	Yes	20	Poor Fertile

Table 5-18. Fertility of *leu* M23a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of initiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M23a X *his*-2(T51M152(t))A	I	Yes	15	Few Small	Yes	20	Poor Fertile
*leu*M23a X *arg*-5(27947)**A**	II	Yes	15	Few Small	Yes	20	Poor Fertile
*leu*M23a X *trp*-1(10575)A	III	Yes	16	Many Large	Yes	22	Good Fertile
*leu*M23a X *pyr*-1(H263)**A**	IV	Yes	13	Many Large	Yes	22	Good Fertile
*leu*M23a X *trp*-5(A420)A	V	Yes	13	Many Large	Yes	22	Good Fertile
*leu*M23a X *ad*-1(3254)A	VI	Yes	14	Many Medium	Yes	22	Fertile
*leu*M23a X *arg*-10(B317)A	VII	Yes	14	Many Medium	Yes	21	Fertile
*leu*M23a X *nt*(65001)A	VII	Yes	14	Many Medium	Yes	21	Fertile

Table 5-19. Fertility of *leu* M25a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M25a X *his*-2(T51M152(t))A	I	Yes	16	Many Medium	Yes	22	Fertile
*leu*M25a X *arg*-5(27947)A	II	Yes	14	Few Small	Yes	24	Poor Fertile
*leu*M25a X *trp*-1(10575)A	III	Yes	15	Many Large	Yes	20	Good Fertile
*leu*M25a X *pyr*-1(H263)A	IV	Yes	14	Many Large	Yes	20	Good Fertile
*leu*M25a X *trp*-5(A420)A	V	Yes	14	Many Large	Yes	20	Good Fertile
*leu*M25a X *ad*-1(3254)A	VI	Yes	13	Few Small	Yes	24	Poor Fertile
*leu*M25a X *arg*-10(B317)A	VII	Yes	13	Few Small	Yes	24	Poor Fertile
*leu*M25a X *nt*(65001)A	VII	Yes	13	Many Medium	Yes	21	Fertile

Table 5-20. Fertility of *leu* M30a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M30a x *his*-2(t51M152(t))A	I	Yes	13	Few Small	Yes	26	Poor Fertile
*leu*M30a x *arg*-5(27947)A	II	Yes	15	Few Small	Yes	25	Poor Fertile
*leu*M30a x *trp*-1(10575)A	III	Yes	16	Many Large	Yes	20	Good Fertile
*leu*M30a x *pyr*-1(H263)A	IV	Yes	15	Many Large	Yes	20	Good Fertile
*leu*M30a x *trp*-5(A420)A	V	Yes	13	Many Large	Yes	20	Good Fertile
*leu*M30a x *ad*-1(3254)A	VI	Yes	13	Few Small	Yes	27	Poor Fertile
*leu*M30a x *arg*-10(B317)A	VII	Yes	14	Few Small	Yes	25	Poor Fertile
*leu*M30a x *nt*(65001)A	VII	Yes	14	Many Medium	Yes	25	Fertile

Table 5-21. Fertility of *leu* M39a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M39a x *his*-2(t51M152(t))A	I	Yes	13	Few Small	Yes	23	Poor Fertile
*leu*M39a x *arg*-5(27947)A	II	Yes	13	Few Small	Yes	24	Poor Fertile
*leu*M39a x *trp*-1(10575)A	III	Yes	13	Many Large	Yes	25	Good Fertile
*leu*M39a x *pyr*-1(H263)A	IV	Yes	14	Many Large	Yes	25	Good Fertile
*leu*M39a x *trp*-5(A420)A	V	Yes	14	Many Large	Yes	25	Good Fertile
*leu*M39a x *ad*-1(3254)A	VI	Yes	16	Many Medium	Yes	25	Fertile
*leu*M39a x *arg*-10(B317)A	VII	Yes	12	Many Medium	Yes	24	Fertile
*leu*M39a x *nt*(65001)A	VII	Yes	14	Many Medium	Yes	24	Fertile

Table 5-22. Fertility of *leu* M47a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M47a X *his*-2(T51M152(t))A	I	Yes	15	Many Medium	Yes	23	Fertile
*leu*M47a X *arg*-5(27947)A	II	Yes	15	Many Medium	Yes	25	Fertile
*leu*M47a X *trp*-1(10575)A	III	Yes	15	Many Large	Yes	25	Good Fertile
*leu*M47a X *pyr*-1(H263)A	IV	Yes	14	Many Large	Yes	24	Good Fertile
*leu*M47a X *trp*-5(A420)A	V	Yes	13	Many Large	Yes	24	Good Fertile
*leu*M47a X *ad*-1(3254)A	VI	Yes	13	Few Small	Yes	24	Poor Fertile
*leu*M47a X *arg*-10(B317)A	VII	Yes	14	Few Small	Yes	22	Poor Fertile
*leu*M47a X *nt*(65001)A	VII	Yes	14	Few Small	Yes	24	Poor Fertile

Table 5-23. Fertility of *leu* M63a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M63a X *his*-2(T51M152(t))A	I	Yes	15	Many Large	Yes	23	Good Fertile
*leu*M63a X *arg*-5(27947)A	II	Yes	14	Few Small	Yes	23	Poor Fertile
*leu*M63a X *trp*-1(10575)A	III	Yes	13	Many Large	Yes	24	Good Fertile
*leu*M63a X *pyr*-1(H263)A	IV	Yes	13	Many Large	Yes	24	Good Fertile
*leu*M63a X *trp*-5(A420)A	V	Yes	12	Many Large	Yes	24	Good Fertile
*leu*M63a X *ad*-1(3254)A	VI	Yes	14	Few Small	Yes	24	Poor Fertile
*leu*M63a X *arg*-10(B317)A	VII	Yes	11	Few Small	Yes	25	Poor Fertile
*leu*M63a X *nt*(65001)A	VII	Yes	11	Many Medium	Yes	23	Fertile

Table 5-24. Fertility of *leu* M146a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M146a X *his*-2(T51M152(t))A	I	Yes	11	Many Medium	Yes	22	Fertile
*leu*M146a X *leu*-5(27947)A	II	Yes	11	Few Small	Yes	22	Poor Fertile
*leu*M146a X *trp*-1(10575)A	III	Yes	12	Many Large	Yes	21	Good Fertile
*leu*M146a X *pyr*-1(H263)A	IV	Yes	12	Many Large	Yes	23	Good Fertile
*leu*M146a X *trp*-5(A420)A	V	Yes	12	Many Large	Yes	24	Good Fertile
*leu*M146a X *ad*-1(3254)A	VI	Yes	14	Many Medium	Yes	21	Fertile
*leu*M146a X *arg*-10(B317)A	VII	Yes	14	Many Medium	Yes	21	Fertile
*leu*M146a X *nt*(65001)A	VII	Yes	12	Few Small	Yes	25	Poor Fertile

Table 5-25. Fertility of *leu* M202a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of inifiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M202a X *his*-2(T51M152(t))A	I	Yes	13	Many Medium	Yes	21	Fertile
*leu*M202a X *arg*-5(27947)A	II	Yes	14	Few Small	Yes	22	Poor Fertile
*leu*M202a X *trp*-1(10575)A	III	Yes	14	Many Large	Yes	23	Good Fertile
*leu*M202a X *pyr*-1(H263)A	IV	Yes	12	Many Large	Yes	23	Good Fertile
*leu*M202a X *trp*-5(A420)A	V	Yes	15	Many Large	Yes	23	Good Fertile
*leu*M202a X *ad*-1(3254)A	VI	Yes	12	Many Medium	Yes	23	Fertile
*leu*M202a X *arg*-10(B317)A	VII	Yes	11	Many Medium	Yes	24	Fertile
*leu*M202a X *nt*(65001)A	VII	Yes	10	Few Small	Yes	20	Poor Fertile

Table 5-26. Fertility of *leu* M262a with standard markers.

Name of the cross	Linkage group of marker	Whether perithecia formed (Yes/No)	Days of initiation of perithecia	Frequency and size of perithecia	Whether spore shed (Yes/No)	Sheding days	Fertility
*leu*M262a X *his*-2(T51M152(t))A	I	Yes	14	Many Medium	Yes	23	Fertile
*leu*M262a X *arg*-5(27947)A	II	Yes	15	Many Medium	Yes	24	Fertile
*leu*M262a X *trp*-1(10575)A	III	Yes	13	Many Large	Yes	24	Good Fertile
*leu*M262a X *pyr*-1(H263)A	IV	Yes	13	Many Large	Yes	25	Good Fertile
*leu*M262a X *trp*-5(A420)A	V	Yes	12	Many Medium	Yes	23	Fertile
*leu*M262a X *ad*-1(3254)A	VI	Yes	11	Few Small	Yes	25	Poor Fertile
*leu*M262a X *arg*-10(B317)A	VII	Yes	12	Few Small	Yes	25	Poor Fertile
*leu*M262a X *nt*(65001)A	VII	Yes	12	Many Medium	Yes	25	Fertile

5.7. STUDY OF LINKAGE

For the study of linkage the mutants (*leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a, *leu* M262a) were crossed with the mutants of 7 linkage groups [*his*-2(T51M152(t))AI, *arg*-5(27947)AII, *trp*-1(10575)AIII, *pyr*-1(H263)AIV, *trp*-5(A420)AV, *ad*-1(3254)AVI and *arg*-10(B317)AVII]. The crosses were performed in Westergaard's crossing medium with required supplements. The spores from the crosses were spread on SM supplemented plate. They were kept at 58°C for 50 minutes and then inoculated at 25°C for 12-16 hours. The germinating and growing spores were isolated randomly and incubated into small tube containing VM supplemented medium with necessary amino acid. The cultures were incubated at 25°C for 4-6 days. They were tested on SM and SM supplemented plates. Four types of progeny were found:

- ⇨ Mutant
- ⇨ Marker
- ⇨ Marker + Mutant
- ⇨ Wild

Then the linkage was studied by analyzing the percentage of recombinant and parental types (Tables 5-27 to 5-42 and Figs. 5-17 to 5-35).

5.7.1. Determination of genetic map of *leu* M1a auxotroph

Table 5-27. Linkage of *leu* M1a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M1a X *his*-2(T51M152(t))A	105	80	25	23.809	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M1a X *arg*-5(27947)A	110	80	30	27.272	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M1a X *trp*-1(10575)A	112	85	27	24.107	Not linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M1a X *pyr*-1(H263)A	101	92	9	***8.910***	Linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M1a X *trp*-5(A420)A	104	81	23	22.115	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M1a X *ad*-1(3254)A	110	75	35	31.818	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M1a X *arg*-10(B317)A	105	71	34	32.380	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 3 and serial no. 4 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 3

Mutants : Wild (recombinants)
85 : 27
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4} \times 3$
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(85-84)^2}{84} + \frac{(27-28)^2}{28} = 0.011+0.035 = 0.046$$

Value of probability lies between 0.90-0.80 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M1a and *trp*-1 (10575)A. So, there is no linkage between them.

Serial No. 4

Mutants : Wild (recombinants)
92 : 9
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(92-75.75)^2}{75.75} + \frac{(9-25.25)^2}{25.25} = 3.485+10.457 = 13.942$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M1a and *pyr*-1 (H263)A.

Determination of genetic map of *leu* M1a auxotroph:

Crossing name: *leu* M1a x *pyr*-1(H263)A

pyr-1(H263)A	+
pyr-1(H263)A +	+ *leu* M1a
+	*leu* M1a

$$\textbf{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{9+11}{101} x\ 100 \text{ centimorgan}$$

= 19.801 centimorgan

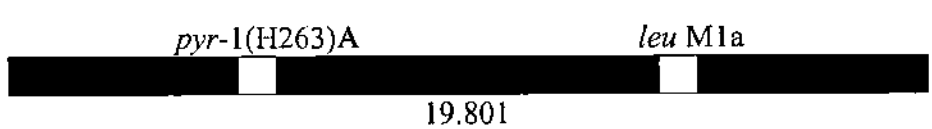


Fig. 5-17. Genetic map of *leu* M1a auxotroph.

5.7.2. Determination of genetic map of *leu* M3a auxotroph

Table 5-28. Linkage of *leu* M3a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M3a X *his*-2(T51M152(t))A	102	72	30	29.411	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M3a X *arg*-5(27947)A	110	73	37	33.636	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M3a X *trp*-1(10575)A	101	68	33	32.673	Not linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M3a X *pyr*-1(H263)A	105	94	11	*10.476*	Linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M3a X *trp*-5(A420)A	103	78	25	24.271	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M3a X *ad*-1(3254)A	110	75	35	31.818	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M3a X *arg*-10(B317)A	108	80	28	25.925	Not linked with *arg-10* of linkage group VII

Checking the segregation of serial no. 5 and serial no. 4 with chi-square:

$$\chi^2=\frac{(o_1-e_1)^2}{e_1}+\frac{(o_2-e_2)^2}{e_2}$$

Serial No. 5

Mutants : Wild (recombinants)
78 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(78-77.25)^2}{77.25} + \frac{(25-25.75)^2}{25.75} = 0.007+0.021 = 0.028$$

Value of probability lies between 0.90-0.80 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M3a and *trp*-5 (A420)A. So, there is no linkage between them.

Serial No. 4

Mutants : Wild (recombinants)
94 : 11
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(94-78.75)^2}{78.75} + \frac{(11-26.25)^2}{26.25} = 2.953+8.859 = 11.812$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M3a and *pyr*-1 (H263)A.

Determination of genetic map of *leu* M3a auxotroph:

Crossing name: *leu* M3a x *pyr*-1(H263)A

pyr-1(H263)A	+
pyr-1(H263)A +	+ *leu* M3a
+	*leu* M3a

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$
$$= \frac{9+11}{105} x\ 100 \text{ centimorgan}$$
$$= 19.047 \text{ centimorgan}$$

Fig. 5-18. Genetic map of *leu* M3a auxotroph.

5.7.3. Determination of genetic map of *leu* M4a auxotroph

Table 5-29. Linkage of *leu* M4a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M4a X *his*-2(T51M152(t))A	100	90	10	***10.0***	Linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M4a X *arg*-5(27947)A	110	84	26	23.636	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M4a X *trp*-1(10575)A	98	67	31	31.632	Not linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M4a X *pyr*-1(H263)A	95	66	29	30.526	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M4a X *trp*-5(A420)A	102	72	30	29.411	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M4a X *ad*-1(3254)A	106	70	36	33.962	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M4a X *arg*-10(B317)A	99	71	28	28.282	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 2 and serial no. 1 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 2

Mutants : Wild (recombinants)
84 : 26
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4} \times 3$
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(84-82.5)^2}{82.5} + \frac{(26-27.5)^2}{27.58} = 0.027+0.081 = 0.108$$

Value of probability lies between 0.80-0.70 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M4a and *arg*-5 (27947)A. So, there is no linkage between them.

Serial No. 1

Mutants : Wild (recombinants)
90 : 10
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(90-75)^2}{75} + \frac{(10-25)^2}{25} = 3.0+9.0 = 12.0$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M4a and *his*-2 (T51M152(t))A.

Determination of genetic map of *leu* M4a auxotroph:

Crossing name: *leu* M4a x *his*-2 (T51M152(t))A

leu M4a	+
leu M4a +	+ *his*-2 (T51M152(t))A
+	*his*-2 (T51M152(t))A

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{10+15}{100} x\ 100 \text{ centimorgan}$$

= 25.0 centimorgan

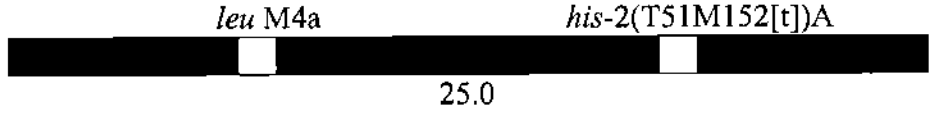


Fig. 5-19. Genetic map of *leu* M4a auxotroph.

5.7.4. Determination of genetic map of *leu* M8a auxotroph

Table 5-30. Linkage of *leu* M8a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M8a X *his*-2(T51M152(t))A	103	92	11	***10.679***	Linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M8a X *arg*-5(27947)A	102	78	24	23.529	Not linked with arg-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M8a X *trp*-1(10575)A	100	70	30	30.0	Not linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M8a X *pyr*-1(H263)A	105	71	34	32.380	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M8a X *trp*-5(A420)A	97	66	31	31.958	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M8a X *ad*-1(3254)A	100	72	28	28.0	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M8a X *arg*-10(B317)A	101	70	31	30.693	Not linked with *arg*-10oflinkage group VII

Checking the segregation of serial no. 2 and serial no. 1 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 2

Mutants : Wild (recombinants)
78 : 24
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(78-76.5)^2}{76.5} + \frac{(24-25.5)^2}{25.5} = 0.294+0.882 = 1.176$$

Value of probability lies between 0.30-0.20 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M8a and *arg*-5 (27947)A. So, there is no linkage between them.

Serial No. 1

Mutants : Wild (recombinants)
92 : 11
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(92-77.25)^2}{77.25} + \frac{(11-25.75)^2}{25.75} = 2.816+8.448 = 11.264$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3:1 which is hampered due to linkage between *leu* M8a and *his*-2 (T51M152(t))A.

Determination of genetic map of *leu* M8a auxotroph:

Crossing name: *leu* M8a x *his*-2 (T51M152(t))A

leu M8a	+
leu M8a +	+ *his*-2 (T51M152(t))A
+	*his*-2 (T51M152(t))A

$$\textbf{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{11+13}{103} x\ 100 \text{ centimorgan}$$

= 23.300 centimorgan

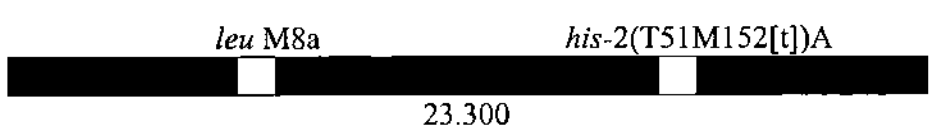


Fig. 5-20. Genetic map of *leu* M8a auxotroph.

5.7.5. Determination of genetic map of *leu* M9a auxotroph

Table 5-31. Linkage of *leu* M9a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M9a X *his*-2(T51M152(t))A	95	60	35	36.842	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M9a X *arg*-5(27947)A	105	80	25	23.809	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M9a X *trp*-1(10575)A	108	85	23	21.296	Not linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M9a X *pyr*-1(H263)A	104	96	8	***7.692***	Linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M9a X *trp*-5(A420)A	98	50	48	48.979	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M9a X *ad*-1(3254)A	99	59	40	40.404	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M9a X *arg*-10(B317)A	97	65	32	32.989	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 3 and serial no. 4 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 3

Mutants : Wild (recombinants)
85 : 23
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4} \times 3$
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(85-81)^2}{81} + \frac{(23-27)^2}{27} = 0.197+0.592 = 0.789$$

Value of probability lies between 0.50-0.30 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M9a and *trp*-1 (10575)A. So, there is no linkage between them.

Serial No. 4

Mutants : Wild (recombinants)
96 : 8
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(96-78)^2}{78} + \frac{(8-26)^2}{26} = 4.153+12.461 = 16.614$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M9a and *pyr*-1 (H263)A.

Determination of genetic map of *leu* M9a auxotroph:

Crossing name: *leu* M9a x *pyr*-1(H263)A

pyr-1(H263)A	+
pyr-1(H263)A +	+ *leu* M9a
+	*leu* M9a

$$\textbf{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$
$$= \frac{8+12}{104} x\ 100 \text{ centimorgan}$$
$$= 19.230 \text{ centimorgan}$$

Fig. 5-21. Genetic map of *leu* M9a auxotroph.

5.7.6. Determination of genetic map of *leu* M11a auxotroph

Table 5-32. Linkage of *leu* M11a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M11a X *his*-2(T51M152(t))A	101	70	31	30.693	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M11a X *arg*-5(27947)A	105	80	25	23.809	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M11a X *trp*-1(10575)A	102	92	10	*9.803*	Linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M11a X *pyr*-1(H263)A	100	70	30	30.0	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M11a X *trp*-5(A420)A	99	72	27	27.272	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M11a X *ad*-1(3254)A	98	70	28	28.571	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M11a X *arg*-10(B317)A	104	78	26	25.0	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 2 and serial no. 3 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 3

Mutants : Wild (recombinants)
80 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(80-78.75)^2}{80} + \frac{(25-26.25)^2}{26.25} = 0.019+0.059 = 0.078$$

Value of probability lies between 0.80-0.70 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M11a and *arg*-5 (27947)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
92 : 10
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(92-76.5)^2}{76.5} + \frac{(10-25.5)^2}{25.5} = 3.140+9.421 = 12.561$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M11a and *trp*-1 (105751)A.

Determination of genetic map of *leu* M11a auxotroph:

Crossing name: *leu* M11a x *trp*-1 (105751)A

leu M11a	+
leu M11a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\textbf{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{10+15}{102} x\ 100 \text{ centimorgan}$$

= 24.509 centimorgan

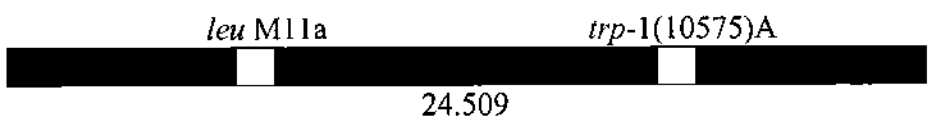


Fig. 5-22. Genetic map of *leu* M11a auxotroph.

5.7.7. Determination of genetic map of *leu* M23a auxotroph

Table 5-33. Linkage of *leu* M23a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M23a X *his*-2(T51M152(t))A	99	70	31	30.693	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M23a X *arg*-5(27947)A	104	80	25	23.809	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M23a X *trp*-1(10575)A	101	92	10	*9.803*	Linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M23a X *pyr*-1(H263)A	102	70	30	30.0	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M23a X *trp*-5(A420)A	99	72	27	27.272	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M23a X *ad*-1(3254)A	98	70	28	28.571	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M23a X *arg*-10(B317)A	104	78	26	25.0	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 2 and serial no. 3 with chi-square:

$$\chi^2=\frac{(o_1-e_1)^2}{e_1}+\frac{(o_2-e_2)^2}{e_2}$$

Serial No. 2

Mutants : Wild (recombinants)
79 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(79-78)^2}{78} + \frac{(25-26)^2}{26} = 0.012+0.038 = 0.050$$

Value of probability lies between 0.90-0.80 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M23a and *arg*-5 (27947)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
90 : 11
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(90-75.75)^2}{75.75} + \frac{(11-25.25)^2}{25.25} = 2.680+8.042 = 10.722$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M23a and *trp*-1 (10575)A.

Determination of genetic map of *leu* M23a auxotroph:

Crossing name: *leu* M23a x *trp*-1 (10575)A

leu M23a	+
leu M23a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{11+28}{101} x\ 100 \text{ centimorgan}$$

$$= 38.613 \text{ centimorgan}$$

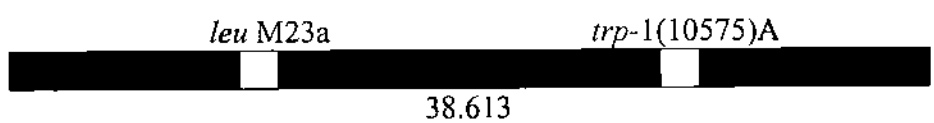


Fig. 5-23. Genetic map of *leu* M23a auxotroph.

5.7.8. Determination of genetic map of *leu* M25a auxotroph

Table 5-34. Linkage of *leu* M25a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M25a X *his*-2(T51M152(t))A	101	67	34	33.663	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M25a X *arg*-5(27947)A	105	75	30	28.571	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M25a X *trp*-1(10575)A	100	70	30	30.0	Not linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M25a X *pyr*-1(H263)A	106	94	12	***11.320***	Linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M25a X *trp*-5(A420)A	102	77	25	24.509	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M25a X *ad*-1(3254)A	101	72	29	28.712	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M25a X *arg*-10(B317)A	100	65	35	35.0	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 4 and serial no. 5 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 5

Mutants : Wild (recombinants)
77 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4} \times 3$
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(77-76.5)^2}{76.5} + \frac{(25-25.5)^2}{25.5} = 0.003+0.009 = 0.012$$

Value of probability lies between 0.95-0.90 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M25a and *trp*-5 (A420)A. So, there is no linkage between them.

Serial No. 4

Mutants : Wild (recombinants)
94 : 12
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(94-79.5)^2}{79.5} + \frac{(12-26.5)^2}{26.5} = 2.644+7.933 = 10.577$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M25a and *pyr*-1 (H263)A.

Determination of genetic map of *leu* M25a auxotroph:

Crossing name: *leu* M25a x *pyr*-1(H263)A

pyr-1(H263)A	+
pyr-1(H263)A +	+ *leu* M25a
+	*leu* M25a

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$
$$= \frac{12+18}{106} x\ 100 \text{ centimorgan}$$
$$= 28.301 \text{ centimorgan}$$

Fig. 5-24. Genetic map of *leu* M25a auxotroph.

5.7.9. Determination of genetic map of *leu* M30a auxotroph

Table 5-35. Linkage of *leu* M30a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M30a X *his*-2(T51M152(t))A	107	77	30	28.037	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M30a X *arg*-5(27947)A	99	70	29	29.292	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M30a X *trp*-1(10575)A	101	92	9	***8.910***	Linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M30a X *pyr*-1(H263)A	110	83	27	24.545	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M30a X *trp*-5(A420)A	100	70	30	30.0	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M30a X *ad*-1(3254)A	102	70	32	31.372	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M30a X *arg*-10(B317)A	100	69	31	31.0	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 3 and serial no. 4 with chi-square:

$$\chi^2=\frac{(o_1-e_1)^2}{e_1}+\frac{(o_2-e_2)^2}{e_2}$$

Serial No. 4

Mutants : Wild (recombinants)
83 : 27
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(83-82.5)^2}{82.5} + \frac{(27-27.5)^2}{27.5} = 0.003+0.009 = 0.012$$

Value of probability lies between 0.95-0.90 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M30a and *pyr*-1 (H263)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
92 : 9
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(92-75.75)^2}{75.75} + \frac{(9-25.25)^2}{25.25} = 3.485+10.457 = 13.942$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M30a and *trp*-1 (10575)A.

Determination of genetic map of *leu* M30a auxotroph:

Crossing name: *leu* M30a x *trp*-1 (10575)A

leu M30a	+
leu M30a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{9+12}{101} x\ 100 \text{ centimorgan}$$

= 20.792 centimorgan

Fig. 5-25. Genetic map of *leu* M30a auxotroph.

5.7.10. Determination of genetic map of *leu* M39a auxotroph

Table 5-36. Linkage of *leu* M39a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M39a X *his*-2(T51M152(t))A	101	67	34	33.66	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M39a X *arg*-5(27947)A	105	80	25	23.80	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M39a X *trp*-1(10575)A	105	95	10	*9.523*	Linked with *trp*-1 of linkage group **III**
4	*pyr*-1(H263)A	IV	*leu*M39a X *pyr*-1(H263)A	102	70	32	31.37	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M39a X *trp*-5(A420)A	101	72	29	28.71	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M39a X *ad*-1(3254)A	100	69	31	31.0	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M39a X *arg*-10(B317)A	98	66	32	32.65	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 2 and serial no. 3 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 2

Mutants : Wild (recombinants)
80 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(80-78.75)^2}{78.75} + \frac{(25-26.25)^2}{26.25} = 0.019+0.059 = 0.078$$

Value of probability lies between 0.80-0.70 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M39a and *arg*-5 (27947)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
95 : 10
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(95-78.75)^2}{78.75} + \frac{(10-26.25)^2}{26.25} = 3.353+10.059 = 13.412$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M39a and *trp*-1 (10575)A.

Determination of genetic map of *leu* M39a auxotroph:

Crossing name: *leu* M39a x *trp*-1(10575)A

leu M39a	+
leu M39a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\textbf{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{10+16}{105} x\ 100 \text{ centimorgan}$$

= 24.761 centimorgan

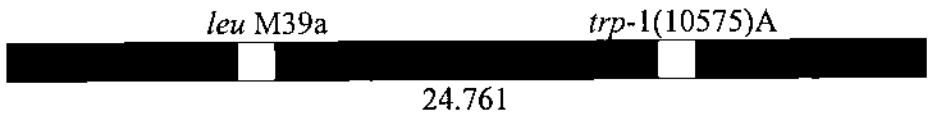


Fig. 5-26. Genetic map of *leu* M39a auxotroph.

5.7.11. Determination of genetic map of *leu* M47a auxotroph

Table 5-37. Linkage of *leu* M47a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M47a X *his*-2(T51M152(t))A	103	70	33	32.03	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M47a X *arg*-5(27947)A	102	57	45	44.11	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M47a X *trp*-1(10575)A	104	92	12	*11.53*	Linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M47a X *pyr*-1(H263)A	99	69	30	30.30	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M47a X *trp*-5(A420)A	103	68	35	33.98	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M47a X *ad*-1(3254)A	105	82	23	21.90	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M47a X *arg*-10(B317)A	100	70	30	30.0	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 3 and serial no. 6 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 6

Mutants : Wild (recombinants)
82 : 23
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4} \times 3$
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(82-78.75)^2}{78.75} + \frac{(23-26.25)^2}{26.25} = 0.0134+0.402 = 0.536$$

Value of probability lies between 0.50-0.30 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M47a and *ad*-1 (3254)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
95 : 12
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(92-78)^2}{78} + \frac{(12-26)^2}{26} = 2.512+7.538 = 10.050$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M47a and *trp*-1 (10575)A.

Determination of genetic map of *leu* M47a auxotroph:

Crossing name: *leu* M47a x *trp*-1(10575)A

leu M47a	+
leu M47a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\textbf{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{12+16}{104} x\ 100 \text{ centimorgan}$$

$$= 26.923 \text{ centimorgan}$$

Fig. 5-27. Genetic map of *leu* M47a auxotroph.

5.7.12. Determination of genetic map of *leu* M63a auxotroph

Table 5-38. Linkage of *leu* M63a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M63a X *his*-2(T51M152(t))A	101	67	34	33.663	Not linked with *his*-2 of linkage group **I**
2	*arg*-5(27947)A	II	*leu*M63a X *arg*-5(27947)A	105	75	30	28.571	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	**III**	*leu*M63a X *trp*-1(10575)A	108	100	8	*7.40*	Linked with *trp*-1 of linkage group **III**
4	*pyr*-1(H263)A	IV	*leu*M63a X *pyr*-1(H263)A	100	68	32	32.00	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M63a X *trp*-5(A420)A	102	69	33	32.35	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M63a X *ad*-1(3254)A	101	72	29	28.712	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M63a X *arg*-10(B317)A	98	74	24	24.48	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 3 and serial no. 7 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 7

Mutants : Wild (recombinants)
74 : 24
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4} \times 3$
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(74-73.5)^2}{73.5} + \frac{(24-24.5)^2}{24.5} = 0.340+1.020 = 1.360$$

Value of probability lies between 0.30-0.20 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M63a and *arg*-10 (B317)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
100 : 8
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(100-81)^2}{81} + \frac{(8-27)^2}{27} = 4.456+13.370 = 17.826$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M63a and *trp*-1 (10575)A.

Determination of genetic map of *leu* M63a auxotroph:

Crossing name: *leu* M63a x *trp*-1 (10575)A

leu M63a	+
leu M63a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{8+13}{108} x\ 100 \text{ centimorgan}$$

= 19.444 centimorgan

Fig. 5-28. Genetic map of *leu* M63a auxotroph.

5.7.13. Determination of genetic map of *leu* M146a auxotroph

Table 5-39. Linkage of *leu* M146a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M146a X *his*-2(T51M152(t))A	100	68	32	32.0	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M146a X *arg*-5(27947)A	101	68	33	32.67	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M146a X *trp*-1(10575)A	109	97	12	***11.00***	Linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M146a X *pyr*-1(H263)A	102	70	32	31.37	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M146a X *trp*-5(A420)A	102	68	34	33.33	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M146a X *ad*-1(3254)A	107	82	25	23.36	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M146a X *arg*-10(B317)A	101	67	34	33.66	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 3 and serial no. 6 with chi-square:

$$\chi^2 = \frac{(o_1 - e_1)^2}{e_1} + \frac{(o_2 - e_2)^2}{e_2}$$

Serial No. 6

Mutants : Wild (recombinants)
82 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4} \times 3$
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(82-80.25)^2}{80.25} + \frac{(25-26.75)^2}{26.75} = 0.116+0.350 = 0.466$$

Value of probability lies between 0.50-0.30 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M146a and *ad*-1 (3254)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
97 : 12
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(97-81.75)^2}{81.75} + \frac{(12-27.25)^2}{27.25} = 2.844+8.534 = 11.378$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M146a and *trp*-1 (10575)A.

Determination of genetic map of *leu* M146a auxotroph:

Crossing name: *leu* M146a x *trp*-1 (10575)A

leu M146a	+
leu M146a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{12+15}{109} x\ 100 \text{ centimorgan}$$

$$= 24.770 \text{ centimorgan}$$

Fig. 5-29. Genetic map of *leu* M146a auxotroph.

5.7.14. Determination of genetic map of *leu* M202a auxotroph

Table 5-40. Linkage of *leu* M202a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M202a X *his*-2(T51M152(t))A	102	60	42	41.17	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M202a X *arg*-5(27947)A	103	69	34	33.0	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M202a X *trp*-1(10575)A	102	89	13	*12.74*	Linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M202a X *pyr*-1(H263)A	102	77	25	24.50	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M202a X *trp*-5(A420)A	102	58	44	43.13	Not linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M202a X *ad*-1(3254)A	97	60	37	38.14	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M202a X *arg*-10(B317)A	99	65	34	34.34	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 3 and serial no. 4 with chi-square:

$$\chi^2=\frac{(o_1-e_1)^2}{e_1}+\frac{(o_2-e_2)^2}{e_2}$$

Serial No. 4

Mutants : Wild (recombinants)
77 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(77-76.5)^2}{76.5} + \frac{(25-25.5)^2}{25.5} = 0.003+0.009 = 0.0012$$

Value of probability lies between 0.98-0.95 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M202a and *pyr*-1 (H263)A. So, there is no linkage between them.

Serial No. 3

Mutants : Wild (recombinants)
89 : 13
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(89-76.5)^2}{76.5} + \frac{(13-25.5)^2}{25.5} = 2.042+6.127 = 8.169$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M202a and *trp*-1 (10575)A.

Determination of genetic map of *leu* M202a auxotroph:

Crossing name: *leu* M202a x *trp*-1 (10575)A

leu M202a	+
leu M202a +	+ *trp*-1 (10575)A
+	*trp*-1 (10575)A

$$\textbf{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{13+19}{102} x\ 100 \text{ centimorgan}$$

$$= 31.372 \text{ centimorgan}$$

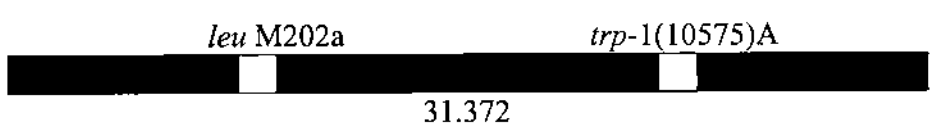


Fig. 5-30. Genetic map of *leu* M202a auxotroph.

5.7.15. Determination of genetic map of *leu* M262a auxotroph

Table 5-41. Linkage of *leu* M262a auxotroph.

Serial No.	Marker Used	Linkage group	Name of the cross	Total isolates	No. of mutant	No.of wild recom-binant	% of wild recom-binant	Inference of Linkage
1	*his*-2(T51M152(t))A	I	*leu*M262a X *his*-2(T51M152(t))A	101	69	32	31.68	Not linked with *his*-2 of linkage group I
2	*arg*-5(27947)A	II	*leu*M262a X *arg*-5(27947)A	104	70	34	32.69	Not linked with *arg*-5 of linkage group II
3	*trp*-1(10575)A	III	*leu*M262a X *trp*-1(10575)A	100	69	31	31.0	Not linked with *trp*-1 of linkage group III
4	*pyr*-1(H263)A	IV	*leu*M262a X *pyr*-1(H263)A	101	68	33	32.67	Not linked with *pyr*-1 of linkage group IV
5	*trp*-5(A420)A	V	*leu*M262a X *trp*-5(A420)A	106	94	12	*11.32*	Linked with *trp*-5 of linkage group V
6	*ad*-1(3254)A	VI	*leu*M262a X *ad*-1(3254)A	102	77	25	24.50	Not linked with *ad*-1 of linkage group VI
7	*arg*-10(B317)A	VII	*leu*M262a X *arg*-10(B317)A	96	65	31	32.29	Not linked with *arg*-10 of linkage group VII

Checking the segregation of serial no. 5 and serial no. 6 with chi-square:

$$\chi^2=\frac{(o_1-e_1)^2}{e_1}+\frac{(o_2-e_2)^2}{e_2}$$

Serial No. 6

Mutants : Wild (recombinants)
77 : 25
free segregation ratio = 3 : 1

Here,
O_1=Observed value (no of mutants)
O_2= Observed value (no of wild)
e_1= Expected value $\frac{\text{Total isolates}}{4}$ x 3
e_2= Expected value $\frac{\text{Total isolates}}{4}$

$$\chi^2 = \frac{(77-76.5)^2}{76.5} + \frac{(25-25.5)^2}{25.5} = 0.003+0.009 = 0.0012$$

Value of probability lies between 0.98-0.95 (in chi square table) indicating non-significance. Therefore, the data is acceptable for free segregation (3 : 1) of the mutant *leu* M262a and *ad*-1 (3254)A. So, there is no linkage between them.

Serial No. 5

Mutants : Wild (recombinants)
94 : 12
free segregation ratio = 3 : 1

$$\chi^2 = \frac{(94-79.5)^2}{79.5} + \frac{(12-26.5)^2}{26.5} = 2.644+7.933 = 10.577$$

Value of probability is much higher than tabular chi-square at 0.5 level and significant in the ratio 3 : 1 which is hampered due to linkage between *leu* M262a and *trp*-5 (A420)A.

Determination of genetic map of *leu* M262a auxotroph:

Crossing name: *leu* M262a x *trp*-5 (A420)A

trp-5 (A420)A	+
trp-5 (A420)A +	+ *leu* M262A
+	*leu* M262A

$$\text{Distance} = \frac{\text{Total number of recombinant}}{\text{Total progenies}} x\ 100 \text{ centimorgan}$$

$$= \frac{12+16}{106} x\ 100 \text{ centimorgan}$$

$$= 26.415 \text{ centimorgan}$$

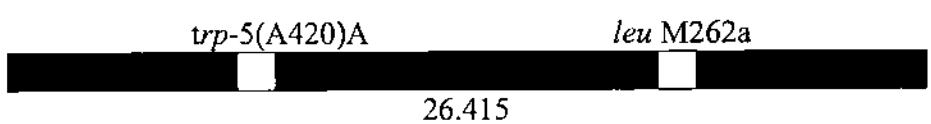


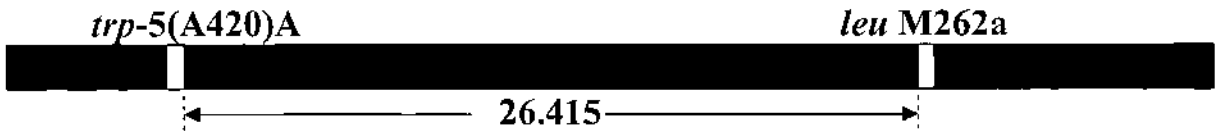
Fig. 5-31. Genetic map of *leu* M262a auxotroph.

5.7.16. Genetic map of *leucine* auxotrophs

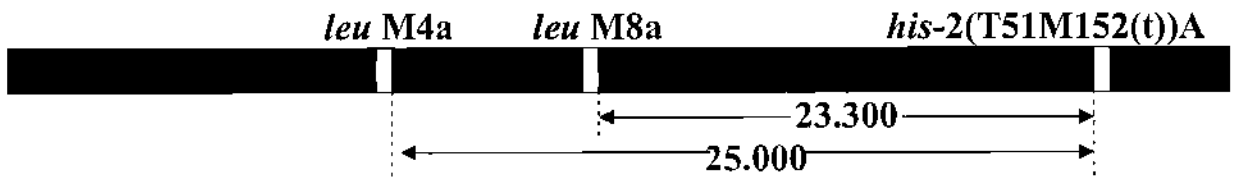
Distance of the mutants with the markers are tabulated in Table 5-42. The genetic map of newly induced 15 *leucine* auxotrophs are shown in Fig. 5-32. The gene order and the distances of the newly induced 15 *leucine* auxotrophs are given in Table 5-43. Finally, the distances of the newly induced 15 *leucine* auxotrophs with *leu*-1, *leu*-2, *leu*-3 & *leu*-5 standard markers are shown in Fig. 5-33.

Table 5-42. Distance of the mutants with the markers.

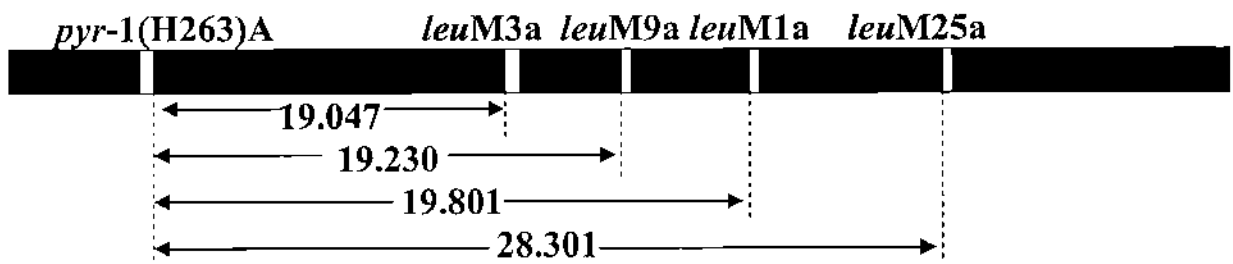
Mutant used	Marker Used	Linkage group of the marker	Distance of the mutant with marker
leu M1a	*pyr*-1(H263)A	IV	*pyr*-1(H263)A ← 19.801 → *leu* M1a
leu M3a	*pyr*-1(H263)A	IV	*pyr*-1(H263)A ← 19.047 → *leu* M3a
leu M4a	*his*-2(T51M152(t))A	I	*leu* M4a ← 25.000 → *his*-2(T51M152(t))A
leu M8a	*his*-2(T51M152(t))A	I	*leu* M8a ← 23.300 → *his*-2(T51M152(t))A
leu M9a	*pyr*-1(H263)A	IV	*pyr*-1(H263)A ← 19.230 → *leu* M9a
leu M11a	*trp*-1(10575)A	III	*leu* M11a ← 38.613 → *trp*-1(10575)A
leu M23a	*trp*-5(A420)A	III	*leu* M23a ← 24.509 → *trp*-1(10575)A
leu M25a	*pyr*-1(H263)A	IV	*pyr*-1(H263)A ← 28.301 → *leu* M25a
leu M30a	*trp*-1(10575)A	III	*leu* M30a ← 20.792 → *trp*-1(10575)A
leu M39a	*trp*-1(10575)A	III	*leu* M39a ← 24.761 → *trp*-1(10575)A
leu M47a	*trp*-1(10575)A	III	*leu* M47a ← 26.923 → *trp*-1(10575)A
leu M63a	*trp*-1(10575)A	III	*leu* M63a ← 19.444 → *trp*-1(10575)A
leu M146a	*trp*-1(10575)A	III	*leu* M146a ← 24.770 → *trp*-1(10575)A
leu M202a	*trp*-1(10575)A	III	*leu* M202a ← 31.372 → *trp*-1(10575)A
leu M262a	*trp*-5(A420)A	V	*trp*-5(A420)A ← 26.415 → *leu* M262A



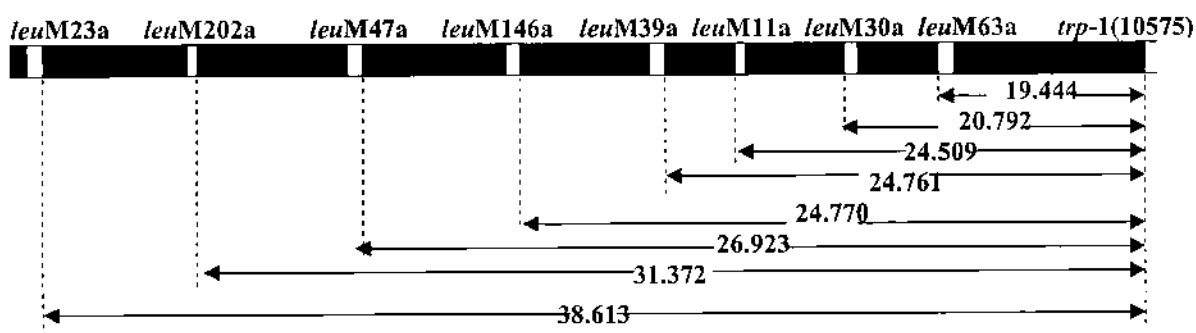
Genetic map of *leu* M262a with *trp*-5(A420)A of linkage group-V.



Genetic map of *leu* M4a & *leu* M8a with *his*-2(T51M152(t))A of linkage group-I.



Genetic map of *leu* M1a, *leu* M3a, *leu* M9a & *leu* M25a with *pyr*-1(H263)A of linkage group-IV.



Genetic map of *leu* M11a, *leu* M23a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* 146a & *leu* M202a with *trp*-1(10575)A of linkage group-III.

Fig. 5-32. Genetic map of newly induced 15 *leucine* auxotrophs.

Table 5-43. The gene order and distances of the newly induced 15 *leucine* auxotrophs.

Name of the cross with their linkage group	No. of total spores	No. of germinating spores	No. of growing spores	Wild	Marker recombinant	True wild recombinant	Pseudo wild recombinant	Gene order and distance of the two mutants
leu M4a x *leu*-3(R156)AI	5003	5000	3	2	1	2	O	*leu*-3(R156)A — *leu* M4a: 0.119 CM
leu M8a x *leu*-3(R156)AI	5112	5107	5	3	2	3	O	*leu*-3(R156)A — *leu* M8a: 0.195 CM
leu M11a x *leu*-1(33757)AIII	6107	6100	7	3	4	3	O	*leu*-1(33757)A — *leu* M11a: 0.229 CM
leu M23a x *leu*-1(33757)AIII	7122	7117	5	4	1	4	O	*leu*-1(33757)A — *leu* M23a: 0.140 CM
leu M30a x *leu*-1(33757)AIII	5227	5223	3	1	1	2	O	*leu*-1(33757)A — *leu* M30a: 0.114 CM
leu M39a x *leu*-1(33757)AIII	5915	5912	3	1	2	1	O	*leu*-1(33757)A — *leu* M39a: 0.101 CM
leu M47a x *leu*-1(33757)AIII	6213	6209	4	2	1	3	O	*leu*-1(33757)A — *leu* M47a: 0.128 CM
leu M63a x *leu*-1(33757)AIII	7107	7100	7	3	4	3	O	*leu*-1(33757)A — *leu* M63a: 0.196 CM

leu M146a x *leu*-1(33757)AIII	7529	7520	9	6	3	6	O	*leu*-1(33757)A *leu* M146a 0.239 CM
leu M202a x *leu*-1(33757)AIII	7845	7840	5	4	1	4	O	*leu*-1(3757)A *leu* M202a 0.127 CM
leu M1a x *leu*-2(37501)AIV	7001	6994	7	1	6	1	O	*leu*-2(37501)A *leu* M1a 0.199 CM
leu M3a x *leu*-2(37501)AIV	7110	7100	10	6	4	6	O	*leu*-2(37501)A *leu* M3a 0.281 CM
leu M9a x *leu*-2(37501)AIV	6915	6910	5	4	1	4	O	*leu*-2(37501)A *leu* M9a 0.144 CM
leu M25a x *leu*-2(37501)AIV	7225	7219	6	2	4	2	O	*leu*-2(37501)A *leu* M25a 0.166 CM
leu M262a x *leu*-5(45208(t))AV	6550	6547	3	2	1	2	O	*leu*-5(45208(t))A *leu* M262a 0.091 CM

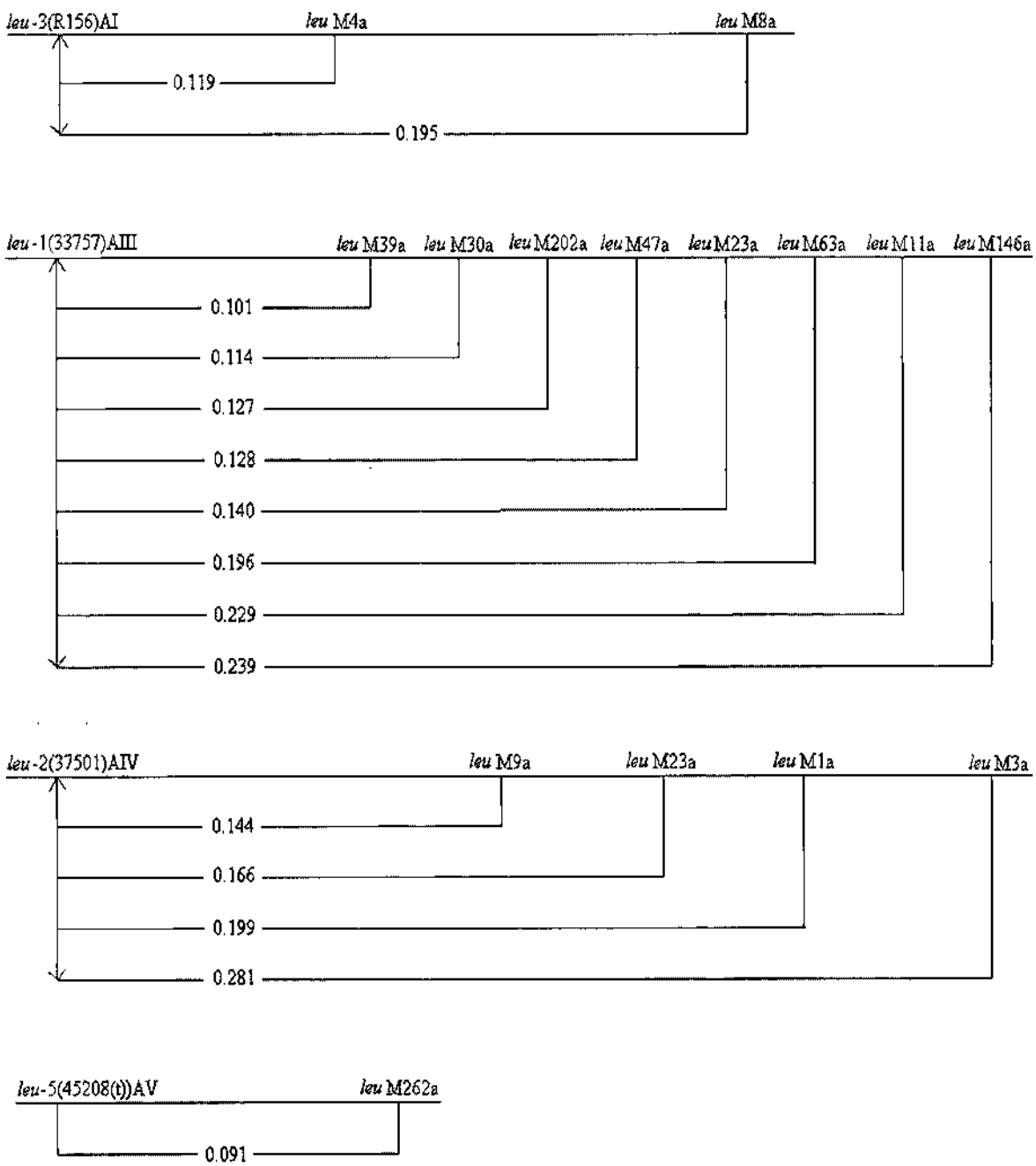


Fig. 5-33. Distances of the newly induced 15 *leucine* auxotrophs with *leu*-1, *leu*-2, *leu*-3 & *leu*-5 standard markers.

CHAPTER VI

DISCUSSION

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DISCUSSION

Hereditary genotypic variation arises from the variation in the genetic elements responsible for heredity, the genes, which may undergo changes known as mutation. The changed form of a gene may thereafter be transmitted and entered into a variety of combinations with other genes, producing genotypic variety. Spontaneous mutation is very infrequent in nature. However, Auerbach (1959) reported spontaneous mutation in dry conidia of *Neurospora crassa*. Ultra-violet ray, an important physical mutagenic agent with low penetrating power, was used quite safely in the laboratory. UV of 254 nm absorbs greatly by purines and pyrimidines which then became very reactive. The UV thus induce the formation of dimers which perturb the DNA double helix and interfere with accurate DNA replication and again make occasional errors during the process that cells possess for the repair of damaged DNA. Thereby the mutation is produced (Gardner 1981).

In the present investigation, fresh conidia (4-5 days old) of wild type *Neurospora crassa* Em`a'(5297) were induced with UV irradiation (254nm) for different periods such as 45 sec, 50 sec & 1 minute at a distance of 6 inches. UV-ray can alter nutritional requirements i.e. the capacities for synthesis of most of the B-vitamins and most of the essential amino acids (Beadle and Tatum 1945). A selective method of UV induction and isolation of the *leucine* auxotrophs resulted in 28 mutants from Em'a'(5297) by following technique of filtration enrichment method (Catcheside 1954 ; Ahmed and Catcheside 1960).

A total of five groups of nutritionally altered biochemical mutants called *tryptophan, leucine, lysine, histidine & adenine* were found in the investigation. Out of a total of 79 biochemical mutants, only 26 were *tryptopham*, 28 were *leucine*, 10 were *lysine*, 9 were *histidine* and 6 were *adenine* mutants. Classification and nomenclature of UV induced biochemical mutants are tabulated in Table 5-4. Then the 28 leucine

mutants were tested on SM media and SM supplemented with *leucine* for finding their growth requirements. Those cultures which did not grow on SM media, but grow on SM supplemented with leucine are termed as the *leucine* mutants (Table 5-9).

Complementation test were performed with 28 *leucine* mutants in all possible pair-wise combination in solid VM media, in liquid VM media and in hanging drop methods (Fig. 5-2). All the 28 mutants tested for complementation with the standard 5 *leucine* markers [*leu*-I(33757)IIIa, *leu*-2(37501)IVa, *leu*-3(R157)Ia, *leu*-4(R359)IVa & *leu*-5(45208(t)Va] are shown in Fig. 5-3. A new quick and decisive method "hanging drop method" of interallelic heterocaryosis of *leucine* mutants of *Neurospora crassa* was also performed. In this method the conidia of two mutants were kept in close contact within the medium at a controlled temparature. Six *leucine* mutants (*leu* M2a, *leu* M6a, *leu* M8a, *leu* M9a, *leu* M31a & *leu* M52a) belong to locus *leu*-1(33757)IIIa. One *leucine* mutant (*leu* M51a) belongs to locus *leu*-2(37501)IVa, three *leucine* mutants (*leu* M7a, *leu* M59a & *leu* M62a) belong to locus *leu*-3(R156)Ia, Three *leucine* mutants(*leu* M5a, *leu* M42a & *leu* M138a) belong to locus *leu*-4(R359)IVa. With the *leu*-5(45208(t)Va, all the *leucine* mutants showed positive result producing no mutant for *leu*-5. The rest 15 mutants showed positive complementation with all the available 5 *leucine* standard markers.

In *Neurospora crassa* hetarocaryon between two biochemical mutants of the same mating type is formed when the deffective region of the genes are not overlapped. The 28 *leucine* mutants were tested with each others in all pair-wise combinations for complementing ability (Fig. 5-2). From the complementation matrix (Fig. 5-7) complementation map is prepared (Fig 5-8). Roman numerals I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII & XIV represent the 14 groups of mutants. The map has 11 complons designated by A, B, C, D, E, F, G, H, I, J & K. The complementation map of 15 mutants (*leu* M1a, *leu* M3a, *leu* M4a, *leu* M8a, *leu* M9a, *leu* M11a, *leu* M23a, *leu* M25a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a, *leu* M202a & *leu* M262a) is linear and complex and only continuous defects were found within all mutants. This shows a slight variation with the finding of Ahmad *et al* 1981. In case of *lysine*-5 mutants, it was shown that the mutants fall into 17 groups. It

was also found that 10 mutants were defected in one complon, 2 mutants were defected in 2 complons and 3 mutants were defected in four complons and the complementation map became very complex. It is observed from this study that the organization of *leucine* mutants is not as complex as *lysine*-5. No non complementing mutants were found. All the mutants were complementing at least with other mutants. But the ability of formation of large number of mutants (non-complementing) is attributed to other loci e.g. *adenine*-4 Woodward *et al* (1958), *trp*-3 Ahmad *et al* (1960), *pan*-2 Case *et al* (1960), *arginine*-1, 6 & 10 Catcheside (1960), *methionine*-2 Murray (1960), *trp*-3 Lacy *et al* (1961) and *pyrimidine*-3 Woodward (1962).

The most interesting part of the current study was to examine the cellulytic activity of the gastro-intestinal extracts of common garden snail (*Helix sp*). Two samples were used to make one gastric extracts from stomach and the other was intestinal extracts from intestine. The mycelia of selected biochemical mutants those were grown on cellophone paper were treated separetly with both of gastric and intestinal extracts. The mutants were treated in different concentrations of sorbitol and snail extract to find out the concentration in which the maximum activity of enzyme observed. After 4-5 hours of treatments the cell wall of the mycelia were digested off in most plates and masses of spherical protoplasts were seen moving in the extracts. From the study, it is concluded that the enzymatic activity of gastric extract gave the best result than that of the intestinal extracts and the ratio of 1:4 is better than the other ratios (Table 5-7).

The fungal cell wall is composed mainly of cellulose and chitin. Besides, a number of chemicals, viz. glucan (a polymer of glucose), mannon (a polymer of mannose), chitosan, aminosugars, callose (a glucose polymer), heteropoly sacchaarides, protein, lignin, lipids and various inorganic materials are present (Alexopoulos 1962; Biligrami & Verma 1981). Different enzymes are responsible for the digestion of different chemicals in the cell wall. Giaja (1922); Holden and Tracey (1950) & Myers and Northcote (1958) reported thirty or more enzymes among which more than twenty are carbohydrases. Since the extracts digest the fungal cell wall as

observed during the present study, it may contain a number of carbohydrases, proteinases and amylases.

The next research work was the fusion of protoplast of different mutants of *Neurospora crassa*. After the successful enzymatic isolation of protoplasts and their fusion, a large number of studies on protoplasts are going on by different workers using various types of plants ranging from bacteria, fungi, bryophytes, gymnosperms to angiosperms. In this work an attempt was made to isolate protoplasts and then to fuse the isolated protoplast of different selected biochemical mutants (*leu* M9a , *leu* M30a & *leu* M51a) of *Neurospora crassa* among themselves. Two defective mutants overcame their defects mutually. The biochemical mutants were found lack of synthesis of *leucine*. So leucine must be supplemented to the medium for their growth. But when the protoplasts of the two biochemical mutants fused with 1% PEG solutions, hybrids were formed and then overcame their defects mutually and grew in the unsupplemented SM medium.

In this study three *leucine* mutants were taken for protoplast fusion experiments purpose. The protoplasts of *leu* M9a, *leu* M30a & *leu* M51a were taken in Petri dishes containing 1% PEG (Poly Ethylene Glycol) supplemented SM media. The complementation study between three *leucine* requiring mutants is described in Table 5-8. After 48 hours, observation of the formation of several colonies on SM medium indicated positive complementation. When these colonies were isolated in the test tubes containing VM medium, the culture showed wild like growth which indicates positive complementation. These mutants did not grow separately in the PEG unsupplemented SM medium due to the defects in leucine biosynthesis (Table 5-9), but growth occurred as a result of protoplast fusion of those mutants. The fusion of two protoplasts including the nuclei resulted the formation of heterocaryon. The resulting growth is due to the complementary effects of the two nuclear genotypes in a common cytoplasm. This result agrees with the finding of Mozmader & Hadiuzzaman (1998). They isolated protoplast from the auxotrophs A10a and A63a of *trp*-1 locus in *Neurospora crassa* with the help of snail gut enzyme and novozyme-234.

Further modifications of this procedure may improve the routine isolation of protoplasts in *Neurospora crassa*. This will then permit many genetic analysis and manipulation of this potential fungi using the technology of protoplast fusion. So it is concluded that the snail gut enzyme extracts can be used successfully in protoplast isolation and hybrid formation by fusion of the protoplasts of two mutants of heterogenic organisms. Heterocaryons are variable in their vigour. These variations in the vigour of heterocaryon suggests that besides the heterocaryon compatibility controlling loci C, D and W; other loci exist which may influence the growth of heterocaryon in *Neurospora crassa* (Howlett *et al* 1993, Perkins *et al* 1993 , De Serres *et al* 1995 & Mozmader 1997).

The fertility and mating type of those 28 leucine mutants were checked by crossing them with Em`A' and Em`a' (Tables 5-12 to 5-26). All the mutants were made fertile with Em`A' and their mating types are `a' and were not changed during mutation. Different mutants behave differently. Some were highly fertile, some were poor in their fertility. No sterile cross was found, however the crosses were highly fertile. Perithecia were formed within 7-10 days and spores were shed after 15-20 days. In case of poor fertility, perithecia were formed after 10-15 days and spores shed after 22-25 days (Table 5-10). Spores from the fertile crosses were spread, isolated and classified into mutant and wild types. Their segregation data are put in Table 5-11. From the table, it is observed that the wild types and mutant progenies are segregating in 1:1 ratio. This finding suggests that the *leucine* auxotrophs might be the reason of changes in the nuclear gene and not due to any gene in the cytoplasm or due to any suppression.

From 28 *leucine* auxotrophs, 15 *leucine* mutants were selected for linkage study. Linkage study of 15 *leucine* mutants were undertaken by crossing with standard markers of all the seven linkage groups. Crossing were fertile in Westergaard's crossing medium, but in some crosses spore shedding was not found. In Suyama's crossing medium some crosses showed perithecia and spore shedding. Poor fertility was the main problem of linkage study (Tables 5-27 to 5-41).

From the study (Table 6-42), it seems that eight *leucine* auxotrophs (*leu* M11a, *leu* M23a, *leu* M30a, *leu* M39a, *leu* M47a, *leu* M63a, *leu* M146a & *leu* M202a) were found to be linked with *trp*-1(10575)A of linkage group III. Four *leucine* auxotrophs (*leu* M1a, *leu* M3a, *leu* M9a & *leu* M25a) were found to be linked with *pyr*-1(H263)A of linkage group IV. Two *leucine* auxotrophs (*leu* M4a & *leu* M8a) were found to be linked with *his*-2(T51M152(t)A of linkage group I and only one mutant *leu* M262a was formed to be linked with *trp*-5(A420)A of linkage group V. Four linkage maps were constituted showing the distances of 15 *leucine* auxotrophs (Fig. 5-32). The mapping was performed in those cases where the complete analysis was possible following Ahmad's quick method (Ahmad 1962).

It is shown from the genetic map that the mutant *leu* M63a is the nearest (19.444 CM) and *leu* M23a is the furthest (38.613 CM) from *trp*-1(10575)A (Fig. 5-32). On the other hand, *leu* M3a is the nearest (19.047 CM) and *leu* M25a is the furthest (28.301 CM) from *pyr*-1(H263)A.

The distances between the newly induced 15 *leucine* mutants with four *leucine* standard markers [*leu*-1(33757)IIIA, *leu*-2(37501)IVA, *leu*-3(R156)IA & *leu*-5(45208(t)VA] were also shown. It is found from the genetic map that the mutant *leu* M39a is the nearest (0.101 CM) and *leu* M146a is the furthest (0.239 CM) from *leu*-1(33757)IIIA and *leu* M9a is the nearest (0.144CM) and *leu* M3a is the furthest (0.281 CM) from *leu*-2(37501)IVA (Fig. 5-33).

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