PHYSIOLOGICAL DIVERSITY AMONGST TRYPTOPHAN AUXOTROPHS IN

Neurospora crassa

A DISSERTATION SUBMITTED FOR THE DEGREE OF

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FACULTY OF BIOLOGICAL SCIENCES, UNIVERSITY OF DHAKA

BY

SALINA PARVIN BEAUTY

Department of Botany
University of Dhaka
Dhaka-1000, Bangladesh

February, 2014

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CERTIFICATE

This is to certify that this thesis contains the results of research on **Physiological diversity amongst tryptophan auxotrophs in *Neurospora crassa*** and has been carried out by Salina Parvin Beauty under my supervision. It is further certified that the work presented here is original and suitable for submission as a Ph.D. thesis.

Dr. Tahsina Rahim
Professor
Department of Botany
University of Dhaka

DECLARATION

I hereby declare that this thesis entitled **"Physiological diversity amongst tryptophan auxotrophs in *Neurospora crassa*"** has been composed by me and all the works presented herein are my own. I further declare that this work has not been submitted anywhere for any academic degree.

February, 2014 **Salina Parvin Beauty**

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LIST OF ABBREVIATION

%	=	Percentage
AA	=	anthranilic acid
ade-2	=	adenine-2 mutant
al	=	albino mutant
anth	=	anthranilic acid mutants
Anth Syn	=	Anthranilic synthetase
arg-10	=	argine-10 auxotroph
b	=	buffer
BSA	=	Bovin Serum Albumin
C	=	conidia formed
CA	=	Chrosmic acid
CDRP	=	1- (O-caxboxy phenylamino-1-deoxyribulose-5 phosphate)
CG	=	Conidia germinating
cm	=	Centimeter
CMCase	=	Carboxymethyl cellulose
con- band	=	conidial band mutant
Conc	=	Concentration
Em	=	Emerson stock
Em ‘a’	=	Emerson stock mating type ‘a’
Em ‘A’	=	Emerson stock mating type ‘A’
EMS	=	Ethylmethane sulphonate
FCG	=	Few conidia germinating
fl	=	fluffy mutant

FM	=	Few mycelia
FMG	=	Few mycelia growing
g	=	gram
G	=	Glutamine
his-7	=	histidine-7 mutant
hrs	=	hours
i.e.	=	idest (that is)
iv-1	=	isoleucine-valine-1 auxotroph
ind	=	indole mutant
InGP	=	Indole-3-glycerol phosphate
leu-1	=	leucine-1 mutant
IU	=	International unit
lys	=	lysine mutant
M	=	mole
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimole
NA	=	Nicotinic acid
NG	=	No growth
nm	=	Nanometre
OD	=	Optical density
PR	=	Phosphoribosyl
PRA	=	Phosphoribosyl anthranilate

PRPP	=	Phosphoribosyl pyrophosphate
r/m	=	relative mobility
ro	=	*ropy* mutant
SA	=	Shikimic acid
Sec	=	Second
SM	=	Sorbose minimal medium
TEMED	=	Tetra methyl ethylene diamine
trp	=	*tryptophan* auxotroph
trp-1	=	*tryptophan*-1 -auxotroph
trp-2	=	*tryptophan*-2 -auxotroph
trp-3	=	*tryptophan*-3 -auxotroph
trp-4	=	*tryptophan*-4 -auxotroph
trp-5	=	*tryptophan*-5 –auxotroph
UV	=	Ultra Violet
vg	=	*vigorous* mutant
VM	=	Vogel’s minimal medium
WCM	=	Westergaard’s crossing medium

BIO SYNTHESIS OF TRYPTOPHAN

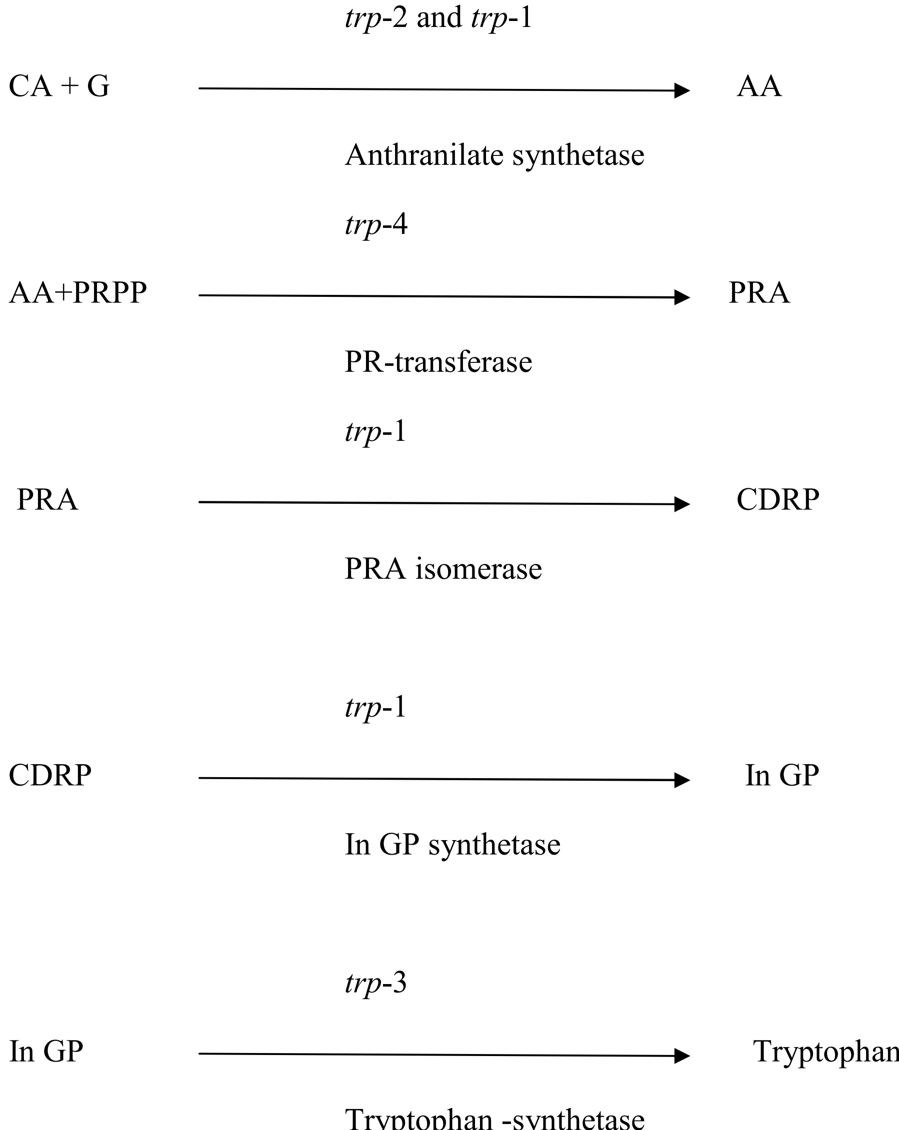


Fig 1. Intermediates, genes and enzymes in tryptophan biosynthesis of *Neurospora crassa*.

CA = Chorismic acid, G = Glutamine, AA = Anthranilic acid, PRPP = 5-phosphoribosy-1-pyrophosphate, PRA = N-(5-phosphoribosyl) anthranilic acid, CDRP = 1 (carboxyphenylamino-1-deoxyribulose-5-phosphate), InGP = Indole-3-glycerol Phosphate, PR = phosphoribosyl.

ABSTRACT

Conidia of Ema of *Neurospora crassa* were treated with 1.168% concentration of formaldehyde (HCHO) for 10 minutes and 3% concentration of ethyl-methane sulphonate (EMS) for 12 hours and produced 4 types (*albino*, *vigorous*, *fluffy* and *ropy*) and 5 types (*buff*, *conidial-band*, *albino*, *ropy* and *fluffy*) of morphological mutants respectively. Mutants showed variation in their conidial germination, linear mycelial growth and weight of dry mycelia. UV irradiation of conidia of Ema at a wave length of 254 nm for 90 seconds produced 6 types (*albino*, *buff*, *conidial-band*, *fluffy*, *ropy* and *vigorous*) of morphological and 3 types (*tryptophan*, *arginine* and *leucine*) of biochemical mutants.

Complementation test of all the seventeen *tryptophan* mutants were made with all the *tryptophan* standard markers *trp*-1a (10575), *trp*-2a (75001), *trp*-3a(C83), *trp*-4a (Y2198), and *trp*-5a (A420). It was found that four mutants belong to *trp*-1, seven mutants belong to *trp*-2, two mutants belong to *trp*-3, two mutants belong to *trp*-4 and two mutants belong to *trp*-5.

On the basis of their specific growth requirements physiological diversity was detected amongst the seventeen *tryptophan* auxotrophs. The mutants fall into three categories namely anthranilic acid requiring (9 mutants), indole requiring (6 mutants) and tryptophan requiring (2 mutants).

Inter allelic complementation studies of seventeen *trp* auxotrophs reveal that *trp*-1 auxotrophs comprises two heterocaryon groups (I and II) and two complons (A and B). *trp*-2 auxotrophs comprises three groups (I, II, III) and two complons (A and B). Auxotrophs of *trp*-3, *trp*-4 and *trp*-5 do not show any inter allelic complementation and no heterocaryon grouping amongst themselves.

Linkage studies of seventeen auxotrophs with marker representatives of all the seven linkage groups reveal that *trp*-B13a, *trp*-B21a, *trp*-B41a and *trp*-B51a are linked with *leu*-1 of linkage group-III; *trp*-B8a, *trp*-12a, *trp*-B14a, *trp*-B22a, *trp*-B28a, *trp*-B38a and

trp-B42a are linked with *trp*-2 of linkage group VI; *trp*-B17a and *trp*-B54a are linked with *arg*-2 of linkage group IV; *trp*-B5a and *trp*-B55a are linked with *leu*-5 of linkage group-5; *trp*-B11a and *trp*-B33a is linked with *leu*-3 of linkage group 1 and *arg*-5 of linkage group II respectively.

Fine structure map of *trp*-1 with *leu*-1(33757) as a marker comprises about 10.120 centimorgan and fine structure map of *trp*-2 with *lys*-5(DS6-85) comprises 3.235 centimorgan.

Cross feeding test of seventeen *trp* auxotrophs reveals three groups, Group-A, Group-B and Group-C. Nine anthranilic utilizing mutants belong to Group-A (B5a, B8a, B12a, B14a, B22a, B28a, B38a, B42a and B55a). Six indole utilizing mutants belong to Group-B (B11a, B13a, B17a, B41a, B51a and B54a). Two tryptophan utilizing mutants belong to Group-C (B21a and B33a). Group-A mutants grow in the filtrate of Group-B and Group-C. Group-B mutants grow poorly in the filtrate of Group-C only. Group-C mutants do not grow in the filtrate of either Group-A or Group-B mutants.

INTRODUCTION

Neurospora crassa is an important excellent and model genetic material. This filamentous fungus belongs to the Class-*Ascomycetes*, Sub-Class-*Pyrenomycitadae*, Order-*Xylariales*, Family-*Sordariaceae,* (Burnett 1975). It is genetically and biochemically the most thoroughly studied of any eucaryotic organism that are haploid and non pathogenic. For understanding gene action in chemical term Beadle and Tatum used *Neurospora crassa* for the first time in 1941 for genetic control of biochemical reaction.

Initiated by the investigation of Beadle and Tatum (1941) it is now known that DNA determine the structure of protein and it controls the biochemical activities of cell by determining the structure of specific enzyme and more over specific molecules of DNA determine the specific amino acid sequence of specific protein chain.

Neurospora crassa has proved to be an important genetic material for its profuse growth on synthetic media, haploid nature and linear arrangement of its ascospores. It can be used for any classical as well as molecular investigations and as a result the genetics of *Neurospora crassa* have been studied fairly intensively during the recent years (Yassin and Wheals 1992, Kuldau *et al.* 1993, Perkins and Kinsey 1993 Aranson *et al.* 1994, Maloisel *et al.* 1994, Atkinson *et al*. 1995, Kappor *et al.* 1995, McDonald *et al.* 1995, Mohiuddin 1996, Mozmader and Tahsina 1997, Mozmader *et al.* 1986, 1998, 1999 and 2000).

The fungus produces large number of identical, haploid and uninucleate conidia and as such it is used for the investigation of mutations. Since conidia can be exposed to a wide variety of mutagens and which are of particular value in mutation studies. (Stadler *et al.* 1993 and Watters *et al.* 1995, Rahman *et al.* 1998, Lutfar and Mozmader 1999, Mozmader *et al.* 2000, Haque *et al.* 2001).

Artificial induction of mutation is one of the criteria to study the organization and mode of action of genes. A good number of physical (X-ray, UV-ray) and chemical mutagens (EMS, HCHO, Phenol etc) are used by the geneticists for induction of mutation. (Thomb and Steinberg 1939, Auerbach and Robson 1946). Chemical mutagens have the ability to penetrate cells and to alter DNA (Fishbein *et al.* 1970).

Ultraviolet light has very weak penetrating power and as such it is very effective as a mutagenic agent in microorganism specially in *Nurospora crassa*. The maximum adsorption of UV by DNA is at a wave length of 254 nm. Maximum mutagenecity also occurs at this wave length.

Ethylmethane sulphonate is a well known chemical mutagen. Which induce high frequency of mutation in both microorganism and higher plants. Several workers used EMS as mutagen (Marki and Morea 1970, Gupta and Sharma 1990, Cid *et al.* 1994, Hossain 1995, Mustafa 1995, Keya 1998, Haque *et al.* 2001).

Complementation is a process in which two defective mutants mutually overcome their defects. In *Neurospora crassa* heterocaryon 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 mutants that produce the same or similar phenotype are in the same gene or in different genes.

Linkage was first demonstrated in *Neurospora crassa* by Lindegren (1993). He published linkage maps of linkage group I and II, In *Neurospora crassa* there are seven haploid chromosomes and seven linkage groups and each chromosome corresponds to a linkage group (Verma and Wann 1983). The study of linkage is basic to genetic analysis and help us to produce genetic map (Barratt *et al.* 1945).

Recombination is also an important basic genetic process. Recombination between linked genes occurs when homologous chromosomes come together and pair intimately at one stage of sexual cycle. The process involved in the exchange of information between the paired chromosomes. On the basis of frequency of recombinants the distances between any two linked genes are determined and thereby it helps to construct a genetic fine structure map which is turn reveals the organization of a specific gene.

Tryptophan, β indole, α amino propionic acid is one of the most important and costly aromatic amino acid which is characterized by the presence of the benzene ring in its structure.

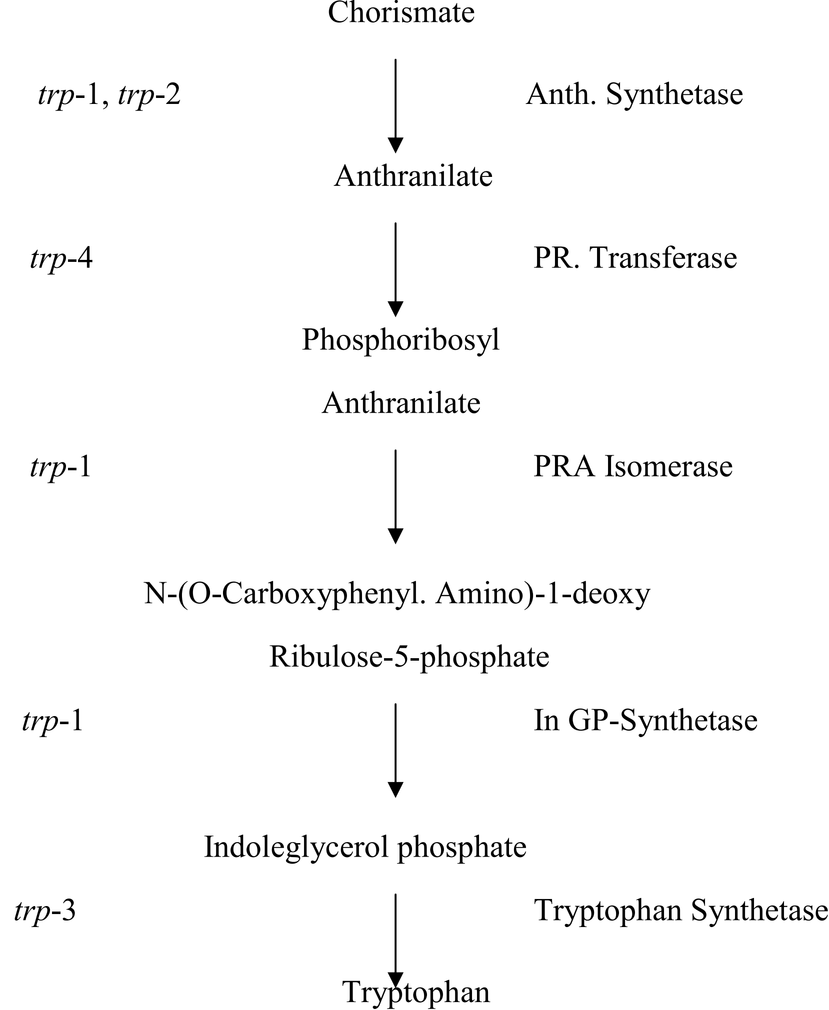
$CH_2 - C - CO_2H$

NH_2

N

Tryptophan is the second abundant of the common amino acids, generally constituting one percent less of the average protein. Some protein contains no tryptophan residues while others contain as much as 1.7 percent of tryptophan (Yanofsky *et al*. 1967, Nichols *et al*. 1981). Tryptophan biosynthesis in *Neurospora crassa* is controlled by 5 loci (Ahmad *et al*. 1968, Matchet 1974, Matchet and DeMoss 1975). Some mutants for *trp*-1 locus utilize anthranilic acid while others fail to do so but utilize indole for growth (Tatum and Bonner 1944, Ahmad and Catcheside 1960). Mutants for *trp*-2 locus were reported to utilize anthranilic acid, indole, tryptophan and kynuremine (Bonner 1946, DeMoss and Wegman 1965), *trp*-3 could use indole or tryptophan (Mitchell and Lein 1948, Ahmad and Catcheside 1960), *trp*-4 (Newmeyer 1951) utilizes anthranilic acid (Ahmad *et al*. 1968).

The biosynthesis of tryptophan from chorismic acid in micro organism believed to proceed as out lined below.



From the figure it shows that tryptophan is metabolized from chorismic acid in five steps in *Neurospora crassa*. *trp*-1 specifies components which shows anthranilate synthetase, PRA isomerase and In GP Synthetase activities. *trp*-2 specifies the component which catalyzes the anthranilic synthetase reaction in combination with *trp*-1 gene product. *trp*-3 catalyzes step 5 and *trp*-4 catalyzes step 2.

"No to leaves are equal" this important saying is true in nature because diversity exists almost everywhere in natural populations as well as in induced mutants. Diversity is of prime importance because it supplies the raw material of evolution. It also greatly helps us to improve our important plants of interest and as a result the plant diversity has been studied fairly intensively during the recent years (Pandey and Shukla 2005, Van Vuuren *et al*. 2006, Shukla 2009, Isbell *et al*. 2011, Isbell and Wilsey. 2011, Warren and Kramer *et al*. 2011 and Ratnadass *et al*. 2012).

In *Neurospora crassa* diversity was also found by (Ahmad and Catcheside 1960) in tryptophan mutants. The mutants were first classified physiologically into three nutritional groups (a) those (tryptophan mutants) which grew only when tryptophan was supplied, (b) those (indole mutants) which grew on indole as well as on tryptophan and (c) those (Anthranilic mutants) which grew on anthranilic acid as well as on indole and tryptophan. Next the nutritional group was further subdivided by heterocaryon test. Those test disclosed one group of tryptophan mutants, three groups of indole mutants and two groups of anthranilic mutants. Complementation between alleles occurred only in *trp*-1 and *trp*-3 mutants. The complementation map of *trp*-1 was simple but complementation map of *trp*-3 was complex. The mutants belonged to four genetic Loci: *trp*-1, *trp*-2, *trp*-3 and *trp*-4.

The *trp*-1 locus in *Neurospora crassa* was found to have two classes of mutants, one utilizing anthranilic acid and the other utilizing indole. The mutants fell into nine complementation groups (Ahmad *et al*. 1964).

Suyama *et al.* (1964) found that tryptophan mutants were of two types CRM^+ and CRM^-. The mutants fell into nine complementation groups.

Mutants for locus *trp*-3 were indole utilizing and indole non utilizing that is tryptophan utilizing. Complementation test revealed thirteen groups (Ahmad and Islam 1969).

Tryptophan mutants were all indole utilizing. The mutants fell into four complementation groups. Recombination studies on the mutants revealed that they belonged to the same locus *trp*-3 (Ahmad and Islam 1969)

Diversity was detected in all of the above mutants studied but there exists discrepancies.

From the above findings it is clear that the tryptophan mutants are very important and worth investigating. Therefore, a plan has been made to see the diversity amongst newly induced tryptophan mutants. The aim of the present investigation was based on the following heads:

I) Induction of morphological and biochemical mutants by U.V irradiation, EMS and formaldehyde in *Neurospora crassa* (Ema) will be performed.

II) Characterization of induced mutants by observing the growth pattern, conidiation, morphological differences and weight of dry mycelia and specificity of growth in different media will be done. (Haque *et al*. 2001).

III) Linkage study of their mutants will be made by crossing with standard markers of all the linkage groups and isolating and counting of the progenies. Triple-point interallelic crosses will be made by crossing a double mutant (a mutant with a marker) with different mutants for making a genetic fine structure map (Ahmad *et al.* 1964).

IV) Complementation study of the induced mutants will be undertaken by using heterocaryosis.

V) Cross-feeding test will be undertaken with different auxotrophs for elucidation of biochemical differences among themselves (Mozmader *et al*. 2000).

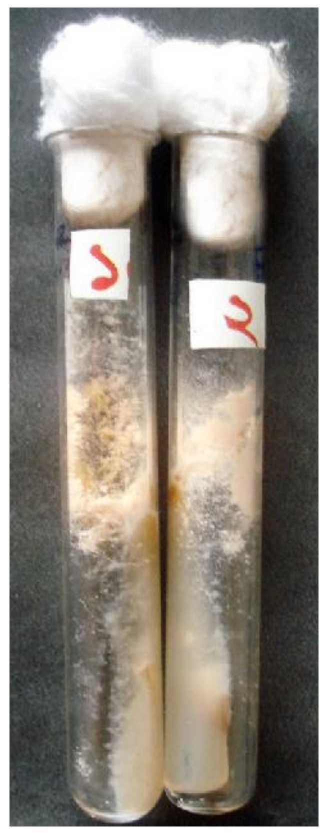


Fig.1. Ema & EmA

Fig.2. A Fertile Cross of Ema & EmA

MATERIALS

The experimental material was *Neurospora crassa* an ascomycetes fungus. It exists as soil saprophyte. It can grow on sugarcane bagasse, carbon vegetation or in bakeries. It is called “Red Bread Mold” (Sher and Dodge, 1927). This fungus possesses intertwined segmented filaments called hyphae. Each hyphal segment contains a haploid nucleus with seven chromosomes. It reproduces asexually by haploid spores called conidia and sexually by the contact of hyphae of two opposite mating types or strains known as Em ‘A’ and Em ‘a’.

Culture Emerson ‘a’ (Ema) (5297), Emerson ‘A’ (Ema) (5296) and some markers were used for this investigation. Ema was used for treating conidia with mutagen. EmA and Ema both are used for checking the mating types of the mutants and extracts. Markers were used for the study of linkage.

The wild types and markers were received from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical College Center U.S.A. The following strains of *Neurospora crassa* were kindly supplied by Prof. T.I.M.A.Mozmader from Genetic stock, Microbial Genetics Laboratory, Dept of Botany, University of Dhaka.

2.1 WILD TYPES

I. Emerson ‘A’ (5296)
II. Emerson ‘a’ (5297)

2.2 STANDARD MARKERS

The following markers tabulated in Table 1 were used for the linkage study and triple point interallelic crosses.

Table 1. List of Markers used for investigation

Markers With Number	Mating Types	Position of the gene	Linkage Group
1 *leu-3* (R 156)	A,a	IL	I
2 *his*-2 (T51 M152 t)	A,a	IR	I
3 *arg*-5 (27947)	A,a	IIR	II
4 *trp*-3 {C83(td1)}	A,a	IIR	II
5 *leu*-1 (33757)	A,a	IIIR	III
6 *trp*-1 (10575)	A,a	IIIR	III
7 *arg*-2 (33442)	A,a	IVR	IV
8 *trp*-4 (Y2198)	A,a	IVR	IV
9 *leu*-5 {45208(t)}	A,a	VR	V
10 *trp*-5 (A420)	A,a	VR	V
11 *trp*-2 (75001)	A,a	VIR	VI
12 *lys*-5 (DS6-85)	A,a	VIR	VI
13 *arg*-10 (B317)	A,a	VIIR	VII

2.3 MUTANTS

The Following seventeen *tryptophan* mutants given in Table-2 were induced with UV rays (254 nm wave length) using filtration enrichment method.

Table 2. List of *tryptophan* Mutants.

SL No	Number of Mutants	Name of auxotrophs
1	B 5	*tryptophan* auxotroph
2	B 8	*tryptophan* auxotroph
3	B 11	*tryptophan* auxotroph
4	B 12	*tryptophan* auxotroph
5	B 13	*tryptophan* auxotroph
6	B 14	*tryptophan* auxotroph
7	B 17	*tryptophan* auxotroph
8	B 21	*ttyptophan* auxotroph
9	B 22	*tryptophan* auxotroph
10	B 28	*tryptophan* auxotroph
11	B 33	*tryptophan* auxotroph
12	B 38	*tryptophan* auxotroph
13	B 41	*tryptophan* auxotroph
14	B 42	*ttyptophan* auxotroph
15	B 51	*tryptophan* auxotroph
16	B 54	*tryptophan* auxotroph
17	B 55	*tryptophan* auxotroph

2.4 APPARATUS AND GLASSWARES WHICH WERE USED FOR THE PRESENT RESEARCH WORKS ARE AS FOLLOWS.

APPARATUS

1. Air Pump.
2. Autoclave (Hirayame Manufacturing Corporation. Japan)
3. Centrifuge.
4. Electric balance.
5. Fluorescence Microscope.
6. Incubator.
7. Inoculation Chamber
8. Microscope
9. Oven
10. Refrigerator
11. Shaker Gy Rotary
12. Stand with Clamp
13. Thermometer
14. Ultra-Violet Lamp (254 nm Wave Length)
15. Water bath with circotherm.

GLASSWARES

Name	Made	Size	Purpose
1. Test Tubes	Pyrex	0.5″x 2″	Isolating Tubes
2. Test Tubes	Pyrex	0.5″x 3″	Sub culturing Tubes
3. Test Tubes	Pyrex	5/8″x 5″	Stock Tubes
4. Test Tubes	Pyrex	1″x 6″	Crossing Tubes
5. Test Tubes	Pyrex	1.5″x 9″	Radiation Tubes
6. Petridishes	Pyrex	1.5″dia	Irradiation
7. Petridishes	Pyrex	3″dia	Plating
8. Flasks (Erlenmyer)	Pyrex	100 ml	Media and Water etc.
9. Flasks (Erlenmyer)	Pyrex	250 ml	Media and Water etc.
10. Flasks (Erlenmyer)	Pyrex	500 ml	Media and Water etc.
11. Flasks (Erlenmyer)	Pyrex	1000 ml	Media and Water etc.
12. Pipette (Erlenmyer)	Pyrex		Dropping Solution, water

2.5 CHEMICALS USED FOR THE RESEARCH WORK

1. Agar (Bacto)

2. Ammonium nitrate [NH_4NO_3]

3. Biotin [$C_{10}H_{16}N_2O_3S$]

4. Boric Acid [H_3Bo_4] anhydride

5. Calcium Chloride [$CaCl_2$, $6H_2O$]

6. Chloroform [$CHCI_3$]

7. Citric Acid [$C(OH)COOH)(CH_2COOH)H_2O$]

8. Copper sulphate [$CuSo_4$, $7H_20$]

9. Ferrous sulphate [$FeSo_4$,$7H_20$]

10. Glucose ($C_6H_{12}O_6$)

11. Magnesium sulphate [$MgSo_4$, $7H_2O$]

12. Manganese sulphate [$MnSo_4$, $7H_20$]

13. Methanol [CH_30H]

14. Potassium Di-hydrogen phosphate [KH_2PO_4]

15. Potassium nitrate [KNO_3]

16. Sodium chloride [NaCI]

17. Sodium citrate [$NaH_2C_6H_5O_7$, $2H_2O$]

18. Sodium molybdate [Na_2MoO_4, $2H_2O$]

19. Sorbose [OCH_2 $(CHOH)_3$]

20. Sucrose [$C_{12}H_{22}O_{11}$]

21. Zinc sulphate [$ZnSO_4$, $7H_2O$]

2.6 SUPPLEMENTS USED FOR THE RESEARCH WORK

1. Adenine
2. Arginine
3. Histidine
4. Leucine
5. Lysine
6. Tryptophan
7. Anthranilic acid
8. Indole
9. Nicotinic acid

2.7 MUTAGEN

PHYSICAL MUTAGEN

a) UV irradiation of 254 nm

CHEMICAL MUTAGEN

b) Formaldehyde
c) EMS

2.8 STAINS

a) Cotton Blue
b) Acridine Orange

MEDIA

Wild type strains of *Neurospora crassa* can be grown on a suitable mixture of mineral salt in water plus a carbon source (sucrose, glucose) and the vitamin biotin (Beadle and Tatum 1945, Anonymous 1966).

To prepare different medium various stock solutions were needed. Conical flask was used for keeping the 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. All the stock solution was kept in refrigerator. All media were sterilized in the autoclave at 120^0C under 15 lb pressures for 20 minutes.

3.1 Composition of Trace elements solution per 100ml.

1.	Distilled water.	95 ml
2.	Citric Acid [$C(OH)(COOH)(CH_2COOH)\ H_2O$]	5 g
3.	Zinc sulphate [$ZnSO_4$, 7H2O]	5 g
4.	Ferrous sulphate [FeSo4, $7H_2O$]	1.0 g
5.	Copper sulphate [$CuS0_4$, $7H_20$]	0.25 g
6.	Manganese Sulphate [$MnS0_4$, $7H_20$]	0.65 g
7.	Boric Acid [H_3B0_4] anhydride	0.05 g
8.	Sodium molybdate [$NaMoO_4$, $2H_20$]	0.05 g
9.	Chloroform[$CHCI_3$]	1 ml

This solution needed for Vogel's stock solution.

3.2 Composition of Biotin solution per 100 ml

1.	Distilled water.	100 ml
2.	Biotin	10 g

This solution was needed for Vogel's stock solution and Westergaard's stock solution.

3.3 Vogel's stock solution

Vogel's stock solution was essential for the preparation of Vogel's minimal medium and Sorbose minimal medium.

Composition of Vogel's stock solution per litre

1.	Distilled Water	770 ml
2.	Sodium citrate [Na H_2 C_6 H_5 O_7, $2H_2O$]	63.5
3.	Potassium dihydrogen phosphate [KH_2PO_4]	125 g
4.	Ammonium nitrate [NH_4NO_3]	50 g
5.	Magnesium sulphate [$MgSo_4$, $7H_2O$]	5 g
6.	Calcium chloride [$CaCl_2$, $6H_2O$]	3.75 g
7.	Trace elements solution	2.5 g
8.	Biotin solution	1.25 g
9.	Chloroform	2 ml

1000 ml conical flask was used for keeping the stock solution. Calcium chloride was added first to the distilled water, and then other chemicals were added one after another.

3.4 Westergaard's stock solution

It was essential for the Preparation of Westergaard's crossing medium.

Composition of Westergaard's stock solution per litre.

No.	Component	Amount
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 [$MgSo_4$, $7H_2O$]	4.1 g
5.	Calcium chloride [$CaCl_2$, $6H_2O$]	0.8 g
6.	Sodium chloride[NaCl]	0.4 g
7.	Biotin solution	15 drops
8.	Chloroform	2 ml

3.5 Stock solution of amino acids

This solution was required for preparation of supplemented medium. 100 mg of amino acid dissolved in 100 ml of sterilized distilled water. The stock solution was named after the amino acid used, e.g. tryptophan solution, adenine solution, leucine solution etc. In case of culture and crossing media the amino acid supplements were added at the time of their preparation. The amino acid solution was also kept in 100 ml conical flask. For frequent use solution were kept in test tubes fitted with sterilizes pipettes. The solutions in the pipette were used drop wise.

3.6 Vogel's minimal medium

Vogel's minimal medium (VM) (Vogel 1956) was used for culturing *Neurospora crassa* in the test tubes and counting the spores on plate. For plating 10ml of medium was taken in large test tubes (6″), 2-4 ml medium was taken in small test tube (2.5″-3″) for maintaining cultures and growing spores. Amino acid supplements were used in culturing medium according to the specific type of mutants. The supplemented media were named after the name of the amino acid used e.g. leucine medium, adenine medium etc.

Composition of Vogel's minimal medium (VM) per litre for culturing

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

This medium is used for sub culturing and heterocaryon test but in case of plating 2% of agar was used and in case of linear growth 4% agar was used. 2% glucose was used in lieu of sucrose. 100 mg of required amino acid was used for supplemented VM per liter.

3.7 Sorbose minimal medium

Sorbose minimal medium was used for plating. It restricts growth so that colonies do not run into each other which could happen in VM. The rapid linear growth rate of *Neurospora crassa* was a serious drawback when individual colonies and single spore needed to be isolated. The problem was circumvented by the finding that when certain chemicals, notably the ketohexose sorbose had been added to the culture media a restricted colonial growth habit results (Tatum *et al.* 1949). The importance of the ratio between other carbon sources and sorbose had been investigated (Tatum *et al*. 1949 Brockman and De Serres, 1964, Pittenger 1964). For that reason supplemented sorbose minimal medium as well as non supplemented sorbose minimal medium were used for plating. This medium was also used for minimal test of wild type and mutant.

Composition of sorbose minimal (SM) medium per litre

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

Sorbose was added after boiling all other constituents to avoid caramelization.

3.8 Medium for Crossing

Westergaard's crossing medium was used for crossing. It restricts the vegetative growth but enhances sexual reproduction.

Composition of Westergaard's crossing medium per litre.

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

Necessary Supplements were used while mutants with wild or marker were crossed and mutants with mutants were crossed. The filter papers measuring 1.5″ x 2.5″were folded three times to make two furrows and introduced into the test tube (8″). Then 6-8 ml of the medium was poured in each crossing tubes. The medium remained in the furrow of the folded paper when the tubes were slanted.

3.9 Suyama's Crossing Medium

In case of sterile crosses, sucrose was used at the rate of 0.2 g per 100 ml media and the percentage of the amino acid supplement was increased to 50 mg per 100 ml (Suyama *et al*. 1958).

Composition of Suyama's crossing medium per litre.

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

The above two cases, amino acid was added when necessary. For example in case of tryptophan mutant, 20 mg of tryptophan per 100 ml medium was generally used.

METHODS

4.1 Stock Maintaining

Usually 6 inches tubes containing 4 ml of required medium were used for maintaining the stock of wild type, marker and mutant. After incubation period the inoculated tubes were preserved in the refrigerator at ± 40^0 C.

4.2 Sub culturing

Sub culturing was important to obtain fresh cultures and for preservation. Generally 2.5″-3″ tubes were used for this purpose. 2 ml sterilized Vogel's minimal medium or Vogel's supplemented medium (Where supplement was necessary) were used for sub culturing. Wild type (Ema and EmA) and morphological mutant was sub cultured in Vogel's minimal medium. Biological mutants and markers were sub cultured in supplemented VM. Particular amino acid supplement was used for particular biochemical mutants and markers.

Sub culturing was done in a sterilized inoculation chamber with at most care to avoid any kind of infection. The chamber, inoculation needle was 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 transfer the inoculum. Inoculum was transferred from old stock to new medium. The culture tube then incubated at 25^0C for 4-5 days for the growth and formation of conidia.

4.3 Induction of Mutation with formaldehyde

Obtaining of Fresh Culture

Ema was cultured in VM tubes 4 times after an interval of 4 days in each time. A culture of 5 days old was used for treating conidia.

Preparation of solution of formaldehyde

Formaldehyde solutions of different concentration were made by serial dilution technique. The concentration of supplied formaldehyde was 37.4 %. 6 bottles marking 1 2 3 4 5 6 were taken, 10 ml of sterilized distilled water was taken in each bottle. 10 ml of 37.4 % formaldehyde solution was added in the first bottle and it was shaken well. It's Concentration was 18.7%. 10 ml of Solution from first bottle was added to the second bottle and was shaken. Thus 10 ml of solution was transferred from second to third, third to fourth, fourth to fifth and from fifth to sixth bottle in the same procedure. The concentration of the solution of 6 bottles were 18.7% , 9.35%, 4.65%, 2.33%, 1.168%, and 0.58%. The last two concentrations were used for treating conidia.

Centrifugation

6 ml of 1.16% and 6 ml of 0.58% formaldehyde solution were taken into two Centrifuged tubes marking 1.16% and 0.58% simultaneously, 1 loop of conidia from Ema was taken into each centrifuge tube. The centrifuge tubes were placed in the centrifuge machine so that the tubes remain opposite to each other. Then they were centrifuged. Inoculation and centrifugation were completed within 5 minutes.

Filtration

The solution of the centrifuge tubes was poured out carefully so that conidia could remain in the bottom of the tubes. 6 ml of sterilized distilled water was added to each centrifuge tube and they were again centrifuged for 3 minutes and the upper solution was poured out. The process of addition of water, centrifugation and pouring out water was repeated 3 times.

Preparation of suspension

10 ml of sterilized distilled water was added to the treated and washed conidia of each centrifuge tube and the tubes were shaken well.

Plating of treating conidia and Incubation

I drop of the suspension was taken in each sterilized Petridish. The plates had been marked 1.16% and 0.58% previously and suspension was added accordingly. 10 ml of molten SM medium was added in each Petridish and the plates were shaken gently for mixing up the suspension and media. The temperature of the medium did not exceed 47°C. The whole experiment was done very carefully to avoid contamination. The plates were incubated in the incubator at 25°C.

Isolation and Incubation

When the growth was visible on the media, the distinctly separated conidial colonies were isolated. A part of the well-separated colony was cut with agar surface by isolating needle and was inoculated into VM culturing media. The cultures were incubated at a temperature of 25°C.

Observation and Classification

The Cultures were observed after 5 days and classified by comparing their characters with wild type Ema. Among the isolate some showed morphological abnormalities after 5-7 days those were classified by comparing their characters such as mycelia, conidia, colour etc with wild type Ema.

Test for biochemical mutants

All the isolates according to their classes were tested on SM and SM supplemented media individually. Those mutants which did not grow on SM but grow on special amino acid supplemented SM are the biochemical mutants.

4.4 INDUCTION OF MUTATION WITH EMS

One sterilized radiation tubes (8″x1″) fitted with pipette were taken. 0.3 ml of EMS with 0.50 M phosphate buffer (p^h8.0) was taken in a radiation tube and added 9.7 ml of sterilized distill water.

10 ml of suspension was made with 1 loop of conidia from 5 days old Ema and the solutions were taken in the radiation tube. The radiation tube was shaken gently for homogenous suspension. The radiation tubes were placed in the water bath at 25^0C. Then the radiation tubes with pipettes were fixed with the plastic tubing of air pump and the pump was started. Continuous air was passed through the suspension for gentle stirring.

The suspension was filtered after 12 hours. Then 10 ml of 0.004 M sodium thio-sulphate was added in the radiation tube for termination of the reaction of EMS. The suspension was plated with molten SM containing leucine, lysine, arginine, histidine & tryptophan.The plates were incubated at 25^0C for the growth of conidial colonies.

After 18 hours and onwards, the well separated conidial colony was isolated in VM containing leucine, lysine, arginine, histidine & tryptophan.The isolates were incubated at 25^0C.

The cultures were observed after 5 days if there were any morphological changes such as mycelia, colour, compared with Ema. The isolates were then classified according to their characters.

All the isolates according to their classes were tested on SM and SM supplemented media. Those mutants which did not grow on SM but grew on amino acid supplemented SM were the Biochemical mutants.

4.5 INDUCTION OF MUTATION BY UV IRRADIATION USING FILTRATION ENRICHMENT METHOD

Obtaining Fresh culture

To obtain fresh culture Ema was cultured 4 times after 4 days intervals in each case. 5 days old culture was used for treating conidia.

Preparation of conidial suspension and irradiation

10 ml of suspension was made with 5 days old conidia of Ema and sterilized distilled water. 2 ml of suspension was transferred to 3 small-sterilized Petridishes and irradiated with UV light having wavelengths of 254 nm for 2, $2^{1/2}$& 3 minutes. Radiation was given at a distance of 15 cm.

The suspension was continuously rotated during irradiation with an wooden handle to ensure an equal dose for all conidia. After completion of irradiation the suspension was stored at room temperature in the dark for 2 hours to prevent photo reactivation.

Repeated filtration

The irradiated conidial suspension was taken in 3 separate large radiation tubes (2.5″ x 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°C. The plastic tubing's 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 Pasteur Pipette inserted into each radiation tube. To secure continued suspension of the conidial suspension and 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 through a cotton filter. 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.

Plating of the filtered conidia

After the final filtration, the filtered conidial suspension was taken in Petridishes. 20 Petridishes were taken for each suspension and 1-2 drops of suspension were given in Petridishes, 10 ml 0f molten SM supplemented with adenine, arginine, leucine, lysine, histidine and tryptophan was used for plating.

Incubation

The Plates were incubated at 25°C. Observations were taken at an interval of 24 hours until some colonies were visible.

Isolation

Separated colonies were isolated into VM supplemented tubes with adenine, arginine, leucine, lysine, histidine and tryptophan medium and incubated at 25°C for 5-6 days.

Observation

Among isolates some showed morphological abnormalities. After 5-7 days those were classified by comparing their characters such as mycelia, conidia, color etc with wild type Ema.

Test for biochemical mutants

All the isolates according to their classes were tested on SM and SM supplemented media individually. Those mutants which did not grow on SM but grow on special amino acid supplemented SM are the biochemical mutants.

4.6 STUDY OF MYCELIAL GROWTH

It was done in liquid Vogel's minimal medium. Selected mutants and Ema was inoculated in the medium of the flask. Each flask contained 50 ml medium. To prevent the formation of conidia flask were continuously shaken.

Weight of the empty filter papers was taken. When the culture was 72 hours old, flasks with culture were boiled in water for 2 minutes and were filtered with filter paper of known weight. The filter papers with mycelia were dried in an oven and the weights of filter papers with culture were taken. Those were done with control.

4.7 STUDY OF REQUIRED TIME FOR CONIDIAL GERMINATION AND RADIAL GROWTH

It was done in VM medium. Fresh conidia of 4-6 days old cultures of selected morphological mutants and Ema were inoculated at the center of the VM plate. Germination of the conidia was observed under the microscope after half an hour interval.
After completion of germination the plates were incubated at 25°C for growth. Radial growth of the culture and Ema were measured from inoculated point in cm at 6 hours interval.

4.8 CROSSING

Neurospora crassa strains are heterothallic with 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, Westergaard and Mitchell 1947, Anonymous 1966) Westergaard's crossing medium was usually used for crossing. In some cases, when the crosses were sterile i.e. perithecia did not form, then Suyama's crossing medium was used (Suyama *et al*. 1958).

Crossing was made in a test tube (6″) containing 5-7 ml of Westergaard's crossing medium. One strain was put on one through of the filter paper pleat and at 25°C which is the optimum temperature for sexual reproduction.

Fertile mutants formed perithecia within 5-8 days and in rare cases after 10-12 days of incubation. It was observed that the more delay in the formation of perithecia, the less in the number of perithecia formed in a cross.

Further the greater the concentration of amino acid supplement the earlier were the formation of perithecia and also the higher number of perithecia. The perithecia generally liberated spores within 15-30 days.

The mutants were crossed with wild types Ema and EmA for checking the mating types and they were crossed with biological markers of seven linkage groups for determination of linkage group.

4.9 COMPLEMENTATION TEST

Complementation tests were done in order to classify the mutants based on their positive and negative complementation.

The tests were done among the mutants of same mating type. For these tests always fresh cultures of 4 to 6 days and fresh medium were used as suggested for better accuracy. Tests for complementation compatibility were made on Vogel's minimal both on solid and liquid medium.

(a) Solid medium

Test for complementation compatibility was performed on VM in small tubes. Complementation tests were carried out between two mutants of the same mating type. Conidia of one strain were put at two places on VM slant in small tubes. Next conidia from another strain were superimposed in the same position, where previously taken conidia were placed. The complementation tests and the controls were done in duplicate.

After this test the tubes were kept in incubator at 25^0C. Observations of control and complementation tests were taken after 2, 4 and 7 days.

(b) Liquid medium

Final checking for complementation test was done in liquid VM contained in small tubes. Observations in these cases were taken as in solid medium and final results were taken after 2, 4 and 7 days.

4.10 METHOD FOR THE DETERMINATION OF LINKAGE GROUP

The method of determination of linkage group has been described under the following title.

Sub culturing of induced mutants and markers

The mutants and markers were sub cultured four times after 4 days interval to improve their fertility.

Crossing and Observation

Mutants were crossed with seven markers and incubated at 25°C. The fertile crosses formed perithecia in 5-8 days and shedded spores within 15-30 days.

Spreading of spores

About 10 ml sterilized medium preserved in 20 ml tube was 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 angles was boiled in water and kept for spreading. One drop of sterilized water was taken on the medium of the Petridish. A loop needle were burnt red hot and then cooled in sterilized distilled water. A loop of spores were taken from the desired cross with the help of this needle and were transferred to water drop on the SM plate. The spreading needle was then cooled in sterilized distilled water plate and 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 Petridish. The prepared Petridish was given a heat shock at 58°C for 50 minutes and was then incubated at 25°C for 12-16 hours.

Counting of spores from SM Plate

After incubation period the spreading SM plates were examined under stereoscopic microscope and the germinating spores, growing spores, non growing spores were counted. Parallel lines were drawn with a glass marking pencil on the back of the lower lid of the Petridish. The spores were counted from one end of the space within the two parallel lines to the other and by slowly moving the plate in one direction. The next space was then focused on completion of the previous one and the spores were counted by moving the plate in the direction opposite of the preceding one.

Isolation of single spores

On completion of counting the spores (both growing and germinating) separately 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.

Plate test of the isolates of mutant

After 4 days isolates were analyzed. The ratio between the mutant isolates and wild like isolates was calculated. The isolates were tested on SM and supplemented SM plates.

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 Ema and EmA. If perithecia formed with Ema the mating type of the extracts would be 'A' and if perithecia formed with EmA it would be 'a'.

4.11 EXTRACTION OF DOUBLE MUTANTS BY SINGLE SPORE CULTURE METHOD

Mutant and opposite mating type linked marker were crossed in necessary supplemented crossing tubes. The ascospores from the desired cross were spread on necessary supplemented plate. The plate was given a heat shock of 58°C for 50 minutes and was then incubated at 25°C for 12-16 hours. A number of growing spores were isolated with the help of an isolating needle in the VM supplement tubes. Necessary precautions were taken for correct and complete isolation. The single spore isolates were incubated at 25°C

for 4-5 days to from good conidia. These were then tested to get the double mutant. Completely non-growing ones on VM as well as on single supplemented VM and growing in the double supplemented VM plates were the desired double mutants.

4.12 DETERMINATION OF MATING TYPE OF DOUBLE MUTANT

The double mutants thus obtained were next crossed with wild type both Ema and EmA in Westergaard's crossing medium containing desired supplements. If perithecia formed with Ema the mating type of extracts would be 'A' and if perithecia formed with EmA it would be 'a'.

4.13 TRIPLE POINT CROSS

The cross between two parents having three mutated characters is known as triple point cross.

To do a triple-point cross two parents were taken, one of which was a double mutant having a marker and a mutant and the other a different mutant of the same gene, the distance and order of which to be determined.

4.14 SPORE ANALYSIS OF TRIPLE POINT INTERALLELIC CROSS AND PREPARATIONOF FINE STRUCTURE MAP

The triple point interallelic crosses are very helpful for determining order of mutants as well as their distances and two construct the fine structure map accurately. Fine structure maps are constructed on the basis of recombination frequency data obtained. Fine structure maps are very important to represent the exact distance and order of different mutants on the linkage group.

4.15 DETERMINATION OF ORDER AND DISTANCE OF THE MUTANTS FROM TRIPLE-POINT CROSSES.

Spores were spread from triple-point cross on SM plates with necessary supplement. After giving the plates a heat shock at 58°C for 50 minutes, incubated in 25°C bath for 16 hours. The growing spores were then isolated with the help of an isolating needle in VM supplemented tube. The single isolates were tested on VM and VM supplemented plates after 4 or 5 days. The distance between the mutants and marker (Included in the Cross) was calculated out from the following formula:

$$\text{Distance} = \frac{(\text{TWR}+\text{MR}) \times 2 \times 100}{\text{T}} \quad \text{(Centimorgan)}$$

Here,

TWR = Number of true Wild recombinants.

MR = Number of marker recombinants.

T = Total number of spore.

When the distance and order of the mutant were obtained as above, they were represented in a linear arrangement and that was the linkage map.

4.16 TRUE WILD AND PSEUDO WILD DIFFERENTIATION

The isolates, which were found to be wild, were crossed with Ema and EmA in the crossing medium containing no supplement. The spores from this cross were spread on VM plate and growing and germinating spores were examined. If there was no germinating spore but only growing spores. It was concluded that the Wild isolate was true Wild. In case where germinating spores were found. 10 germinating spores were isolated in supplemented tubes and classified into wild and mutants. The Wild isolate was called pseudo Wild.

4.17 CR0SS - FEEDING TEST

Biological tests for the detection of inter mediate compounds in culture filtrate of the *Neurospora crassa.* A 100 ml flask containing 50 ml VM supplemented with 1% *tryptophan*. After sterilization it was inoculated with the 5 days old *tryptophan* mutant and incubated for 72 hours on a GY-Rotary shaker at 25°C. The mycelia were filtered off. One ml of the filtrate was poured into each of 1.5 x 12.5 cm tubes. One drop of fresh Vogel's stock solution was added to each tube and sterilized. After cooling the tubes were inoculated in duplicate with each mutant under investigation. Observations for growth were taken after 24, 48, 72 and 168 hour's incubation at 25°C.

RESULTS

5.1 INDUCTION OF MUTATION WITH FORMALDEHYDE (HCHO) SOLUTION

The concentration of supplied formaldehyde was 37.4%.
Different concentrations of formaldehyde solution were prepared with sterilized distilled water by serial dilution technique.

1.168% concentration was used for treating conidia.

4 groups of morphological mutants were found Table 3

Table 3. Classification of induced mutants with1.16% solution of HCHO

Name of the mutants	Number of mutants obtained
albino	12
vigorous	3
fluffy	2
ropy	5

5.2 Induction of mutation with EMS :

Concentration of EMS was prepared with sterilized distilled water Table 4.

Table 4. Concentration of EMS solution.

Amount of EMS	Amount of sterilize distilled water	Concentration of solution %
0.3 ml	9.7 ml	3

The suitable concentration of ethyl methane sulphonate was 3% which was used for treating conidia of Ema. 5 groups of morphological mutants were found. Mutants were shown in Table 5.

Table 5. Classification of the EMS induced mutants using 3% solution

Name of the mutants	Number of mutants obtained
buff	8
conidial- band	5
albino	9
ropy	3
fluffy	2

5.3 INDUCTION OF MUTATION WITH FILTRATION ENRICHMEN METHOD OF CATCHESIDE:

5 days old conidia of Ema were irradiated with UV light having wave length of 254 nm for 90 seconds. Radiation was given at a distance of 12 cm. Media supplemented with arginine, histidine, leucine, lysine and tryptophan were used for plating the irradiated conidia and isolating conidial cultures respectively. The isolates were incubated at 25^{0}C. After 5 days they were observed and classified according to their morphological and nutritional requirements.

Classification of mutants induced with UV

All the isolates were tested in the non-supplemented SM plates. Which grew well were prototrophs. The cultures that showed changes in its morphology comparing with Ema of the same age were taken as morphological mutants. The mutants were different from Ema in coloration of conidia, mode of conidiation and mycelial growth etc. Six (6) groups of morphological mutants (*albino, buff, conidial-band, fluffy, ropy, and vigorous*) were classified and has been shown in Table 6 and Fig.3.

The cultures that did not grow in the SM media were selected for further test for biochemical mutants. The result of plate test for biochemical mutants was given in Table 7.

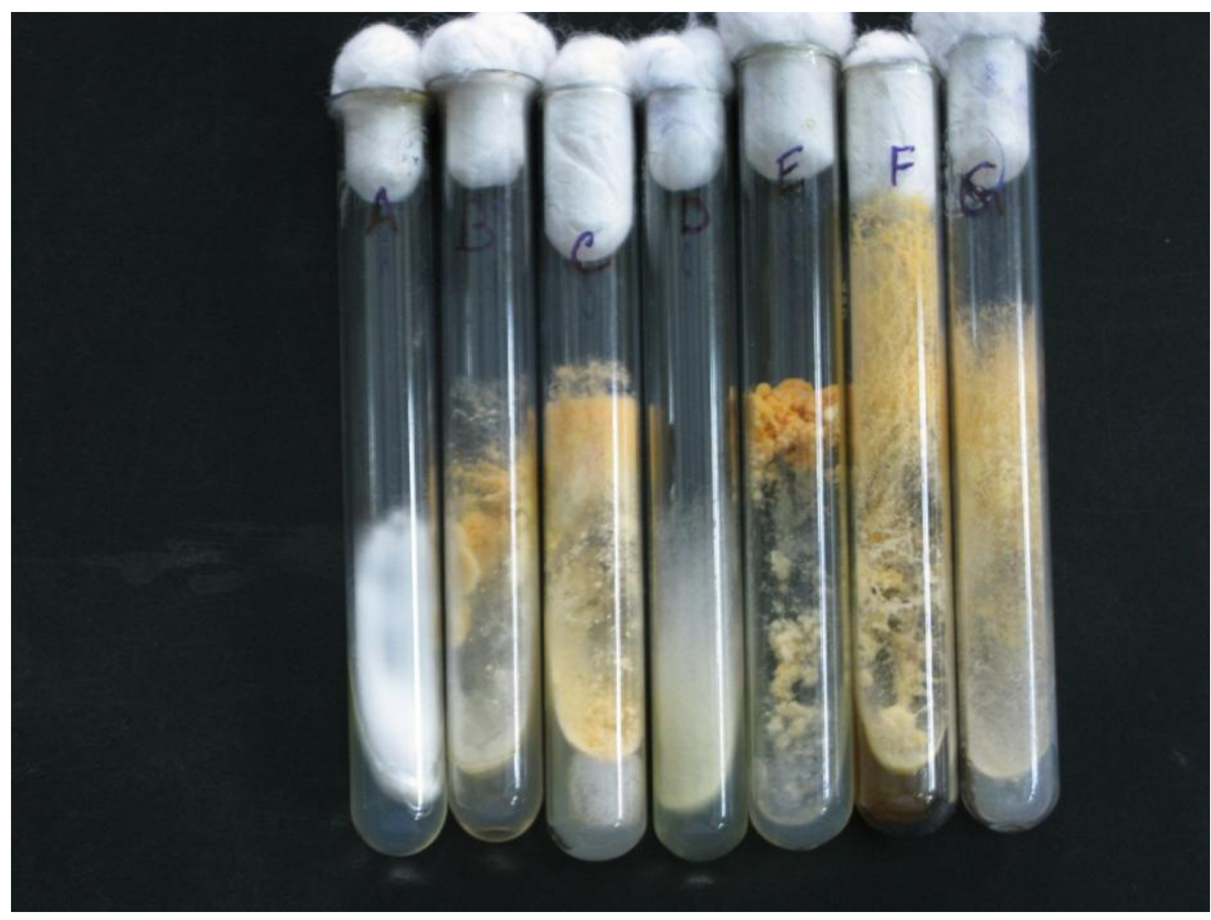


Fig. 3. Induced Morphological Mutants and Wild type (Ema)

A. ***albino***

B. ***buff***

C. ***conidial-band***

D. ***fluffy***

E. ropy

F. vigorous

***G.* Ema**

Table 6. Characteristics, classification and nomenclature of UV induced morphological mutants.

Name of the groups	Characteristics of the mutants	Number of the mutants	Name of the mutants
A	Mycelia and conidia are colourless	6	*albino*
B	Growth checked and buff coloured conidia	2	*buff*
C	Conidia form a band like structure at the top	3	*Conidial-band*
D	Profuse mycelial growth, few conidia formed	3	*fluffy*
E	Mycelia form rope like structure & conidia pink	6	*ropy*
F	Profuse conidial and mycelial growth reach the plug	5	*Vigorous*

Table 7. Growth of induced biochemical mutants on plates containing tryptophan,leucine, histidine, arginine, lysine.

Culture used	SM+trp	SM+leu	SM+his	SM+arg	SM+lys	Inference
B 5	●	○	○	○	○	*trp*
B 6	○	●	○	○	○	*leu*
B 8	●	○	○	○	○	*trp*
B 9	○	○	○	●	○	*arg*
B 11	●	○	○	○	○	*trp*
B 12	●	○	○	○	○	*trp*
B 13	●	○	○	○	○	*trp*
B 14	●	○	○	○	○	*trp*
B 15	○	●	○	○	○	*leu*
B 17	●	○	○	○	○	*trp*
B 20	○	●	○	○	○	*leu*
B 21	●	○	○	○	○	*trp*
B 22	●	○	○	○	○	*trp*
B 28	●	○	○	○	○	*trp*
B 33	●	○	○	○	○	*trp*
B 38	●	○	○	○	○	*trp*
B 41	●	○	○	○	○	*trp*
B 42	●	○	○	○	○	*trp*
B 51	●	○	○	○	○	*trp*
B 54	●	○	○	○	○	*trp*
B 55	●	○	○	○	○	*trp*

Note: ● → Indicates growth
○ → Indicates no growth

trp = tryptophan mutant
leu = leucine
his = histidine
arg = arginine
lys = lysine
SM = sorbose minimal media

Classification of bio-chemical mutants

1. *tryptophan* = Seventeen mutants (B5, B8, B11, B12, B13, B14, B17, B21, B22, B28, B33, B38, B41, B42, B51, B54, B55)
2. *leucine* = Three mutants (B5, B6, B20)
3. *arginine* = One mutant (B9)

Further test of seventeen *tryptophan* mutants on the following plates SM, SM + anthranilic acid, SM + indole and SM + tryptophan were done to find out the physiological diversity amongst *tryptophan* auxotrophs. Result was shown in Table 8.

Table 8. Growth of mutants on SM + anthranilic acid, SM + indole and SM + tryptophan plates.

Serial no	Mutants no	SM	SM+aa	SM+ind	SM+trp	Inference
1	B5	○	●	●	●	*anthranilic acid*
2	B8	○	●	●	●	*anthranilic acid*
3	B11	○	○	●	●	*indole*
4	B12	○	●	●	●	*anthranilic acid*
5	B13	○	○	●	●	*indole*
6	B14	○	●	●	●	*anthranilic acid*
7	B17	○	○	●	●	*indole*
8	B21	○	○	○	●	*tryptophan*
9	B22	○	●	●	●	*anthranilic acid*
10	B28	○	●	●	●	*anthranilic acid*
11	B33	○	○	○	●	*tryptophan*
12	B38	○	●	●	●	*anthranilic acid*
13	B41	○	○	●	●	*indole*
14	B42	○	●	●	●	*anthranilic acid*
15	B51	○	○	●	●	*indole*
16	B54	○	○	●	●	*indole*
17	B55	○	●	●	●	*anthranilic acid*

Notes:

○→no growth, ●→ growth, a→ anthranilic acid, ind→ indole, trp → tryptophan

Classification of *tryptophan* auxotrophs

anthranilic acid mutants = Nine (B5, B8, B12, B14, B22, B28, B38, B42, B55)

indole mutants = Six (B11, B13, B17, B41, B51, B54)

tryptophan mutants = Two (B21, B33)

5.4 Study of required time for germination of selected morphological mutants and Ema.

Fresh conidia of 4-6 days old cultures of selected morphological mutants and Ema were inoculated at the center of the VM plate. Germination of the conidia was observed under the microscope after three hours and then after one hour interval (Table 9)

Table 9. Germination of some selected morphological mutants on VM (Vogel's Minimal Media)

Name of the Cultures	Observation of germination after the following hours							
	3	4	5	6	7	8	9	10
vigorous	NG	NG	FCG	CG	MG	G	G	VG
ropy	NG	NG	NG	NG	FCG	CG	MG	MG
albino	NG	NG	NG	NG	NG	NG	NG	FCG
fluffy	NG	NG	NG	NG	NG	NG	FCG	CG
con-band	NG	NG	NG	FCG	FMG	MG	G	G
buff	NG	NG	NG	NG	FCG	FCG	FCG	CG
Ema	NG	NG	FCG	CG	FMG	MG	G	G

Notes:

NG = Not germinated

FCG = Few conidia germinated

CG = Conidia germinated

FMG = Few mycelial growth

MG = Mycelial growth

G = Growth

VG = Vigorous Growth

5.5 Study of radial growth of selected morphological mutants and Ema.

Fresh conidia of 5-6 days old cultures of selected morphological mutants and Ema were inoculated at the center of the VM plate. After completion of germination the plate were incubated at 25^{0}C for growth. Radial growth of the cultures and Ema were measured from inoculated point in cm at 3 hours interval. (Table10)

Table 10. Radial Growth of Ema and morphological mutants on VM.

Name of Cultures	Observation of radial growth in centimeter after the following hours.									
	12		15		18		21		24	
		Mean		Mean		Mean		Mean		Mean
vigorous	2.7	2.8	2.9	3	4.0	4.1	Full growth	Full growth	Full growth	Full growth
	2.9		3.1		4.2					
ropy	1.1	1.05	1.3	1.25	1.5	1.45	1.7	1.7	1.9	1.95
	1.0		1.2		1.4		1.7		2.0	
albino	CG	CG	MG	MG	0.1	0.15	0.3	0.35	0.4	**0.45**
	CG		MG		0.2		0.4		0.5	
fluffy	MG	MG	0.8	0.75	1.1	1.05	1.6	1.55	1.8	1.75
	MG		0.7		1.0		1.5		1.7	
con-band	1.1	1.2	1.7	1.7	2.9	2.85	3.4	3.4	4.0	4.0
	1.3		1.7		2.8		3.4		4.0	
buff	1.1	1.15	1.6	1.65	2.3	2.35	2.6	2.65	3.6	3.55
	1.2		1.7		2.4		2.7		3.5	
Ema	1.8	1.7	2.2	2.1	3.4	3.45	4.0	4.0	Full growth	Full growth
	1.6		2.0		3.5		4.0			

Notes

CG = Conidia germinates
MG = Mycelial growth

5.6 Study of linear mycelial growth of Ema and some selected morphological mutants in race tube.

Fresh conidia of 5-6 days old cultures of selected morphological mutants (*vigorous, ropy, albino, fluffy, con-band, buff)* and Ema were inoculated at one end of the race tube containing VM media solidified with 4% agar. Observations were recorded after 6 hours interval up to 96 hours. (Fig. 4. & Table 11)

Fig. 4. Linear mycelial growth of Ema and some selected morphological mutants (in race tube)

Table 11. Linear mycelial growth of Ema and some morphological mutans in race tube.

Name of the cultures	Observation of the growth in centimeter after the following hours															
	6	Mean	12	Mean	18	Mean	24	Mean	30	Mean	36	Mean	42	Mean	48	Mean
	NG		CG		0.1		0.4		0.6		0.7		0.8		0.9	
albino		NG		CG		0.15		0.4		0.5		0.6		0.7		0.8
	NG		CG		0.2		0.4		0.4		0.5		0.6		0.7	
	NG		MG		1.1		3.9		4.8		5.1		5.9		7.6	
fluffy		NG		MG		1.05		3.8		4.5		5.0		5.8		7.0
	NG		MG		1.0		3.7		4.2		4.9		5.7		6.4	
	NG		1.1		1.5		1.7		2.3		2.9		4.2		4.8	
ropy		NG		1.05		1.45		1.8		2.4		3.2		4.1		4.6
	NG		1.0		1.4		1.9		2.5		3.5		4.0		4.4	
	CG		2.9		4.0		5.1		6.5		9.8		12.6		13.9	
vigorous		CG		2.8		4.1		5.0		6.7		9.5		12.7		14.1
	CG		2.7		4.2		4.9		6.9		9.2		12.8		14.3	
	NG		1.1		2.3		2.9		4.4		7.3		10.0		11.5	
buff		NG		1.15		2.35		3.0		4.3		7.0		10.2		11.7
	NG		1.2		2.4		3.1		4.2		6.7		10.4		11.9	
	FCG		1.1		2.9		3.3		4.3		6.8		8.9		9.9	
C-band		FCG		1.2		2.85		3.5		4.6		6.5		9.0		10.0
	FCG		1.3		2.8		3.7		4.9		6.2		9.1		10.1	
	CG		1.8		3.4		4.4		4.9		5.9		8.2		11.0	
Ema		CG		1.7		3.45		4.3		5.0		6.05		8.25		11.25
	CG		1.6		3.5		4.2		5.1		6.2		8.3		11.5	

To be continued…

Name of the cultures	Observation of the growth in centimeter after the following hours															
	54	Mean	60	Mean	66	Mean	72	Mean	78	Mean	84	Mean	90	Mean	96	Mean
	1.5		3.9		4.6		4.6		6.5		7.9		7.9		8.3	
albino		1.3		4.0		4.4		4.8		6.3		7.6		8		**8.5**
	1.1		4.1		4.2		5.0		6.1		7.3		8.1		8.7	
	9.8		11.8		15.1		17.5		20.0		21.7		24.0		26.1	
fluffy		9.5		12.0		15.4		17.9		20.1		21.9		24.2		26.3
	9.2		12.2		15.7		18.3		20.2		22.1		24.4		26.5	
	5.8		5.9		6.8		7.3		7.7		8.5		8.8		9.4	
ropy		5.5		6.0		6.5		7.5		7.9		8.3		9.0		9.5
	5.2		6.1		6.2		7.7		8.1		8.1		9.2		9.6	
	15.9		18.7		20.7	20.8	22.9		24.7		27.3		29.0		32.1	
vigorous		16.2		18.5				23.0		24.9		27.5		29.3		**32.3**
	16.5		18.3		20.9		23.1		25.1		27.7		29.6		32.5	
	13.8		16.1		18.1		20.6		22.6		25.0		26.7		28.9	
buff		14.2		16.3		18.5		20.8		22.5		25.2		26.9		29.4
	14.6		16.5		18.9		21.0		22.4		25.4		27.1		29.9	
	11.3		13.0		14.0		16.0		18.0		19.7		20.1		21.9	
C-band		11.5		13.2		14.7		16.2		18.2		19.5		20.3		22.3
	11.7		13.4		14.4		16.4		18.4		19.3		20.5		22.7	
	13.5		15.7		7.6		19.7		22.8		24.3		26.5		29.2	
Ema		13.55		15.8		7.8		19.6		22.15		24.25		26.45		29.0
	13.6		15.9		8.0		19.6	5	22.5		24.2		26.4		28.8	

Notes:

M = Means

CG = conidia germinated

G = Growth

NG = No growth

MG = Mycelial growth

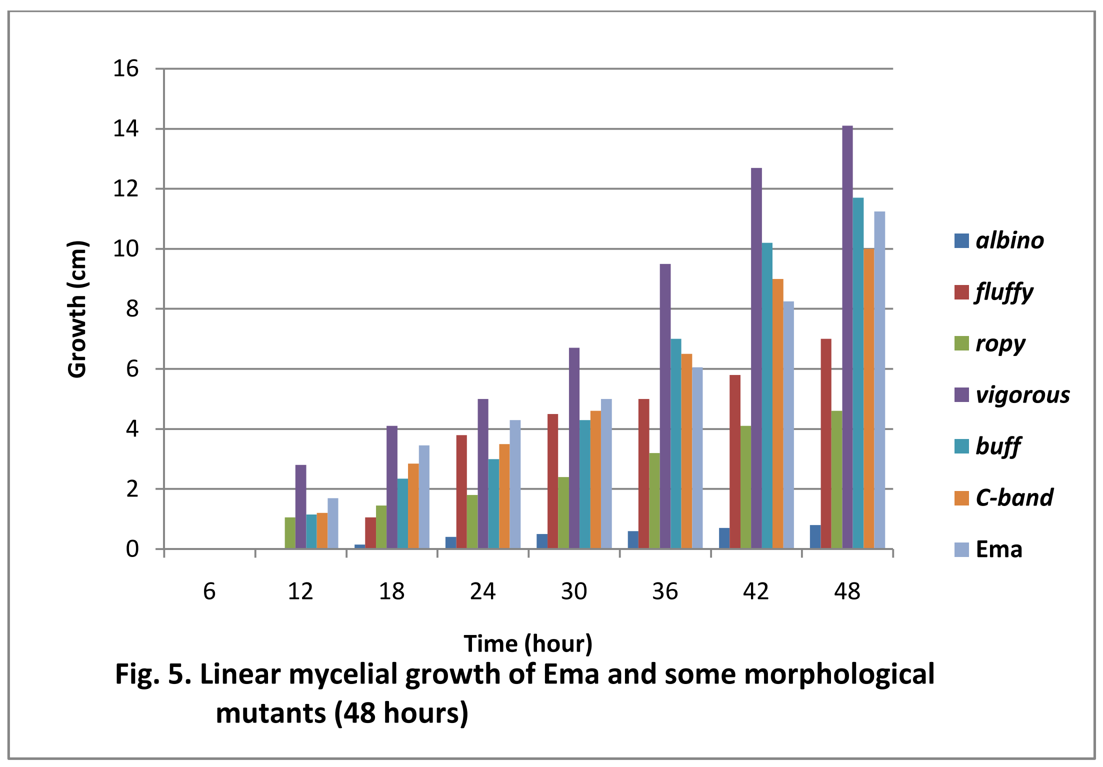


Fig. 5. Linear mycelial growth of Ema and some morphological mutants (48 hours)

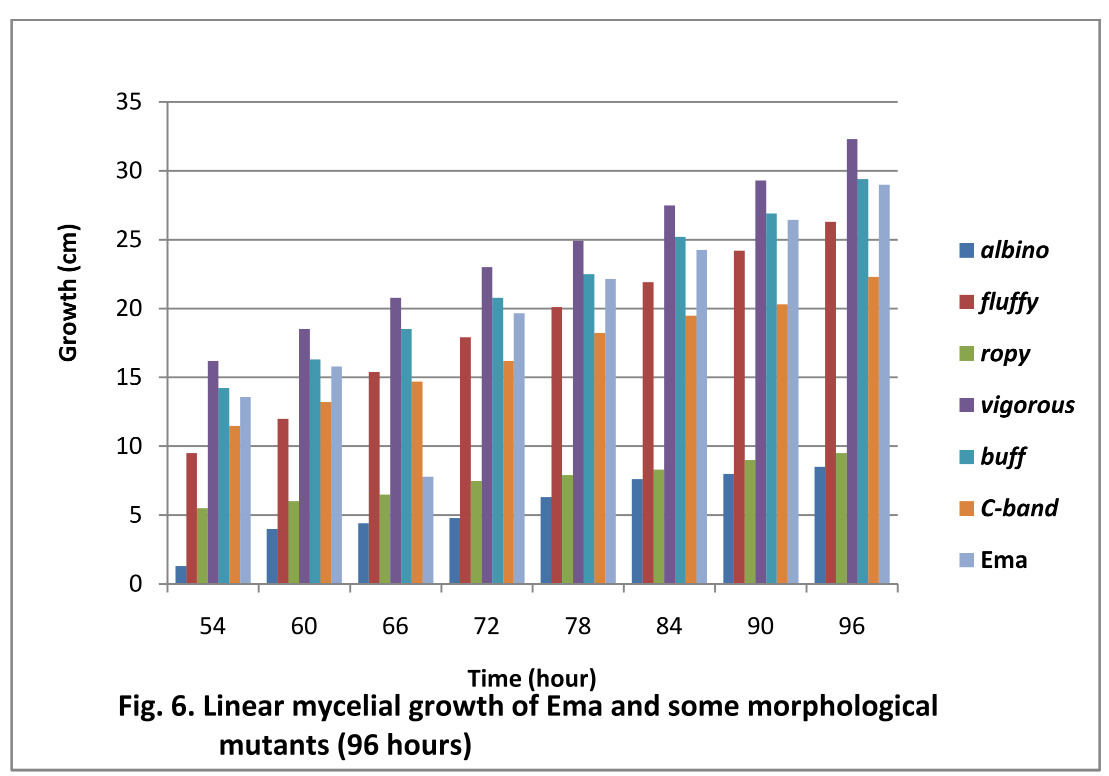


Fig. 6. Linear mycelial growth of Ema and some morphological mutants (96 hours)

5.7 Study of dry mycelial weight of some selected morphological mutants and Ema.

Fresh conidia of 5-6 days old cultures of selected morphological mutants (*vigorous, ropy, albino, fluffy, con-band, buff)* and Ema were inoculated in VM liquid media. The flask were shaken for 96 hours and boiled for 1 minutes. It was filtered and mycelia were dried at 60^{0}Cover night. Mycelial dry weights were measured (Table 12 & Fig.7.)

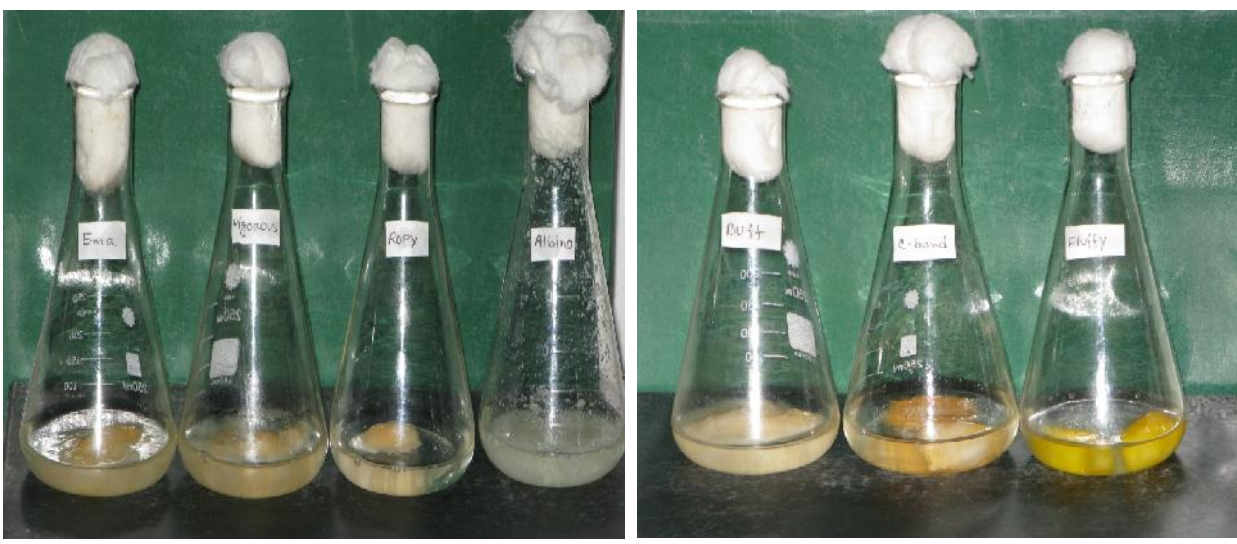


Fig. 7. Growth of mycelia in liquid VM media

Table 12. Dry mycelial weight of some selected morphological mutants and Ema after 72 hours grown in liquid VM

Name of Cultures	Observation of dry mycelial weight in g		
	Weight of the filter 0.80g paper with dried mycelia	Weight of the dried mycelia (g)	Mean (g.)
vigorous	1.16	0.36	**0.33**
	1.10	0.30	
ropy	0.98	0.18	0.17
	0.96	0.16	
albino	0.84	0.04	**0.12**
	1.00	0.20	
fluffy	1.02	0.22	0.23
	1.04	0.24	
con-band	1.04	0.24	0.275
	1.11	0.31	
buff	1.13	0.33	0.30
	1.07	0.27	
Ema	1.10	0.30	0.305
	1.11	0.31	

5.8 Study of mating type and fertility of the selected *tryptophan* mutants with Ema and EmA of *Neurospora crassa.*

All the seventeen *tryptophan* mutants were sub cultured fresh and crossed with Ema and EmA in Westergaard's crossing medium supplemented with tryptophan. The result was given Table 13

Table 13. Mating type and fertility of induced *tryptophan* mutants with Ema and EmA.

Designation of the cross	Perithecia formed yes/no	Days of initiation of perithecia	Frequency of perithecia	Size of Perithecia	Spore shedding days	Fertility	Mating type
B-5 x Ema	No	-	-	-	-	-	
B-5 x EmA	Yes	8	Many	Large	18	Good fertile	a
B-8 x Ema	No	-	-	-	-	-	-
B-8 x EmA	Yes	9	Many	Large	19	Good fertile	a
B-11 x Ema	Yes	12	Few	Medium	21	fertile	A
B-11 x EmA	No	-	-	-	-	-	
B-12 x Ema	No	-	-	-	-	-	-
B-12 x EmA	Yes	8	Many	Large	18	Good fertile	a
B-13 x Ema	Yes	10	Few	Medium	22	Good fertile	A
B-13 x EmA	No	-	-	-	-	-	
B-14 x Ema	No	-	-	-	-	-	-
B-14 x EmA	Yes	8	Many	Large	18	Good fertile	a
B-17 x Ema	Yes	9	Few	Medium	22	fertile	A
B-17 x EmA	No	-	-		-	-	
B-21 x Ema	No	-	-	-	-	-	-
B-21 x EmA	Yes	8	Many	Large	18	Good fertile	a
B-22 x Ema	Yes	8	Many	Large	18	Good fertile	A
B-22 x EmA	No	-	-	-	-	-	
B-28 x Ema	No	-	-	-	-	-	-
B-28 x EmA	Yes	8	Many	Large	18	Good fertile	a
B-33 x Ema	No	-	-	-	-	-	-
B-33 x EmA	Yes	9	Many	Medium	19	fertile	a
B-38 x Ema	No	-	-	-	-	-	-
B-38 x EmA	Yes	8	Many	Large	18	Good fertile	a
B-41 x Ema	Yes	10	Few	Medium	20	fertile	A
B-41 x EmA	No	-	-		-	-	
B-42 x Ema	Yes	8	Many	Large	19	Good fertile	A
B-42 x EmA	No	-	-	-	-	-	
B-51 x Ema	No	-	-	-	-	-	-
B-51 x EmA	Yes	8	Many	Medium	20	fertile	a
B-54 x Ema	No	-	-	-	-	-	-
B-54 x EmA	Yes	10	Few	Medium	22	fertile	a
B-55 x Ema	No	-	-	-	-	-	-
B-55 x EmA	Yes	8	Many	Large	20	Good fertile	a

6.1 Preparation of 'a' Extracts from 'A' mutants

The mutants B11A, B13A, B17A, B22A, B41A and B42A were crossed with Ema. Spores from the crosses were spread on SM plate and isolated germinating spores in VM, supplemented with tryptophan. In each case 20 spores were isolated. After good growth they were tested on SM and SM+ tryptophan plates to classify them into wild and *tryptophan*. The *tryptophan* extracts were again crossed with EmA and Ema separately to obtain 'a' extracts. The following 'a' extracts were obtained and selected.

B11 – Ext 7a, B13 – Ext 6a, B17 – Ext 7a, B22 – Ext 1a, B41 – Ext 7a, B42 –Ext 1a

6.2 Complementation test of all the induced *tryptophan* auxotrophs with all the five standard markers

Complementation tests were first made in tubes (0.2″ x 2″) on freshly prepared VM medium solidified with agar. Conidia from 4-6 day old cultures were used in these tests. To prevent infection, inoculation was done inside an inoculation chamber. These tests were done in duplicate for a check. Controls for the mutants were maintained in VM media in duplicate. The tubes after inoculation were incubated at 25º C for 168 hours. The pairs of mutants which gave doubtful responses were inoculated for heterocaryosis both in solid as well as in liquid VM media. All heterocaryon tests and controls were done in triplicate. Observation were made after 2,4, and 7 days and given in Fig.8.

The seventeen *tryptophan* mutants were tested for heterocaryon formation in both solid and liquid VM medium. Standard tryptophan markers, *trp*-1 (10575)a, *trp*-2 (75001)a, *trp*-3 (C83)a, *trp*-4 (Y2198)a, *trp*-5 (A420)a were used for this test. The tests were observed on 2nd, 4th and 7th day.

Fig.8. Complementation matrix of induced *tryptophan* mutants with standard markers

	Induced *tryptophan* auxotrophs																
Standard markers	B5 ○○	B8 ○○	B11 ○○	B12 ○○	B13 ○○	B14 ○○	B17 ○○	B21 ○○	B22 ○○	B28 ○○	B33 ○○	B38 ○○	B41 ○○	B42 ○○	B51 ○○	B54 ○○	B55 ○○
trp1 ○○	●	●	○	●	●	●	○	●	●	●	●	●	○	●	○	●	●
trp2 ○○	●	○	●	○	●	○	●	●	○	○	●	○	●	○	●	●	●
trp3 ○○	●	●	●	●	●	●	●	○	●	●	○	●	●	●	●	●	●
trp4 ○○	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	○	●
trp5 ○○	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	○

Table 14. Classification of induced *tryptophan* mutants on the basis of complementation with standard markers.

Standard Markers	No. of mutants	Name of the mutants
trp-1	4	B11, B17, B41, B51
trp-2	7	B8, B12, B14, B22, B28, B38, B42
trp-3	2	B21, B33
trp-4	2	B13, B54
trp-5	2	B5, B55

6.3 Interallelic complementation test of all the induced *trp*-1, *trp*-2, *trp*-3, *trp*-4 and *trp*-5 auxotrophs.

Inter allelic complementation tests were first made in tubes (0.2″ x 2″) on freshly prepared VM medium solidified with agar. Conidia from 4-6 day old cultures were used in these tests. To prevent infection, inoculation was done inside an inoculation chamber. These tests were done in duplicate for a check. Controls for the mutants were maintained in VM media in duplicate. The tubes after inoculation were incubated at 25º C for 168 hours. The pairs of mutants which gave doubtful responses were inoculated for heterocaryosis both in solid as well as in liquid VM media. Observations were made after 2, 4, and 7 days. From the test complementation matrix and map were drawn and shown in Fig.9, Fig.10, Fig.11, Fig.12, Fig.13, Fig.14 and Fig.15.

The four *trp*-1 *auxotrophs were* tested in all possible Combination for heterocaryon formation in both solid and liquid VM medium. Result has given in Fig. 9 and Fig. 10

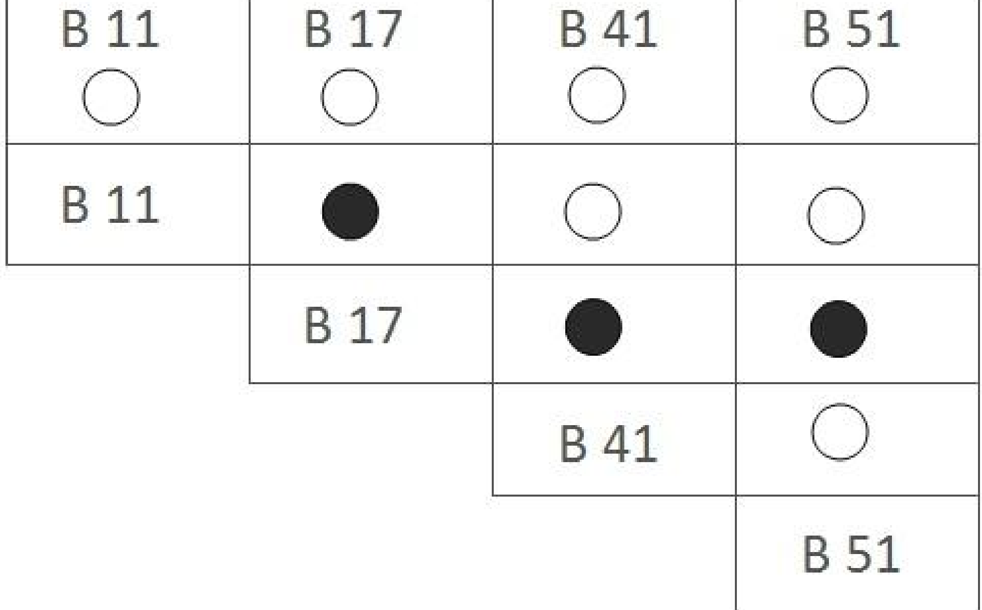


Fig.9. Complementation matrix of four *trp*-1 mutants.

● = Complementation Positive.

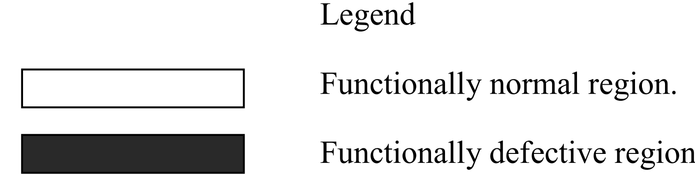
○ = Complementation Negative.

Classification of *trp-1* mutants

On the basis of Complementation test the *trp-1* mutants were classified into two groups.

Group I = B 11, B 41, and B 51 = Three mutants.

Group II = B 17 = One mutant.



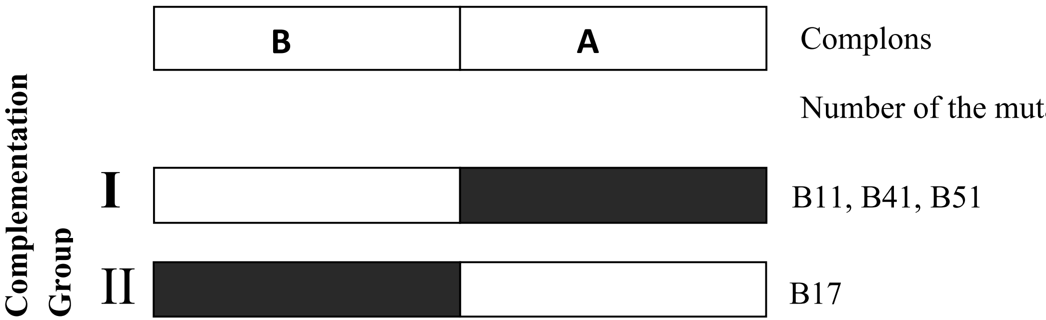


Fig.10. Complementation map of four *trp-1* mutants.

The seven *trp*-2 auxotrophs were tested in all possible complementation for heterocaryon formation in both solid and liquid VM medium. Results has given in Fig.11 and Fig.12

B 8 ○	B 12 ○	B 14 ○	B 22 ○	B 28 ○	B 38 ○	B 42 ○
B 8	○	●	○	○	○	○
	B 12	○	○	○	○	○
		B 14	●	●	●	●
			B 22	○	○	○
				B 28	○	○
					B 38	○
						B 42

Fig. 11. Complementation matrix of seven *trp-2* mutants.

● = Heterocaryon Positive.

○ = Heterocaryon Negative.

Classification of *trp-2* mutants

Group I =B12 = One Mutant.

Group II =B8, B 22, B 28, B 38 and B 42 = Five Mutants.

Group III= B14 = One Mutant.

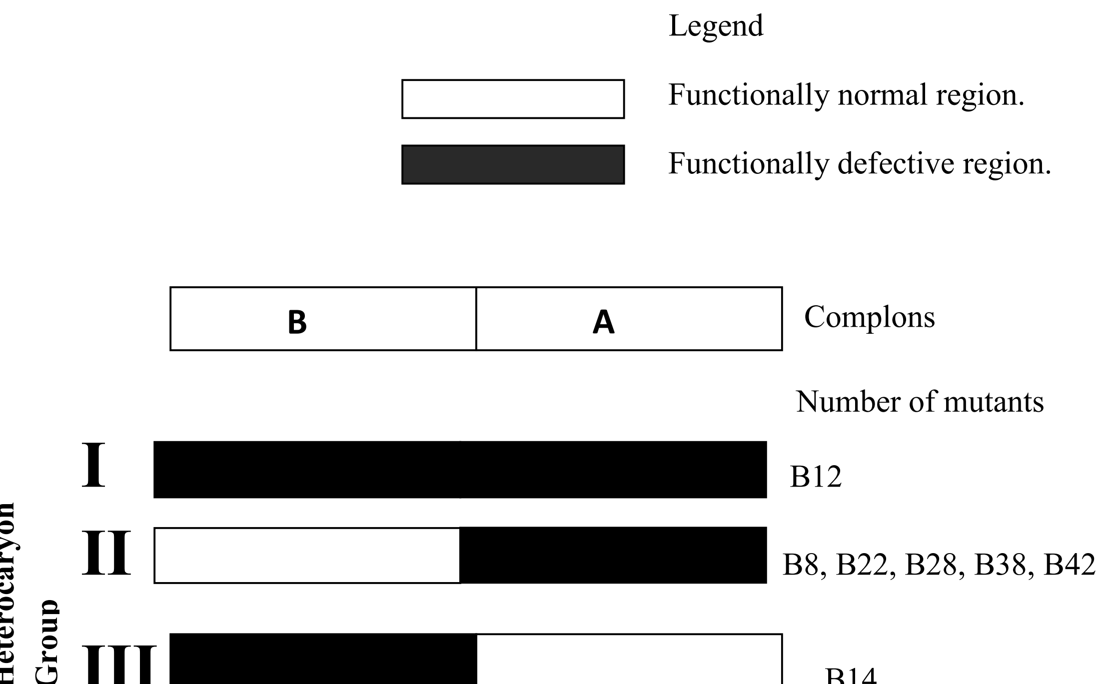


Fig.12. Complementation map of seven *trp-2* mutants.

The two *trp*-3 auxotrophs, two *trp*-4 auxotrophs and two *trp*-5 auxotrophs were tested in all possible combination for heterocaryon formation in both solid and liquid VM medium.

The mutants do not complements with each other and there in no further classification and as such they belong to the same complon. Results are shown in Fig.13, 14 and 15.

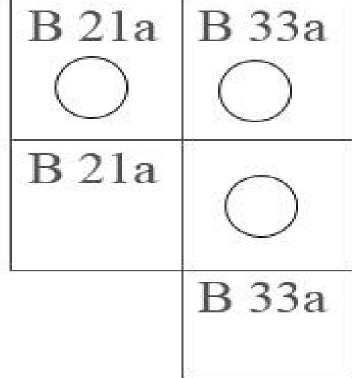


Fig. 13. Complementation Matrix of *trp*-3 mutants.

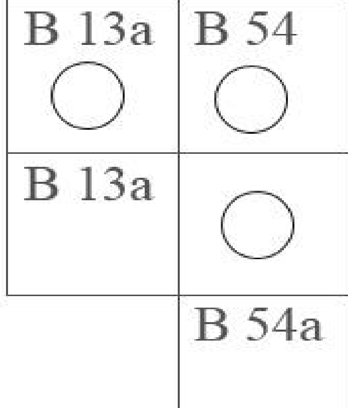


Fig. 14. Complementation Matrix of *trp*-4 mutants.

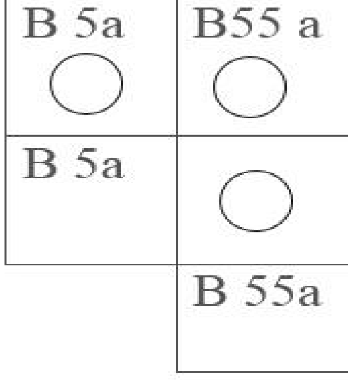


Fig. 15. Complementation Matrix of *trp*-5 mutants

7.1 FERTILITY OF TRYPTOPHAN AUXOTROPHS WITH STANDARD MARKERS

Seventeen tryptophan auxotrophs (B5a, B8a, B11a, B12a, B13a, B14a, B17a, B21a, B22a, B28a, B33a, B38a, B41a, B42a, B51a, B54a, B55a) were crossed with markers of seven linkage groups. *leu*-3(R156)A and *his*-2(T51M152t)A for linkage group I, *arg*-5(27947)A linkage group II, *leu*-I(33757)A linkage group III, *arg*-2(33442)A linkage group IV, *leu*-5{45208(t)}A linkage group V, *trp*-2(75001)A and *lys*-5(DS6-85)A linkage group VI and *arg*-10(B317)A linkage group VII were used. The crosses were made in Westergaard's crossing medium with required supplements. Observations were taken till the crosses shed spores. Data were given in the Table 15 to 31.

Table 15. Fertility of B5 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B5a X *leu*-3A	I	Yes	10	Many Medium	Yes	24	Fertile
B5a X *arg*-5A	II	Yes	8	Many Large	Yes	15	Good Fertile
B5a X *leu*-IA	III	Yes	9	Many Large	Yes	17	Good Fertile
B5a X *arg*-2A	IV	Yes	10	Many Medium	Yes	23	Fertile
B5a X *leu*-5A	V	Yes	8	Many Large	Yes	19	Good Fertile
B5a X *lys*-5A	VI	Yes	9	Many Medium	Yes	22	Fertile
B5a X *arg*-10A	VII	Yes	10	Many Medium	Yes	23	Fertile

Table 16. Fertility of B8 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B8a X *his*-2A	I	Yes	11	Many Medium	Yes	23	Fertile
B8a X *arg*-5A	II	Yes	8	Many Large	Yes	17	Good Fertile
B8a X *leu*-IA	III	Yes	9	Many Large	Yes	18	Good Fertile
B8a X *arg*-2A	IV	Yes	14	Few Small	Yes	29	Poorly Fertile
B8a X *leu*-5A	V	Yes	13	Few Small	Yes	30	Poorly Fertile
B8a X *trp*-2A	VI	Yes	7	Many Large	Yes	19	Good Fertile
B8a X *arg*-10A	VII	Yes	8	Many Large	Yes	18	Good Fertile

Table 17. Fertility of B11 *indole* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B11a X *leu*-3A	I	Yes	8	Many Large	Yes	15	Good Fertile
B11a X *arg*-5A	II	Yes	10	Many Medium	Yes	23	Fertile
B11a X *leu*-IA	III	Yes	11	Many Medium	Yes	24	Fertile
B11a X *arg*-2A	IV	Yes	7	Many Large	Yes	19	Good Fertile
B11a X *leu*-5A	V	Yes	12	Many Medium	Yes	21	Fertile
B11a X *trp*-2A	VI	Yes	11	Many Medium	Yes	22	Fertile
B11a X *arg*-10A	VII	Yes	9	Many Large	Yes	18	Good Fertile

Table 18. Fertility of B12 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B12a X *his*-2A	I	Yes	10	Many Medium	Yes	20	Fertile
B12a X *arg*-5A	II	Yes	8	Many Large	Yes	15	Good Fertile
B12a X *leu*-IA	III	Yes	7	Many Large	Yes	17	Good Fertile
B12a X *arg*-2A	IV	Yes	14	Few Small	Yes	30	Poorly Fertile
B12a X *leu*-5A	V	Yes	13	Few Small	Yes	29	Poorly Fertile
B12a X *trp*-2A	VI	Yes	9	Many Large	Yes	18	Good Fertile
B12a X *arg*-10A	VII	Yes	8	Many Large	Yes	19	Good Fertile

Table 19. Fertility of B13 *indole* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B13a X *his*-2A	I	Yes	10	Many Medium	Yes	21	Fertile
B13a X *arg*-5A	II	Yes	7	Many Large	Yes	16	Good Fertile
B13a X *leu*-IA	III	Yes	8	Many Large	Yes	15	Good Fertile
B13a X *arg*-2A	IV	Yes	12	Many Medium	Yes	22	Fertile
B13a X *leu*-5A	V	Yes	14	Few Small	Yes	26	Poorly Fertile
B13a X *trp*-2A	VI	Yes	9	Many Large	Yes	19	Good Fertile
B13a X *arg*-10A	VII	Yes	8	Many Large	Yes	18	Good Fertile

Table 20. Fertility of B14 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B14a X *leu*-3A	I	Yes	11	Many Medium	Yes	24	Fertile
B14a X *arg*-5A	II	Yes	10	Many Medium	Yes	23	Fertile
B14a X *leu*-IA	III	Yes	8	Many Large	Yes	18	Good Fertile
B14a X *arg*-2A	IV	Yes	12	Many Medium	Yes	23	Fertile
B14a X *leu*-5A	V	Yes	10	Many Medium	Yes	22	Fertile
B14a X *lys*-5A	VI	Yes	8	Many Large	Yes	19	Good Fertile
B14a X *arg*-10A	VII	Yes	12	Many Medium	Yes	20	Fertile

Table 21. Fertility of B17 *indole* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B17a X *leu*-3A	I	Yes	7	Many Large	Yes	19	Good Fertile
B17a X *arg*-5A	II	Yes	13	Few Small	Yes	29	Poorly Fertile
B17a X *leu*-IA	III	Yes	14	Few Small	Yes	25	Poorly Fertile
B17a X *arg*-2A	IV	Yes	8	Many Large	Yes	18	Good Fertile
B17a X *leu*-5A	V	Yes	13	Few Small	Yes	26	Poorly Fertile
B17a X *trp*-2A	VI	Yes	15	Few Small	Yes	30	Poorly Fertile
B17a X *arg*-10A	VII	Yes	9	Many Large	Yes	15	Good Fertile

Table 22. Fertility of B21 *tryptophan* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B21a X *leu*-3A	I	Yes	8	Many Large	Yes	16	Good Fertile
B21a X *arg*-5A	II	Yes	9	Many Large	Yes	19	Good Fertile
B21a X *leu*-IA	III	Yes	7	Many Medium	Yes	17	Good Fertile
B21a X *arg*-2A	IV	Yes	12	Many Medium	Yes	23	Fertile
B21a X *leu*-5A	V	Yes	9	Many Large	Yes	18	Good Fertile
B21a X *lys*-5A	VI	Yes	11	Many Medium	Yes	24	Fertile
B21a X *arg*-10A	VII	Yes	7	Many Large	Yes	15	Good Fertile

Table 23. Fertility of B22 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B22a X *his*-2A	I	Yes	12	Many Medium	Yes	23	Fertile
B22a X *arg*-5A	II	Yes	9	Many Large	Yes	16	Good Fertile
B22a X *leu*-IA	III	Yes	14	Few Small	Yes	29	Poorly Fertile
B22a X *arg*-2A	IV	Yes	11	Many Medium	Yes	21	Fertile
B22a X *leu*-5A	V	Yes	8	Many Large	Yes	18	Good Fertile
B22a X *trp*-2A	VI	Yes	7	Many Large	Yes	17	Good Fertile
B22a X *arg*-10A	VII	Yes	9	Many Large	Yes	15	Good Fertile

Table 24. Fertility of B28 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B28a X *his*-2A	I	Yes	14	Few Small	Yes	30	Poorly Fertile
B28a X *arg*-5A	II	Yes	8	Many Large	Yes	16	Good Fertile
B28a X *leu*-IA	III	Yes	7	Many Large	Yes	19	Good Fertile
B28a X *arg*-2A	IV	Yes	10	Many Medium	Yes	21	Fertile
B28a X *leu*-5A	V	Yes	13	Few Small	Yes	29	Poorly Fertile
B28a X *trp*-2A	VI	Yes	9	Many Large	Yes	18	Good Fertile
B28aa X *arg*-10A	VII	Yes	7	Many Large	Yes	17	Good Fertile

Table 25. Fertility of B33 *tryptophan* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B33a X *leu*-3A	I	Yes	8	Many Large	Yes	15	Good Fertile
B33a X *arg*-5A	II	Yes	7	Many Large	Yes	18	Good Fertile
B33a X *leu*-IA	III	Yes	9	Many Large	Yes	16	Good Fertile
B33a X *arg*-2A	IV	Yes	8	Many Large	Yes	17	Good Fertile
B33a X *leu*-5A	V	Yes	7	Many Large	Yes	15	Good Fertile
B33a X *lys*-5A	VI	Yes	8	Many Large	Yes	19	Good Fertile
B33a X *arg*-10A	VII	Yes	9	Many Large	Yes	16	Good Fertile

Table 26. Fertility of B38 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B38a X *his*-2A	I	Yes	9	Many Large	Yes	16	Good Fertile
B38a X *arg*-5A	II	Yes	8	Many Large	Yes	19	Good Fertile
B38a X *leu*-IA	III	Yes	7	Many Large	Yes	18	Good Fertile
B38a X *arg*-2A	IV	Yes	15	Few Small	Yes	30	Poorly Fertile
B38a X *leu*-5A	V	Yes	13	Few Small	Yes	28	Poorly Fertile
B38a X *trp*-2A	VI	Yes	9	Many Large	Yes	15	Good Fertile
B38a X *arg*-10A	VII	Yes	8	Many Large	Yes	17	Good Fertile

Table 27. Fertility of B41 *indole* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B41a X *leu*-3A	I	Yes	8	Many Large	Yes	19	Good Fertile
B41a X *arg*-5A	II	Yes	15	Few Small	Yes	30	Poorly Fertile
B41a X *leu*-IA	III	Yes	7	Many Large	Yes	15	Good Fertile
B41a X *arg*-2A	IV	Yes	9	Many Large	Yes	17	Good Fertile
B41a X *leu*-5A	V	Yes	8	Many Large	Yes	18	Good Fertile
B41a X *trp*-2A	VI	Yes	12	Many Medium	Yes	22	Fertile
B41a X *arg*-10A	VII	Yes	7	Many Large	Yes	16	Good Fertile

Table 28. Fertility of B42 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B42a X *his*-2A	I	Yes	9	Many Large	Yes	18	Good Fertile
B42a X *arg*-5A	II	Yes	8	Many Large	Yes	15	Good Fertile
B42a X *leu*-IA	III	Yes	10	Many Medium	Yes	21	Fertile
B42a X *arg*-2A	IV	Yes	7	Many Large	Yes	19	Good Fertile
B42a X *leu*-5A	V	Yes	13	Few Small	Yes	27	Poorly Fertile
B42a X *trp*-2A	VI	Yes	8	Many Large	Yes	17	Good Fertile
B42a X *arg*-10A	VII	Yes	7	Many Large	Yes	16	Good Fertile

Table 29. Fertility of B51 *indole* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B51a X *lee*-3A	I	Yes	11	Many Medium	Yes	22	Fertile
B51a X *arg*-5A	II	Yes	7	Many Large	Yes	16	Good Fertile
B51a X *leu*-IA	III	Yes	9	Many Large	Yes	19	Good Fertile
B51a X *arg*-2A	IV	Yes	13	Few Small	Yes	27	Poorly Fertile
B51a X *leu*-5A	V	Yes	8	Many Large	Yes	18	Good Fertile
B51a X *lys*-5A	VI	Yes	11	Many Medium	Yes	23	Fertile
B51a X *arg*-10A	VII	Yes	8	Many Large	Yes	17	Good Fertile

Table 30. Fertility of B54 *indole* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B54a X *his*-2A	I	Yes	8	Many Large	Yes	18	Good Fertile
B54a X *arg*-5A	II	Yes	10	Many Medium	Yes	20	Fertile
B54a X *leu*-IA	III	Yes	9	Many Large	Yes	17	Good Fertile
B54a X *arg*-2A	IV	Yes	7	Many Large	Yes	19	Good Fertile
B54a X *leu*-5A	V	Yes	12	Many Medium	Yes	23	Fertile
B54a X *lys*-5A	VI	Yes	7	Many Large	Yes	16	Good Fertile
B54a X *arg*-10A	VII	Yes	11	Many Medium	Yes	21	Fertile

Table 31. Fertility of B55 *anthranilic* mutant with standard markers

Designation of the cross	Linkage group of markers	Whether perithecia formed yes/no	Days of formation of perithecia	Frequency and size of perithecia	Whether spore shed yes/no	Shedding days	Inference on fertility
B55a X *leu*-3A	I	Yes	7	Many Large	Yes	16	Good Fertile
B55a X *arg*-5A	II	Yes	11	Many Medium	Yes	21	Fertile
B55a X *leu*-IA	III	Yes	9	Many Large	Yes	19	Good Fertile
B55a X *arg*-2A	IV	Yes	10	Many Medium	Yes	23	Fertile
B55a X *leu*-5A	V	Yes	8	Many Large	Yes	18	Good Fertile
B55a X *lys*-5A	VI	Yes	7	Many Large	Yes	17	Good Fertile
B54a X *arg*-10A	VII	Yes	9	Many Large	Yes	15	Good Fertile

8.1 STUDY OF LINKAGE

For the study of linkage, all the mutants (*anth* B5a, *anth* B8a, *anth*B12a, *anth* B14a, *anth* B22a, *anth* B28a, *anth* B38a, *anth* B42a, *anth* B55a, *ind* B11a, *ind* B13a, *ind* B17a, *ind* B41a, *ind* B51a, *ind* B54a, *trp* B21a and *trp* B33a) were crossed with Standard of all the markers of seven linkage groups. *leu*-3(R156)A and *his*-2(T51M152t)A for linkage group I, *arg*-5(27947)A linkage group II, *leu*-I(33757)A linkage group III, *arg*-2(33442)A linkage group IV, *leu*-5{45208(t)}A linkage group V, *trp*-2(75001)A and *lys*-5(DS6-85)A linkage group VI and *arg*-10(B317)A linkage group VII were used. The crosses were made in Westergaard's crossing medium with required supplements. The spores from the crosses were spread on SM plates. They were kept at 58^0 C for 50 minutes and then inoculated at 25^0 C for 12-16 hours. The germinating and growing spores were counted. If the ratio of the percentage of germinating and growing spores is 3:1, there is no linkage between the mutant and the marker. The linkage was studied by analyzing the percentage of germinating and growing spores. The growing spores were Wild recombinants and germinating spores were tryptophan mutants, markers and double mutants of tryptophan + marker. Data were given in Table 32 to 65.

Table 32. Linkage of *anth* B5 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B5a X *leu*-3 (R156)A	I	125	93	32	74.40	25.60	Not linked with *leu*-3 of linkage group I
2	B5a X *arg*-5 (27947)A	II	250	189	61	75.60	24.40	Not linked with *arg*-5 of linkage group II
3	B5a X *leu*-I (33757)A	III	138	105	33	76.09	23.91	Not linked with *leu*-1 of linkage group III
4	B5a X *arg*-2 (33442)A	IV	311	226	85	72.67	27.33	Not linked with *arg*-2 of linkage group IV
5	**B5a X *leu*-5 {45208(t)}A**	**V**	**88**	**75**	**13**	**85.22**	**14.33**	**Linked with *leu*-5 of linkage group V**
6	B5a X *lys*-5 (DS6-85)A	VI	94	68	26	72.34	27.66	Not linked with *lys*-5 of linkage group VI
7	B5a X *arg*-10 (B317)A	VII	450	340	110	75.56	24.44	Not linked with *arg*-10 of linkage group VII

Here,

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

0_1 = Observed mutants
0_2 = Observed Wild recombinants
e_1 = Expected mutants
e_2 = Expected Wild recombinants

Table 33. Checking of segregation of *anth* B5a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B5a x *leu*-3 (R156)A	125	93	32	93.75	31.25	0.006 + 0.018	0.024
2	B5a X *arg*-5 (27947)A	250	189	61	187.50	62.50	0.012 + 0.036	0.048
3	B5a X *leu* - I (33757)A	138	105	33	103.50	34.50	0.021+0.065	0.086
4	B5a X *arg*-2 (33442)A	311	226	85	233.25	77.75	0.225 + 0.676	0.901
5	**B5a X *leu*-5{45208(t)}A**	**88**	**75**	**13**	**66.00**	**22.00**	**1.007 + 3.681**	**4.908**
6	B5a X *lys*-5 (DS6-85)A	94	68	26.00	70.50	23.50	0.088+0.265	0.353
7	B5a X *arg*-10 (B317)A	450	340	110	337.50	112.50	0.018 + 0.055	0.073

The chi-square value for the segregation of the Sl No 1,2,3,4,6 and 7 is 0.024, 0.048, 0.086, 0.353, 0.901, 0.353 and 0.073 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 5 is 4.908, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B5 and *leu-5*{45208(t)}.

Determination of genetic map of *anth* B5 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B5a X *leu-5A*{45208(t)}	88	13	14.33

Order of *anth* B5 and *leu-5*

Distance between *anth* B5 and *leu*-5

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 13}{88} \times 100$

= 29.545 CM

Here
R = Recombinants
N = Total Progenies
X = Distance in CM

leu-5 *anth*B5

29.545

Fig. 16. Genetic map of *anth* B5 and *leu* -5{45208(t)}

Here, 1 Centimorgan = 0.1 Centimeter

Table 34. Linkage of *anth* B8 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B8a X *his*-2 (T51 M 152 t)A	I	738	549	189	74.39	25.60	Not linked with *his*-2 of linkage group I
2	B8a X *arg*-5 (27947)A	II	388	286	102	73.71	26.29	Not linked with *arg*-5 of linkage group II
3	B8a X *leu*-I (33757)A	III	741	551	190	74.35	25.64	Not linked with *leu*-1 of linkage group III
4	B8a X *arg*-2 (33442)A	IV	755	560	195	74.17	25.82	Not linked with *arg*-2 of linkage group IV
5	B8a X *leu*-5 {45208(t)}A	V	748	554	194	74.06	25.93	Not linked with *leu*-5 of linkage group V
6	**B8a X *trp*-2 (75001)A**	**VI**	**205**	**180**	**25**	**87.80**	**12.19**	**Linked with *trp*-2 of linkage group VI**
7	B8a X *arg*-10 (B317)A	VII	406	308	98	75.86	24.14	Not linked with *arg*-10 of linkage group VII

Table 35. Checking of segregation of *anth* B8a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B8a X *his*-2(T51 M 152 t)A	738	549	189	553.5	184.5	0.036 + 0.109	0.145
2	B8a X *arg*-5 (27947)A	388	286	102	291.00	97.00	0.086 + 0.257	0.343
3	B8a X *leu*-I (33757)A	741	551	190	555.75	185.25	0.040 + 0.122	0.162
4	B8a X *arg*-2 (33442)A	755	560	195	566.25	188.75	0.068 + 0.207	0.275
5	B8a X *leu*-5{45208(t)}A	748	554	194	561.00	187.00	0.087 + 0.262	0.349
6	**B8a X *trp*-2 (75001)A**	**205**	**180**	**25**	**153.75**	**51.25**	**4.481 + 13.445**	**17.926**
7	B8a X *arg*-10 (B317)A	406	258	148	304.50	101.50	0.007 + 0.120	0.127

The chi-square value for the segregation of the Sl No 1,2,3,4,5 and 7 is 0.145, 0.343, 0.162, 0.275, 0.349 and 0.127 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 6 is 17.926, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B8 and *trp*-2 (75001).

Determination of genetic map of *anth* B8 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B8a X *trp*-2A (75001)	205	25	12.19

Order of *anth* B8 and *trp*-2 A

trp-2 *anth* B8

Distance between *anth* B8 and *trp*-2 A

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 25}{205} \times 100$

= 24.390 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

trp-2 *anth* B8

24.390

Fig.17. Genetic map of *anth* B8 and *trp*-2 (75001)

Here, 1 Centimorgan = 0.1 Centimeter

Table 36. Linkage of *ind* B11 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	**B11a X *leu*-3 (R156)A**	**I**	**155**	**137**	**18**	**88.39**	**11.61**	**Linked with *leu*-3 of linkage group I**
2	B11a X *arg*-5 (27947)A	II	306	221	85	72.22	27.78	Not linked with *arg*-5 of linkage group II
3	B11a X *leu*-I (33757)A	III	183	136	47	74.32	25.68	Not linked with *leu*-1 of linkage group III
4	B11a X *arg*-2 (33442)A	IV	286	205	81	71.67	28.32	Not linked with *arg*-2 of linkage group IV
5	B11a X *leu*-5 {45208(t)}A	V	284	214	70	75.35	24.65	Not linked with *leu*-5 of linkage group V
6	B11a X *trp*-2 (75001)A	VI	195	143	52	73.33	26.66	Not linked with *trp*-2 of linkage group VI
7	B11a X *arg*-10 (B317)A	VII	262	199	63	75.95	24.04	Not linked with *arg*-10 of linkage group VII

Table 37. Checking of segregation of *ind* B11a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	**B11a X *leu*-3 (R156)A**	**155**	**137**	**18**	**116.25**	**38.75**	**3.703 + 11.111**	**14.814**
2	B11a X *arg*-5 (27947)A	306	221	85	229.50	76.50	0.314 + 0.944	1.258
3	B11a X *leu*-I (33757)A	183	136	47	137.25	45.75	0.011 + 0.034	0.045
4	B11a X *arg*-2 (33442)A	286	205	81	214.50	71.50	0.420 + 1.262	1.682
5	B11a X *leu*-5{45208(t)}A	284	214	70	213.00	71.00	0.004 + 0.014	0.018
6	B11a X *trp*-2 (75001)A	195	143	52	146.25	48.75	0.042 + 0.216	0.258
7	B11a X *arg*-10 (B317)A	262	199	63	196.50	65.50	0.031 + 0.095	0.126

The chi-square value for the segregation of the Sl No 2,3,4,5,6 and 7 is 1.258, 0.045, 1.682, 0.018, 0.258 and 0.126 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 1 is 14.814, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *ind* B11 and *leu*-3 (R156).

Determination of genetic map of *ind* B11 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B11a X *leu*-3A (R156)	155	18	11.61

Order of *ind* B11 and *leu*-3

Distance between *ind* B11 and *leu*-3

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 18}{155} \times 100$

= 23.225 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

ind B11 *leu*-3

23.225

Fig. 18. Genetic map of *ind* B11 and *leu*-3 (R 156)

Here, 1 Centimorgan = 0.1 Centimeter

Table 38. Linkage of *anth* B12 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B12a X *his*-2 (T51 M 152 t)A	I	278	204	74	73.38	26.62	Not linked with *his*-2 of linkage group I
2	B12a X *arg*-5 (27947)A	II	818	617	201	75.43	24.57	Not linked with *arg*-5 of linkage group II
3	B12a X *leu*-I (33757)A	III	265	193	72	72.83	27.16	Not linked with *leu*-I of linkage group III
4	B12a X *arg*-2 (33442)A	IV	527	387	140	73.43	26.57	Not linked with *arg*-2 of linkage group IV
5	B12a X *leu*-5 {45208(t)}A	V	813	593	210	74.94	25.83	Not linked with *leu*-5 of linkage group V
6	**B12a X *trp*-2 (75001)A**	**VI**	**331**	**280**	**51**	**84.59**	**15.41**	**Linked with *trp*-2 of linkage group VI**
7	B12a X *arg*-10 (B317)A	VII	660	480	180	72.73	27.27	Not linked with *arg*-10 of linkage group VII

Table 39. Checking of segregation of *anth* B12a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B12a X *his*-2 (T51 M 152 t)A	278	204	74	208.50	69.50	0.097 + 0.032	0.129
2	B12a X *arg*-5 (27947)A	818	617	201	613.50	204.50	0.019 + 0.059	0.078
3	B12a X *leu*-I (33757)A	265	193	72	198.75	66.25	0.166 + 0.499	0.665
4	B12a X *arg*-2 (33442)A	527	387	140	395.25	131.75	0.175 + 0.516	0.691
5	B12a X *leu*-5{45208(t)}A	813	593	210	609.75	203.25	0.460 + 0.224	0.684
6	**B12a X *trp*-2 (75001)A**	**331**	**280**	**51**	**248.25**	**82.75**	**4.060 + 12.182**	**16.242**
7	B12a X *arg*-10 (B317)A	660	480	180	495.00	165.00	0.454 + 1.363	1.817

The chi-square value for the segregation of the Sl No 1,2,3,4,5, and 7 is 0.129, 0.078, 0.665, 0.691, 0.684 and 1.817 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 6 is 16.242, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B12 and *trp*-2 (75001).

Determination of genetic map of *anth* B12 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B12a X *trp*-2A (75001)	331	51	15.41

Order of *anth* B12 and *trp*-2

trp-2 *anth* B 12

Distance between *anth* B12 and *trp*-2

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 51}{331} \times 100$

= 30.815 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

trp-2 *anth* B12

30.815

Fig. 19. Genetic map of *anth* B12 and *trp*-2 (75001)

Here, 1 Centimorgan = 0.1 Centimeter

Table 40. Linkage of *ind* B13 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B13a X *his*-2 (T51 M 152 t)A	I	278	202	76	72.66	27.34	Not linked with *his*-2 of linkage group I
2	B13a X *arg*-5 (27947)A	II	388	298	19	76.80	23.20	Not linked with *arg*-5 of linkage group II
3	**B13a X *leu*-I (33757)A**	**III**	**410**	**405**	**5**	**98.78**	**1.21**	**Linked with *leu*-1 of linkage group – III**
4	B13a X *arg*-2 (33442)A	IV	379	294	85	77.57	22.43	Not linked with *arg*-2 of linkage group IV
5	B13a X *leu*-5 {45208(t)}A	V	286	207	79	72.38	27.62	Not linked with *leu*-5 of linkage group V
6	B13a X *trp*-2 (75001)A	VI	306	225	81	73.52	26.47	Not linked with *trp*-2 of linkage group VI
7	B13a X *arg*-10 (B317)A	VII	398	293	105	73.62	26.38	Not linked with *arg*-10 of linkage group VII

Table 41. Checking of segregation of *ind* B13a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1}$ _ $\frac{(o_2-e_2)}{e_2}$	x^2
1	B13a X *his*-2 (T51M 152 t)A	278	202	76	208.50	69.50	0.202 + 0.607	0.809
2	B13a X *arg*-5 (27947)A	388	298	90	291.00	97.00	0.168 + 0.505	0.673
3	**B13a X *leu*-I (33757)A**	**410**	**405**	**5**	**307.50**	**102.00**	**30.914 + 92.244**	**123.159**
4	B13a X *arg*-2 (33442)A	379	294	85	284.25	94.75	0.334 + 1.003	1.337
5	B13a X *leu*-5 {45208(t)}A	286	207	79	214.50	71.50	0.262 + 0.786	1.048
6	B13a X *trp*-2 (75001)A	306	215	81	229.50	76.50	0.916 + 0.264	1.180
7	B13a X *arg*-10 (B317)A	398	293	105	298.50	99.50	0.101 + 0.304	0.405

The chi-square value for the segregation of the Sl No 1,2,4,5,6 and 7 is 0.809, 0.673, 1.337, 1.048, 1.180 and 0.405 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 3 is 123.159, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore< linkage is present between *ind* B13 and *leu*-1 (33757).

Determination of genetic map of *ind* B13 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B13a X *leu*-IA (33757)	410	5	1.21

Order of *ind* B13 and *leu*-I

leu-I *ind* B13

Distance between *ind* B13 and *leu*-I

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 5}{410} \times 100$

= 2.439 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

leu-I *ind* B13

2.439

Fig. 20. Genetic map of *ind* B13 and *leu*-I (33757)

Here, 1 Centimorgan = 0.6 Centimeter

Table 42. Linkage of *anth* B14 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B14a X *leu*-3 (R156)A	I	381	285	96	74.80	25.20	Not linked with *leu*-3 of linkage group I
2	B14a X *arg*-5 (27947)A	II	321	236	85	73.52	26.48	Not linked with *arg*-5 of linkage group II
3	B14a X *leu*-I (33757)A	III	153	110	43	71.90	28.10	Not Linked with *leu*-I of linkage group III
4	B14a X *arg*-2 (33442)A	IV	481	355	126	73.80	26.20	Not linked with linkage group –IV
5	B14a X *leu*-5 {45208(t)}A	V	543	395	148	72.74	27.26	Not linked with linkage group –V
6	**B14a X *lys*-5 (DS6-85)A**	**VI**	**165**	**156**	**9**	**94.55**	**5.45**	**Linked with linkage group –VI**
7	B14a X *arg*-10 (B317)A	VII	228	170	58	74.56	25.43	Not linked with linkage group –VII

Table 43. Checking of segregation of *anth* B14a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B14a X *leu*-3 (R156)A	381	285	96	285.75	95.25	0.001 + 0.005	0.006
2	B14a X *arg*-5 (27947)A	321	236	85	240.75	80.25	0.093 + 0.281	0.374
3	B14a X *leu*-I (33757)A	153	110	43	114.75	38.25	0.196 + 0.589	0.785
4	B14a X *arg*-2 (33442)A	481	355	126	360.75	120.25	0.091 + 0.274	0.365
5	B14a X *leu*-5 {45208(t)}A	543	395	148	407.25	135.75	0.368 + 1.105	1.473
6	**B14a X *lys*-5 (DS6-85)A**	**165**	**156**	**9**	**123.75**	**41.25**	**8.404+25.213**	**33.617**
7	B14a X *arg*-10 (B317)A	228	170	58	171.00	57.00	0.005 + 0.017	0.022

The chi-square value for the segregation of the Sl No 1,2,3,4,5 and 7 is 0.006, 0.374, 0.785, 0.365, 1.473 and 0.022 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 6 is 33.617, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B14 and *lys-5*(DS6-85).

Determination of genetic map of *anth* B14 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B14a X *lys-5*A (DS6-85)	165	9	5.45

Order of *anth* B14 and *lys-5*

lys 5 — *anth* B14

Distance between *anth* B14 and *lys-5*

$$\text{Distance} = \frac{2R}{N} \times 100 = X \text{ Centimorgan}$$

$$= \frac{2 \times 9}{165} \times 100$$

= 10.90 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

lys-5 *anth* B14

10.90

Fig. 21. Genetic map of *anth* B14 and *lys-5*(DS6-85)

Here, 1 Centimorgan = 0.1 Centimeter

Table 44. Linkage of *ind* B17 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B17a X *leu*-3 (R156)A	I	535	395	140	73.83	26.16	Not linked with *leu*-3 of linkage group I
2	B17a X *arg*-5 (27947)A	II	433	318	115	73.44	26.55	Not linked with *arg*-5 of linkage group II
3	B17a X *leu*-I (33757)A	III	476	351	125	73.73	26.26	Not linked with *leu*-I of linkage group III
4	**B17a X *arg*-2 (33442)A**	**IV**	**415**	**400**	**15**	**96.39**	**3.61**	**Linked with *arg*-2 of linkage group IV**
5	B17a X *leu*-5 {45208(t)}A	V	613	453	160	73.90	26.10	Not linked with *leu*-5 of linkage group V
6	B17a X *trp*-2 (75001)A	VI	393	290	103	73.79	26.20	Not linked with *trp*-2 of linkage group VI
7	B17a X *arg*-10 (B317)A	VII	748	551	197	73.63	26.33	Not linked with *arg*-10 of linkage group VII

Table 45. Checking of segregation of *ind* B17a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B17a X *leu*-3 (R156)A	535	395	140	401.25	133.75	0.097 + 0.292	0.389
2	B17a X *arg*-5 (27947)A	433	318	115	324.75	108.25	0.140 + 0.420	0.560
3	B17a X *leu*-I (33757)A	476	351	125	357.00	119.00	0.100 + 0.302	0.402
4	**B17a X *arg*-2 (33442)A**	**415**	**400**	**15**	**311.25**	**103.75**	**25.306 + 75.918**	**101.224**
5	B17a X *leu*-5 {45208(t)}A	613	453	160	459.75	153.25	0.099 + 0.297	0.396
6	B17a X *trp*-2 (75001)A	393	290	103	294.75	98.25	0.076 + 0.229	0.305
7	B17a X *arg*-10 (B317)A	748	551	197	561.00	187.00	0.178 + 0.534	0.712

The chi-square value for the segregation of the Sl No 1,2,3,5,6 and 7 is 0.389, 0.560, 0.402, 0.396, 0.305 and 0.712 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 4 is 101.224, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *ind* B17 and *arg*-2 (33442).

Determination of genetic map of *ind* B17 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B17a X *arg*-2A (33442)	415	15	3.61

Order of *ind* B17 and *arg*-2

ind-B 17 *arg*-2

Distance between *ind* B17 and *arg*-2

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 15}{415} \times 100$

= 7.228 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

Ind B17 *arg*-2

7.228

Fig. 22. Genetic map of *ind* B17 and *arg*-2 (33442)

Here, 1 Centimorgan = 0.3 Centimeter

Table 46. Linkage of *trp* B21 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B21a X *leu*-3 (R156)A	I	310	227	83	73.22	26.78	Not linked with *leu*-3 of linkage group I
2	B21a X *arg*-5 (27947)A	II	195	142	53	72.82	27.18	Not linked with *arg*-5 of linkage group II
3	**B21a X *leu*-I (33757)A**	**III**	**167**	**145**	**22**	**86.83**	**13.17**	**Linked with *leu*-I of linkage group III**
4	B21a X *arg*-2 (33442)A	IV	335	249	86	74.32	25.67	Not linked with *arg*-2 of linkage group IV
5	B21a X *leu*-5 {45208(t)}A	V	365	275	90	75.34	24.65	Not linked with *leu*-5 of linkage group V
6	B21a X *lys*-5 (DS6-85)A	VI	385	284	101	73.77	26.23	Not linked with *lys*-5 of linkage group VI
7	B21a X *arg*-10 (B317)A	VII	415	300	115	72.29	27.71	Not linked with *arg*-10 of linkage group VII

Table 47. Checking of segregation of *trp* B21a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B21a X *leu*-3 (R156)A	310	227	83	232.50	77.50	0.130 + 0.390	0.52
2	B21a X *arg*-5 (27947)A	195	142	53	146.25	48.75	0.123 + 0.370	0.493
3	**B21a X *leu*-I (33757)A**	**167**	**145**	**22**	**125.25**	**41.75**	**3.114 + 9.342**	**12.456**
4	B21a X *arg*-2 (33442)A	335	249	86	251.25	83.75	0.020 + 0.060	0.080
5	B21a X *leu*-5 {45208(t)}A	365	275	90	273.75	91.25	0.005 + 0.017	0.022
6	B21a X *lys*-5 (DS6-85)A	385	284	101	288.75	96.25	0.078 + 0.234	0.312
7	B21a X *arg*-10 (B317)A	415	300	115	311.25	103.75	0.406 + 1.219	1.625

The chi-square value for the segregation of the Sl No 1,2,4,5,6 and 7 is 0.052, 0.493, 0.080, 0.022, 0.312 and 1.625 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 3 is 12.456, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *trp* B17 and *leu*-1 (33757).

Determination of genetic map of *trp* B21 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B21a X *leu*-IA (33757)	167	22	13.17

Order of *trp*-B21 and *leu*-I

leu-I *trp*-21

Distance between *trp*-B21 and *leu*-I

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 22}{167} \times 100$

= 26.237 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

leu-I *trp*-B21

26.237

Fig. 23. Genetic map of *trp*-B21 and *leu*-I (33757)

Here, 1 Centimorgan = 0.1 Centimeter

Table 48. Linkage of *anth* B22 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B22a X *his*-2 (T51 M 152 t)A	I	352	263	89	74.71	25.28	Not linked with *his*-2 of linkage group I
2	B22a X *arg*-5 (27947)A	II	486	369	117	75.92	24.07	Not linked with *arg*-5 of linkage group II
3	B22a X *leu*-I (33757)A	III	193	146	47	75.64	24.35	Not linked with *leu*-I of linkage group III
4	B22a X *arg*-2 (33442)A	IV	578	428	150	74.05	25.94	Not linked with *arg*-2 of linkage group IV
5	B22a X *leu*-5 {45208(t)}A	V	518	394	124	76.06	23.93	Not linked with *leu*-5 of linkage group V
6	**B22a X *trp*-2 (75001)A**	**VI**	**456**	**456**	**0**	**100**	**0**	**Linked with *trp*-2 of linkage group VI**
7	B22a X *arg*-10 (B317)A	VII	214	154	60	71.96	28.04	Not linked with *arg*-10 of linkage group VII

Table 49. Checking of segregation of *anth* B22a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} + \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B22a X *his*-2(T51M 152 t)A	352	263	89	264.00	88.00	0.003 + 0.011	0.014
2	B22a X *arg*-5 (27947)A	486	369	117	364.50	121.50	0.055 + 0.166	0.221
3	B22a X *leu*-I (33757)A	193	146	47	144.75	48.25	0.010 + 0.032	0.042
4	B22a X *arg*-2 (33442)A	578	428	150	433.45	144.50	0.068 + 0.209	0.277
5	B22a X *leu*-5 {45208(t)}A	518	394	124	388.50	129.50	0.077 + 0.233	0.310
6	**B22a X *trp*-2 (75001)A**	**456**	**456**	**0**	**342.00**	**114.00**	**38.00 + 114.00**	**152.00**
7	B22a X *arg*-10 (B317)A	214	154	60	160.50	53.50	0.263 + 0.789	1.052

The chi-square value for the segregation of the Sl No 1,2,3,4,5, and 7 is 0.014, 0.221, 0.042, 0.277, 0.310 and 1.052 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 6 is 152.00, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B22 and *trp*-2 (75001).

Determination of genetic map of *anth* B22 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B22a X *trp*-2A (75001)	456	0	0

Order of *anth* B22 and *trp*-2

Distance between *anth* B22 and *trp*-2

$$\text{Distance} = \frac{2R}{N} \times 100 = X \text{ Centimorgan}$$

$$= \frac{2 \times 0}{456} \times 100$$

$$= 0 \text{ CM}$$

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

anth B22
trp-2

Fig. 24. Genetic map of *anth* B22 and *trp*-2 (75001)

Here, 1 Centimorgan = 0.1 Centimeter

Table 50. Linkage of *anth* B28 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B28a X *his*-2 (T51 M 152 t)A	I	604	446	158	73.84	26.16	Not linked with *his*-2 of linkage group I
2	B28a X *arg*-5 (27947)A	II	295	215	80	72.88	27.11	Not linked with *arg*-5 of linkage group II
3	B28a X *leu*-I (33757)A	III	323	236	87	73.06	26.94	Not linked with *leu*-I of linkage group III
4	B28a X *arg*-2 (33442)A	IV	448	333	115	74.33	25.66	Not linked with *arg*-2 of linkage group IV
5	B28a X *leu*-5 {45208(t)}A	V	628	465	163	74.04	25.96	Not linked with *leu*-5 of linkage group V
6	**B28a X *trp*-2 (75001)A**	**VI**	**586**	**586**	**0**	**100**	**0**	**Linked with *trp*-2 linkage group – VI**
7	B28aa X *arg*-10 (B317)A	VII	539	399	140	74.02	25.97	Not linked with *arg*-10 of linkage group VII

Table 51. Checking of segregation of *anth* B28a mutant with chi-square

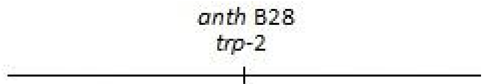
Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B28a X *his*-2 (T51 M 152 t)A	604	446	158	453.00	151.00	0.108 + 0.324	0.432
2	B28a X *arg*-5 (27947)A	295	215	80	221.25	73.75	0.176 + 0.529	0.705
3	B28a X *leu*-I (33757)A	323	236	87	242.25	80.75	0.161 + 0.483	0.644
4	B28a X *arg*-2 (33442)A	448	333	115	336.00	112.00	0.026 + 0.080	0.106
5	B28a X *leu*-5 {45208(t)}A	628	465	163	471.00	157.00	0.076 + 0.229	0.305
6	**B28a X *trp*-2 (75001)A**	**586**	**586**	**0**	**439.50**	**146.50**	**48.833 + 146.500**	**195.333**
7	B28aa X *arg*-10 (B317)A	539	399	140	404.25	134.75	0.068 + 0.204	0.272

The chi-square value for the segregation of the Sl No 1,2,3,4,5, and 7 is 0.432, 0.705, 0.644, 0.106, 0.305 and 0.272 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 6 is 195.33, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B28 and *trp*-2 (75001).

Determination of genetic map of *anth* B28 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B22a X *trp*-2A (75001)	586	0	0

Order of *anth* B28 and *trp*-2



Distance between *anth B*28 and *trp*-2

$$\text{Distance} = \frac{2\,R}{N} \times 100 = X \text{ Centimorgan}$$

$$= \frac{2 \times 0}{586} \times 100$$

= 0 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

anth B28
trp-2

Fig. 25. Genetic map of *anth* B28 and *trp*-2 (75001)

Here, 1 Centimorgan = 0.1 Centimeter

Table 52. Linkage of *trp* B33 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B33a X *leu*-3 (R156)A	I	365	270	95	73.97	26.03	Not linked with *lue*-3 of linkage group I
2	**B33a X *arg*-5 (27947)A**	**II**	**105**	**90**	**15**	**85.71**	**14.29**	**Linked with *arg*-5 of linkage group II**
3	B33a X *leu*-I (33757)A	III	180	133	47	73.89	26.11	Not linked with *leu*-I of linkage group III
4	B33a X *arg*-2 (33442)A	IV	290	220	70	75.86	24.14	Not linked with *arg*-2 of linkage group IV
5	B33a X *leu*-5 {45208(t)}A	V	586	430	156	73.38	26.62	Not linked with *leu*-5 of linkage group V
6	B33a X *lys*-5 (DS6-85)A	VI	405	300	105	74.07	25.93	Not linked with *lys*-5 of linkage group VI
7	B33a X *arg*-10 (B317)A	VII	128	93	35	72.66	27.34	Not linked with *arg*-10 of linkage group VII

Table 53. Checking of segregation of *trp* B33a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B33a X *leu*-3 (R156)A	365	270	95	273.75	91.25	0.044 + 0.154	0.198
2	**B33a X *arg*-5 (27947)A**	**105**	**90**	**15**	**78.75**	**26.25**	**1.607 + 4.821**	**6.428**
3	B33a X *leu*-I (33757)A	180	133	47	135	45	0.029 + 0.088	0.117
4	B33a X *arg*-2 (33442)A	290	220	70	217.5	72.5	0.028 + 0.086	0.114
5	B33a X *leu*-5 {45208(t)}A	586	430	156	439.5	146.5	0.205 + 0.616	0.821
6	B33a X *lys*-5 (DS6-85)A	405	300	105	303.75	101.25	0.046 + 0.138	0.184
7	B33a X *arg*-10 (B317)A	128	93	35	96	32	0.093 + 0.281	0.374

The chi-square value for the segregation of the Sl No 1,3,4,5,6 and 7 is 0.198, 0.117, 0.114, 0.821, 0.184 and 0.374 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 2 is 6.428, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *trp* B33 and *arg*-2 (27947).

Determination of genetic map of *trp* B33 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B22a X *trp*-2A (75001)	105	15	14.29

Order of *trp* B33 and *arg*-5

trp-B 33 *arg*-5

Distance between *trp* B33 and *arg*-5

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 15}{105} \times 100$

= 28.571 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

trp B33 *arg*-5

28.571

Fig. 26. Genetic map of *trp* B33 and *arg*-5 (27947)

Here, 1 Centimorgan = 0.1 Centimeter

Table 54. Linkage of *anth* B38 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B38a X *his*-2 (T51 M 152 t)A	I	590	445	146	75.42	24.75	Not linked with *his*-2 of linkage group I
2	B38a X *arg*-5 (27947)A	II	495	380	115	76.77	23.23	Not linked with *arg*-5 of linkage group II
3	B38a X *leu*-I (33757)A	III	270	205	65	75.93	24.07	Not linked with *leu*-I of linkage group III
4	B38a X *arg*-2 (33442)A	IV	598	446	152	74.58	25.42	Not linked with *arg*-2 of linkage group IV
5	B38a X *leu*-5 {45208(t)}A	V	583	447	136	76.67	23.32	Not linked with *leu*-5 of linkage group V
6	**B38a X *trp*-2 (75001)A**	**VI**	**362**	**362**	**0**	**100**	**0**	**Linked with *trp*-2 of linkage group VI**
7	B38a X *arg*-10 (B317)A	VII	290	210	80	72.41	27.59	Not linked with *arg*-10 of linkage group VII

Table 55. Checking of segregation of *anth* B38a mutant with chi-square

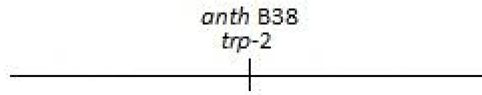
Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} + \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B38a X *his*-2 (T51 M 152 t)A	590	445	146	442.50	147.50	0.014 + 0.015	0.029
2	B38a X *arg*-5 (27947)A	495	380	115	371.25	123.75	0.206 + 0.618	0.824
3	B38a X *leu*-I (33757)A	270	205	65	202.50	67.50	0.030 + 0.092	0.122
4	B38a X *arg*-2 (33442)A	598	446	152	448.50	149.50	0.013 + 0.041	0.054
5	B38a X *leu*-5 {45208(t)}A	583	447	136	437.5	145.75	0.217 + 0.652	0.869
6	**B38a X *trp*-2 (75001)A**	**362**	**362**	**0**	**271.50**	**90.50**	**30.166 + 90.50**	**120.666**
7	B38a X *arg*-10 (B317)A	290	210	80	217.50	72.50	0.258 + 0.775	1.033

The chi-square value for the segregation of the Sl No 1,2,3,4,5, and 7 is 0.029, 0.824, 0.122, 0.054, 0.869 and 1.033 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 6 is 120.666, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B38 and *trp*-2 (75001).

Determination of genetic map of *anth* B38 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B22a X *trp*-2A (75001)	362	0	0

Order of *anth* B38 and *trp*-2



Distance between *anth B*38 and *trp*-2

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 0}{N} \times 100$

= 0 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

anth B38
trp-2

Fig. 27. Genetic map of *anth* B38 and *trp*-2 (75001)

Here, 1 Centimorgan = 0.1 Centimeter

Table 56. Linkage of *ind* B41 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B41a X *leu*-3 (R156)A	I	374	270	104	72.19	27.80	Not linked with *leu*-3 of linkage group I
2	B41a X *arg*-5 (27947)A	II	193	140	53	72.53	27.46	Not linked with *arg*-5 of linkage group II
3	**B41a X *leu*-I (33757)A**	**III**	**111**	**104**	**7**	**93.69**	**6.30**	**Linked with *leu*-I of linkage group III**
4	B41a X *arg*-2 (33442)A	IV	212	152	60	71.69	28.30	Not linked with *arg*-2 of linkage group IV
5	B41a X *leu*-5 {45208(t)}A	V	308	235	73	76.30	23.70	Not linked with *leu*-5 of linkage group V
6	B41a X *trp*-2 (75001)A	VI	235	180	55	76.60	23.40	Not linked with *trp*-2 of linkage group VI
7	B41a X *arg*-10 (B317)A	VII	288	210	78	72.92	27.08	Not linked with *arg*-10 of linkage group VII

Table 57. Checking of segregation of *ind* B41a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B41a X *leu*-3 (R156)A	374	270	104	280.50	93.50	0.393 + 1.179	1.572
2	B41a X *arg*-5 (27947)A	193	140	53	144.75	48.25	0.155 + 0.467	0.622
3	**B41a X *leu*-I (33757)A**	**111**	**104**	**7**	**83.25**	**27.75**	**5.171 + 15.515**	**20.686**
4	B41a X *arg*-2 (33442)A	212	152	60	159.00	53.00	0.308 + 0.924	1.232
5	B41a X *leu*-5 {45208(t)}A	308	235	73	231.00	77.00	0.069 + .207	0.276
6	B41a X *trp*-2 (75001)A	235	180	55	176.25	58.75	0.079 + 0.239	0.318
7	B41a X *arg*-10 (B317)A	288	210	78	216.00	72.00	0.166 + 0.50	0.666

The chi-square value for the segregation of the Sl No 1,2,4,5,6 and 7 is 1.572, 0.622, 1.232, 0.276, 0.318 and 0.666 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 3 is 20.686, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore linkage is present between *ind* B41 and *leu*-1 (33757).

Determination of genetic map of *ind* B41 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B41a X *leu*-IA(33757)	111	7	6.30

Order of *ind* B41 and *leu*-I

Distance between *ind B*41 and *leu*-I

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 7}{111} \times 100$

= 12.612 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

leu-I *ind* B41

12.612

Fig. 28. Genetic map of *ind* B41 and *leu*-I (33757)

Here, 1 Centimorgan = 0.2 Centimeter

Table 58. Linkage of *anth* B42 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B42a X *his*-2 (T51 M 152 t)A	I	374	284	90	75.93	24.06	Not linked with *his*-2 of linkage group I
2	B42a X *arg*-5 (27947)A	II	260	190	70	73.08	26.92	Not linked with *arg*-5 of linkage group II
3	B42a X *leu*-I (33757)A	III	369	265	104	71.82	28.18	Not linked with *leu*-I linkage group III
4	B42a X *arg*-2 (33442)A	IV	340	242	98	71.18	28.82	Not linked with *arg*-2 of linkage group IV
5	B42a X *leu*-5 {45208(t)}A	V	510	379	131	74.31	25.68	Not linked with *leu*-5 of linkage group V
6	**B42a X *trp*-2 (75001)A**	**VI**	**500**	**500**	**0**	**100**	**0**	**Linked with *trp*-2 of linkage group VI**
7	B42a X *arg*-10 (B317)A	VII	332	240	92	72.29	27.71	Not linked with *arg*-10 of linkage group VII

Table 59. Checking of segregation of *anth* B42a mutant with chi-square

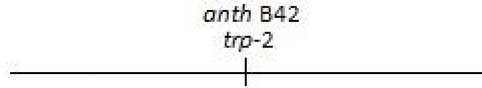
Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1}$ + $\frac{(o_2-e_2)^2}{e_2}$	x^2
1	B42a X *his*-2 (T51 M 152 t)A	374	284	40	280.50	93.50	0.043 + 0.131	0.174
2	B42a X *arg*-5 (27947)A	260	190	70	195.00	65.00	0.128 + 0.384	0.512
3	B42a X *leu*-I (33757)A	369	265	104	276.25	92.25	0.458 + 1.496	1.954
4	B42a X *arg*-2 (33442)A	340	242	98	255.00	85.00	0.662 + 1.988	2.650
5	B42a X *leu*-5 {45208(t)}A	510	379	131	382.50	127.50	0.032 + 0.096	0.128
6	**B42a X *trp*-2 (75001)A**	**500**	**500**	**0**	**375.00**	**125.00**	**41.666 + 125.000**	**166.666**
7	B42a X *arg*-10 (B317)A	332	240	92	249.00	83.00	0.325 + 0.975	1.300

The chi-square value for the segregation of the Sl No 1,2,3,4,5, and 7 is 0.174, 0.512, 1.954, 2.650, 0.128 and 1.300 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 6 is 166.666, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore linkage is present between *anth* B42 and *trp*-2 (75001).

Determination of genetic map of *anth* B42 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B42a X *trp*-2A (75001)	500	0	0

Order of *anth* B42 and *trp*-2



Distance between *anth* B42 and *trp* -2

Distance = $\frac{2R}{N}$ X 100 = X Centimorgan

= $\frac{2 X 0}{500}$ X 100

= 0 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

anth B42
trp-2

Fig. 29. Genetic map of *anth* B42 and *trp*-2 (75001)

Here, 1 Centimorgan = 0.1 Centimeter

Table 60. Linkage of *ind* B51 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B51a X *leu*-3 (R156)A	I	373	275	98	73.73	26.27	Not linked with *lue*-3 of linkage group I
2	B51a X *arg*-5 (27947)A	II	465	345	120	74.19	25.80	Not linked with *arg*-5 of linkage group II
3	**B51a X *leu*-I (33757)A**	**III**	**150**	**135**	**15**	**90**	**10**	**Linked with *leu*-I of linkage group III**
4	B51a X *arg*-2 (33442)A	IV	531	395	136	74.39	25.61	Not linked with *arg*-2 of linkage group IV
5	B51a X *leu*-5 {45208(t)}A	V	510	375	135	73.52	26.47	Not linked with *leu*-5 of linkage group V
6	B51a X *lys*-5 (DS6-85)A	VI	426	310	116	72.77	27.23	Not linked with *lys*-5 of linkage group VI
7	B51a X *arg*-10 (B317)A	VII	280	205	75	73.21	26.79	Not linked with *arg*-10 of linkage group VII

Table 61. Checking of segregation of *ind* B51a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B51a X *leu*-3 (R156)A	373	275	98	279.75	93.25	0.080 + 0.241	0.321
2	B51a X *arg*-5 (27947)A	465	345	120	348.75	116.25	0.040 + 0.120	0.160
3	**B51a X *leu*-I (33757)A**	**150**	**135**	**15**	**112.50**	**37.50**	**4.500 + 13.500**	**18.000**
4	B51a X *arg*-2 (33442)A	531	395	136	398.25	132.75	0.026 + 0.079	0.105
5	B51a X *leu*-5 {45208(t)}A	510	375	135	382.50	127.50	0.147 + 0.441	0.588
6	B51a X *lys*-5 (DS6-85)A	426	310	116	319.50	106.50	0.282 + 0.847	1.129
7	B51a X *arg*-10 (B317)A	280	205	75	210.00	70.00	0.119 + 0.357	0.476

The chi-square value for the segregation of the Sl No 1,2,4,5,6 and 7 is 0.321, 0.160, 0.105, 0.588, 1.129 and 0.476 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 3 is 18.000, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *ind* B51 and *leu*-1 (33757).

Determination of genetic map of *ind* B51 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B41a X *leu*-IA(33757)	150	15	10

Order of *ind* B51 and *leu*-I

leu-I *ind*-B51

Distance between *ind* B51 and *leu* –I

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 15}{150} \times 100$

= 20.00 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

leu-I *ind*- B51

20.00

Fig. 30. Genetic map of *ind* B51 and *leu*-I (33757)

Here, 1 Centimorgan = 0.1 Centimeter

Table 62. Linkage of *ind* B54 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B54a X *his*-2 (T51 M 152 t)A	I	299	214	85	71.58	28.42	Not linked with *his*-2 of linkage group I
2	B54a X *arg*-5 (27947)A	II	450	330	120	73.33	26.68	Not linked with *arg*-5 of linkage group II
3	B54a X *leu*-I (33757)A	III	630	463	167	73.49	26.50	Not linked with *leu*-I of linkage group III
4	**B54a X *arg*-2 (33442)A**	IV	**407**	**400**	**7**	**98.28**	**1.72**	**Linked with *arg*-2 of linkage group IV**
5	B54a X *leu*-5 {45208(t)}A	V	331	246	85	74.32	25.67	Not linked with *leu*-5 of linkage group V
6	B54a X *lys*-5 (DS6-85)A	VI	407	302	105	74.20	25.80	Not linked with *lys*-5 of linkage group VI
7	B54a X *arg*-10 (B317)A	VII	526	387	139	73.57	26.43	Not linked with *arg*-10 of linkage group VII

Table 63. Checking of segregation of *ind* B54a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B54a X *his*-2 (T51 M 152 t)A	299	214	85	224.25	74.75	0.468 + 1.405	1.873
2	B54a X *arg*-5 (27947)A	450	330	120	337.50	112.50	0.166 + 0.50	0.666
3	B54a X *leu*-I (33757)A	630	463	167	472.50	157.50	0.191 + 0.574	0.765
4	**B54a X *arg*-2 (33442)A**	**407**	**400**	**7**	**305.25**	**101.75**	**29.410 + 88.231**	**117.641**
5	B54a X *leu*-5 {45208(t)}A	331	246	85	248.25	82.75	0.020 + 0.061	0.081
6	B54a X *lys*-5 (DS6-85)A	407	302	105	305.25	101.75	0.034 + 0.103	0.137
7	B54a X *arg*-10 (B317)A	526	387	139	394.50	131.50	0.142 + 0.427	0.569

The chi-square value for the segregation of the Sl No 1,2,3,5,6 and 7 is 1.873, 0.666, 0.765, 0.081, 0.137 and 0.569 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 4 is 117.64, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *ind* B54 and *arg*-2 (33442).

Determination of genetic map of *ind* B54 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B54a X *arg*-2A (33442)	407	7	1.72

Order of *ind* B54 and *arg*-2

ind B 54 *arg*-2

Distance between *ind* B54 and *arg* -2

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$= \frac{2 \times 7}{407} \times 100$

= 3.439 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

Ind B54 *arg*-2

3.439

Fig. 31. Genetic map of *ind* B54 and *arg*-2 (33442)

Here, 1 Centimorgan = 0.6 Centimeter

Table 64. Linkage of *anth* B55 mutant

Sl no	Name of the cross & Marker used	Linkage group	No. of viable spore	No. of germinating spore	No. of growing spore	% of germinating spore	% of growing spore	Inference on linkage
1	B55a X *leu*-3 (R156)A	I	538	410	128	76.21	23.79	Not linked with *leu*-3 of linkage group I
2	B55a X *arg*-5 (27947)A	II	580	432	148	74.48	25.52	Not linked with *arg*-5 of linkage group II
3	B55a X *leu*-I (33757)A	III	410	305	105	74.39	25.61	Not linked with *leu*-I of linkage group III
4	B55a X *arg*-2 (33442)A	IV	373	275	98	73.73	26.27	Not linked with *arg*-2 of linkage group IV
5	**B55a X *leu*-5 {45208(t)}A**	**V**	**155**	**135**	**20**	**87.09**	**12.91**	**Linked with *leu*-5 of linkage group V**
6	B55a X *lys*-5 (DS6-85)A	VI	347	252	95	72.62	27.38	Not linked with *lys*-5 of linkage group VI
7	B54a X *arg*-10 (B317)A	VII	450	345	105	76.67	23.33	Not linked with *arg*-10 of linkage group VII

Table 65. Checking of segregation of *anth* B55a mutant with chi-square

Sl no	Cross	Total spore	o_1	o_2	e_1	e_2	$\frac{(o_1-e_1)^2}{e_1} - \frac{(o_2-e_2)^2}{e_2}$	x^2
1	B55a X *leu*-3 (R156)A	538	410	128	403.50	134.50	0.104 + 0.314	0.418
2	B55a X *arg*-5 (27947)A	580	432	148	435.00	145.00	0.020 + 0.062	0.082
3	B55a X *leu*-I (33757)A	410	305	105	307.50	102.50	0.020 + 0.060	0.080
4	B55a X *arg*-2 (33442)A	373	275	98	279.75	93.25	0.080 + 0.241	0.321
5	**B55a X *leu*-5 {45208(t)}A**	**155**	**135**	**20**	**116.25**	**38.75**	**3.024 + 9.072**	**12.096**
6	B55a X *lys*-5 (DS6-85)A	347	252	95	260.25	86.75	0.261 + 0.784	1.045
7	B54a X *arg*-10 (B317)A	450	345	105	337.50	112.50	0.166 + 0.50	0.666

The chi-square value for the segregation of the Sl No 1,2,3,4,6 and 7 is 0.418, 0.082, 0.080, 0.321, 1.045 and 0.666 respectively, which at 1 degree of freedom, shows agreement with the expected ratio (3:1). So, there is no linkage with the above crosses. But the chi-square value for the Sl. No. 5 is 12.096, which is significant and the data is not acceptable for free segregation in the ratio (3:1). Therefore, linkage is present between *anth* B55 and *leu*-5 {45208(t)}.

Determination of genetic map of *anth* B55 mutant

Name of the cross	Total progenies	Wild progenies	% of wild progenies
B55a X *leu*-5A {45208(t)}	155	20	12.91

Order of *anth* B55 and *leu*-5

Distance between *anth* B55 and *leu* -5

Distance = $\frac{2R}{N} \times 100 = X$ Centimorgan

$$\frac{2 \times 20}{155} \times 100 =$$

= 25.806 CM

Here,
R = Recombinants
N = Total Progenies
X = Distance in CM

leu-5 *anth* B55

25.806

Fig. 32. Genetic map of *anth* B55 and *leu*-5 {45208 (t)}

Here, 1 Centimorgan = 0.1 Centimeter

Table 66. Distances of the *tryptophan* auxotrophs with the markers of different linkage groups at a glance.

SL No	Mutant Use	Marked Used	Linkage Group of the Markers	Distance of the Mutant with Markers
1	B5 a	*leu*-5 (45208 (t) A	V	*leu-5* *anth*B5 29.545
2	B8 a	*trp*-2 (75001) A	VI	*trp*-2 *anth*B8 24.390
3	B11 a	*leu*-3 (R156) A	I	*ind* B11 *leu*-3 23.225
4	B12 a	*trp*-2 (75001) A	VI	*trp*-2 *anth*B12 30.815
5	B13 a	*leu*-I (33757) A	III	*leu*-I *ind* B13 2.439
6	B14 a	*lys-5* (DS6-85) A	VI	*lys-5* *anth* B14 10.90
7	B17 a	*arg*-2 (33442) A	IV	*Ind* B17 *arg*-2 7.228
8	B21 a	*leu*-I (33757) A	III	*leu*-I *trp*-B21 26.237
9	B22 a	*trp*-2 (75001) A	VI	*anth* B22 *trp*-2
10	B28 a	*trp*-2 (75001) A	VI	*anth* B28 *trp*-2

11	B33 a	*arg*-5 (27947) A	II	*trp* B33 — *arg*-5; 28.571
12	B38 a	*trp*-2 (75001) A	VI	*anth* B38 *trp*-2
13	B41 a	*leu*-I (33757) A	III	*leu*-I — *ind* B41; 12.612
14	B42 a	*trp*-2 (75001) A	VI	*anth* B42 *trp*-2
15	B51 a	*leu*-I (33757) A	III	*leu*-I — *ind*- B51; 20.00
16	B54 a	*arg*-2 (33442) A	IV	*Ind* B54 — *arg*-2; 3.439
17	B55 a	*leu*-5 (45208 (t) A	V	*leu*-5 — *anth* B55; 25.806

9.1 Obtaining of double mutants for making triple point inter allelic cross.

For the preparation of double mutants the *trp* mutants *trp*-B8a, *trp*-B17a, and *trp*-B21a were crossed with *lys*-5 (DS6-85)A, *arg*-2 (33442)A and *leu*-1 (33757)A respectively. The spores of the linked crosses were spread on SM plate. They were kept at 58^{0}C for 50 minutes and then incubated at 25°C for 16 hours. Growing spores were wild. All germinated spores were either single mutants or double mutants. So all germinated spores were isolated in the VM tube supplemented with *trp*+*lys* or *trp*+*arg* or *trp*+*leu*. After 5-6 days all the isolates were tested on three sets of SM plates containing *trp*, *lys* and *trp*+*lys* / *trp*+ *arg* and *trp*+*leu*. The isolates that grew only in *trp*+*lys* or *trp*+*arg* or *trp*+*leu* plates are the desired double mutants that are shown in Table 67.

Table 67. Double mutants obtained

Sl No	Name of the Cross	No. of double mutant	Designation of double mutants
1	*leu*-1(33757)A x *trp B21a*	2	*leu*-1+*B21* ext 2
			leu-1+*B21* ext 4
2	*lys-5* (DS6-85)A x *trp B8a*	5	*lys-5*+*B8* ext 4
			lys-5+*B8* ext 6
			lys-5+*B8* ext 8
			lys-5+*B8* ext 29
			lys-5+*B8* ext 42
3	*arg-2* (33442)A x *trp B17a*	7	*arg-2*+*B17* ext 12
			arg-2+*B17* ext 15
			arg-2+*B17* ext 16
			arg-2+ *B17* ext 17
			arg-2+*B17* ext 19
			arg-2+*B17* ext 20
			arg-2+*B17* ext 24

9.2 Determination of mating type of double mutants

The mating types of the isolated double mutants were determined by crossing with Ema and EmA.

Table 68. Fertility and mating type of double mutants.

Sl No	Designation of the cross	Whether perithecia formed yes/no	Days of initiation of perithecia	Frequency & size of the perithecia	Shedding days	Fertility	Mating type
1	*leu-1+B21*ext 2 x Ema	No	-	-	-	-	-
	*leu-1+B21*ext 2 x EmA	Yes	10	Many Medium	21	Fertile	a
2	*leu-1+B21*ext 4 x Ema	Yes	8	Many, Large	19	Good Fertile	A
	*leu-1+B21*ext 4 x EmA	No	-	-	-	-	-
3	*lys-5+B8* ext 4 x Ema	Yes	8	Many, Large	20	Good Fertile	A
	lys-5+B8 ext 4 x EmA	No	-	-	-	-	-
4	*lys-5+B8* ext 6 x Ema	No	-	-	-	-	-
	*lys-5+B8*ext 6 x EmA	Yes	8	Many, Large	20	Good Fertile	a
5	*lys-5+B8* ext 8 x Ema	Yes	8	Many, Large	19	Good Fertile	A
	lys-5+B8 ext 8 x EmA	No	-	-	-	-	-
6	*lys-5+B8* ext 29 x Ema	No	-	-	-	-	-
	lys-5+B8 ext 29 x EmA	Yes	9	Many Medium	21	Fertile	a
7	*lys-5+B8* ext 42 x Ema	No	-	-	-	-	-
	lys-5+B8 ext 42 x EmA	Yes	8	Many, Large	19	Good Fertile	a

8	*arg-2+B17e*xt12 x Ema	Yes	8	Many, Large	18	Good Fertile	A
	*arg-2+B17*ext 12xEmA	No	-	-	-	-	-
9	*arg-2+B17e*xt15 x Ema	No	-	-	-	-	-
	*arg-2+B17*ext 15xEmA	Yes	11	Many Medium	23	Fertile	a
10	*arg-2+B17e*xt16 x Ema	No	-	-	-	-	-
	*arg-2+B17*ext 16xEmA	Yes	12	Many Medium	22	Fertile	a
11	*arg-2+B17e*xt17 x Ema	Yes	8	Many, Large	18	Good Fertile	A
	*arg-2+B17*ext 17xEmA	No	-	-	-	-	-
12	*arg-2+B17e*xt19 x Ema	No	-	-	-	-	-
	*arg-2+B17*ext 19xEmA	Yes	9	Many, Large	19	Good Fertile	a
13	*arg-2+B17e*xt20 x Ema	No	-	-	-	-	-
	*arg-2+B17*ext 20xEmA	Yes	7	Many, Large	18	Good Fertile	a
14	*arg-2+B17e*xt24 x Ema	No	-	-	-	-	-
	*arg-2+B17*ext 24xEmA	Yes	12	Many Medium	21	Fertile	a

10.1 Making of triple point inter allelic crosses

Some extracts of the double mutants (which belong to linkage group III, IV and VI) were selected for triple points inter allelic crosses. The double mutants were crossed with other tryptophan mutants of either linkage group III or IV or VI respectively.

Table 69. Selected extracts of double mutants for triple points interallelic crosses.

Sl No	Name of the double mutants
1	*leu*-1 + *B21* ext 4A
2	*arg*-2 + *B17* ext 17A
3	*lys*-5 + *B8* ext 8A

Table 70. Fertility of triple point interallelic crosses with tryptophan auxotrophs.

Sl No	Designation of the cross	Whether perithecia formed yes/no	Days of initiation of perithecia	Frequency & size of the perithecia	Whether spore shed yes/no	Shedding days	Fertility
1	*leu*-1 + *B21* ext 4A x *trp B5a*	Yes	7	Many Large	Yes	17	Good Fertile
2	*leu*-1 + *B21* ext 4A x *trp B13a*	Yes	8	Many Large	Yes	17	Good Fertile
3	*leu*-1 + *B21* ext 4A x *trp B14a*	Yes	7	Many Large	Yes	15	Good Fertile
4	*leu*-1 + *B21* ext 4A x *trp B41a*	Yes	7	Many Large	Yes	15	Good Fertile
5	*leu*-1 + *B21* ext 4A x *trp B51a*	Yes	8	Many Large	Yes	16	Good Fertile
6	*arg-2 +B17* ext17A x *B54a*	Yes	8	Many Large	Yes	16	Good Fertile
7	*lys-5* + *B8* ext 8A x *B12a*	Yes	7	Many Large	Yes	15	Good Fertile
8	*lys-5* + *B8* ext 8A x *B22a*	Yes	7	Many Large	Yes	15	Good Fertile

9	*lys-5* + *B8* ext 8A x *B28a*	Yes	8	Many Large	Yes	15	Good Fertile
10	*lys-5* + *B8* ext 8A x *B38a*	Yes	7	Many Large	Yes	16	Good Fertile
11	*lys-5* + *B8* ext 8A x *B42a*	Yes	7	Many Large	Yes	17	Good Fertile

Fig. 33. A fertile cross of *leu-1* + *B21* ext 4A x *B13a*.

11.1 Analysis of triple point interallelic crosses

The spores after being shed by the crosses were spread on SM plates supplemented with lys/arg/leu separately and heat shocked for 50 minutes at 58^0C bath. The plate was then transferred to 25^0C for 16 hours. The germinated and the growing spores were counted. Separately growing spores were isolated in VM tubes supplemented with trp+lys / trp+arg / trp+leu. After 4-5 days conidia from the isolates were tested on SM and SM+lys / SM+arg / SM+leu supplemented plates. The isolates that did not grow in SM but grew on SM+leu / SM+lys / SM+arg were marker recombinants. The isolates which grew on both SM and SM+lys or SM+arg or SM+leu were wilds. The wild types were further analyzed for true wild and pseudo wild by back crossing them with Ema and EmA. The order and position of the mutants were determined on the basis of the proportion of the wild and markers spores (lys or arg or leu) the distance between two mutants was calculated by the following formula.

$$\text{Distance} = \frac{(MR+TWR) \times 2}{N} \times 100 = X \text{ CM}$$

Here,

D = Distance

MR = Marker recombinant.

TWR = True wild recombinant.

N = Total number of progeny.

CM = Centimorgan.

12.1 Determination of true Wilds and pseudo Wilds of the growing spores (Wilds).

The isolates which were found to be wild, were crossed with Ema and EmA in Westergaard's crossing medium. The spores from this cross were spread on VM/SM plates and growing and germinating spores were examined. If there was no germinating spores but only growing spores, it was concluded that the isolate was true wilds. In case where germinating spores were found are the Pseudo wilds.

The wilds of the triple point crosses were crossed with EmA and Ema. Spores were spread on minimal plates. Presence of germinating spores were observed carefully and recorded in Table 71.

Table 71. Analysis of Wild type spores of B13a and B41a into true Wild and pseudo Wild types.

Name of the Crosses	No. of growing spores analysed	Crosses of growing spores with Ema/EmA	Growing spores present/absent	Germinating spores present/absent	Inference on true wild or pseudo wild
leu-1+*B21*ex-4A X *B13*a	47	1	Present	Present	Pseudo wild
		2	Present	Present	Pseudo wild
		3	Present	Absent	True wild
		4	Present	Present	Pseudo wild
		5	Present	Absent	True wild
		6	Present	Absent	True wild
		7	Present	Absent	True wild
		8	Present	Present	Pseudo wild
		9	Present	Absent	True wild
		10	Present	Absent	True wild
		11	Present	Present	Pseudo wild
		12	Present	Absent	True wild
		13	Present	Present	Pseudo wild
		14	Present	Absent	True wild
		15	Present	Present	Pseudo wild
		16	Present	Present	Pseudo wild
		17	Present	Absent	True wild
		18	Present	Present	Pseudo wild

19	Present	Absent	True wild
20	Present	Present	Pseudo wild
21	Present	Present	Pseudo wild
22	Present	Absent	True wild
23	Present	Present	Pseudo wild
24	Present	Absent	True wild
25	Present	Present	Pseudo wild
26	Present	Absent	True wild
27	Present	Present	Pseudo wild
28	Present	Present	Pseudo wild
29	Present	Absent	True wild
30	Present	Absent	True wild
31	Present	Present	Pseudo wild
32	Present	Present	Pseudo wild
33	Present	Present	Pseudo wild
34	Present	Present	Pseudo wild
35	Present	Absent	True wild
36	Present	Present	Pseudo wild
37	Present	Absent	True wild
38	Present	Present	Pseudo wild
39	Present	Present	Pseudo wild
40	Present	Absent	True wild
41	Present	Present	Pseudo wild
42	Present	Present	Pseudo wild
43	Present	Absent	True wild
44	Present	Absent	True wild
45	Present	Present	Pseudo wild
46	Present	Present	Pseudo wild
47	Present	Present	Pseudo wild

leu-1+*B21*ex-4A X *B41*a	12	2	Present	Absent	True wild
		3	Present	Absent	True wild
		4	Present	Present	Pseudo wild
		5	Present	Present	Pseudo wild
		8	Present	Absent	True wild
		10	Present	Absent	True wild
		11	Present	Present	Pseudo wild
		13	Present	Present	Pseudo wild
		15	Present	Absent	True wild
		17	Present	Present	Pseudo wild
		18	Present	Absent	True wild
		19	Present	Absent	True wild

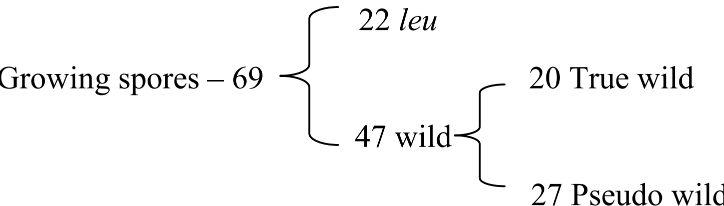
12.2 Determination of order and distance of *trp B13a*and *B21* ext 4A with respect to *leu-1*

Designation of the cross: *leu-1* + *B21* ext 4A x *B13a*

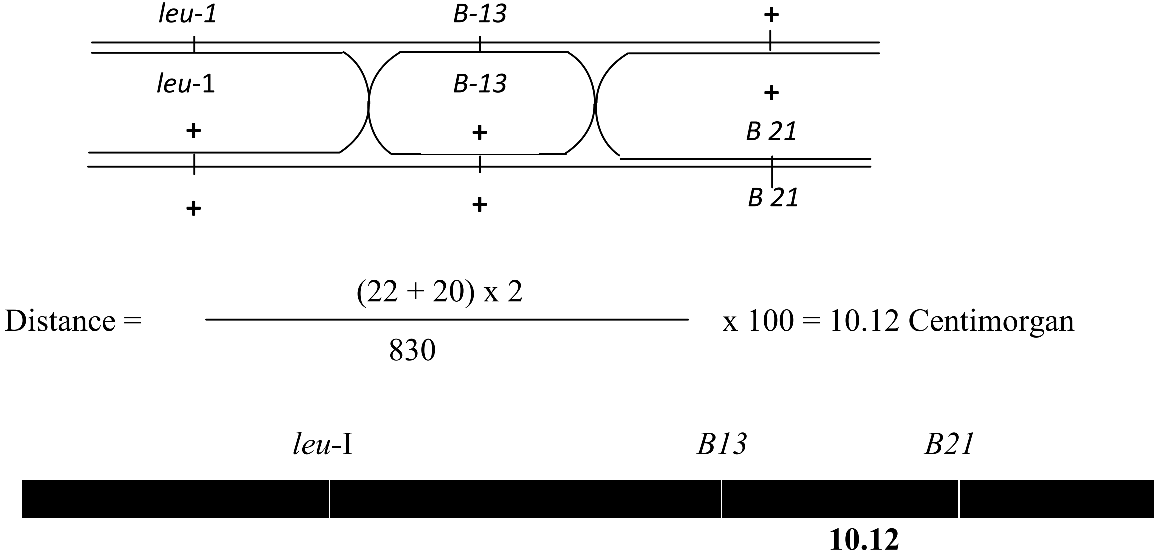
Table 72. Total no of germinating and growing spores on SM + leucine plate from the cross"*leu-1* + *B21* ext 4A x *B13a*"

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
leu-1 + *B21* ext 4A x *B13a*	761	69	830

Analysis of growing spores into *leu-1* and wild type.



Order of the *trpB13* and *trpB21* with respect to *leu*-1



$$\text{Distance} = \frac{(22 + 20) \times 2}{830} \times 100 = 10.12 \text{ Centimorgan}$$

Fig. 34.Order and Distance of *trpB13* and *B21*

Here, 1 Centimorgan = 0.30 Centimete

12.3 Determination of order and distance of *trp B41a*and *B21* ext 4A with respect to *leu-1*

Designation of the cross: *leu-1* + *B21* ext 4A x *B41a*

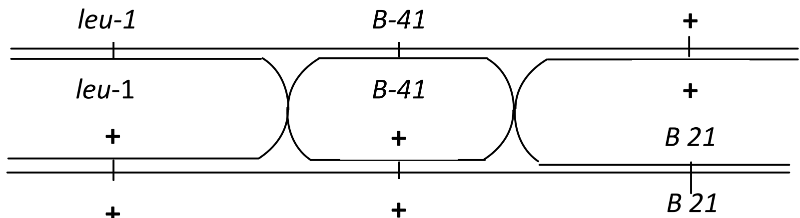
Table 73. Total no of germinating and growing spores on SM + leucine plate from the cross“*leu-1* + *B21* ext 4A x *B41a*”

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
leu-1 + *B21* ext *4A* x *B41a*	548	20	568

Analysis of growing spores into *leu-1* and wild type.

Growing spores – 20
- 8- *leu*
- 12-wild
  - 7- True wild
  - 5- Pseudo wild

Order of the *trpB41* and *trpB21* with respect to *leu*-1



$$\text{Distance} = \frac{(8 + 7) \times 2}{568} \times 100 = 5.281 \text{ Centimorgan}$$

leu-1 *B 41* *B 21*

5.281

Fig.35.order and distance of *trpB41* and *B21*

Here, 1 Centimorgan = 0.30 Centimeter

12.4 Determination of order and distance of *trp B51a*and *B21* ext 4A with respect to *leu-1*

Designation of the cross: *leu-1* + *B21* ext 4A x *B51a*

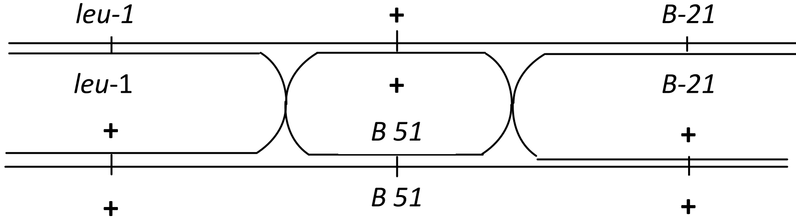
Table 74. Total no of germinating and growing spores on SM + leucine plate from the cross"*leu-1* + *B21* ext 4A x *B51a*"

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
leu-1 + *B21* ext *4A* x *B51a*	414	10	424

Analysis of growing spores into *leu-1* and wild type.

Growing spores – 10
- 6 *leu*
- 4 wild
  - 4 True wild
  - 0 Pseudo wild

Order of the *trpB51* and *trpB21* with respect to *leu*-1



$$\text{Distance} = \frac{(6 + 4) \times 2}{424} \times 100 = 4.71 \text{ Centimorgan}$$

Fig. 36.order and distance of *trpB51* and *B21*

Here, 1 Centimorgan = 0.30 Centimet

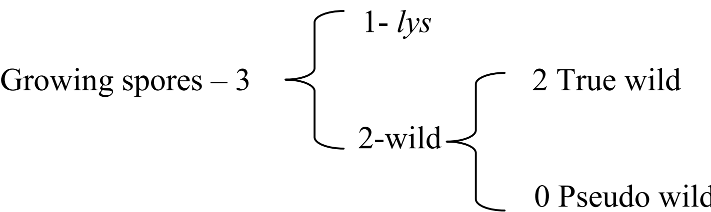
12.5 Determination of order and distance of *trp B12a*and *B8* ext 8A with respect to *lys-5*

Designation of the cross: *lys-5* + *B8* ext 8A x *B12a*

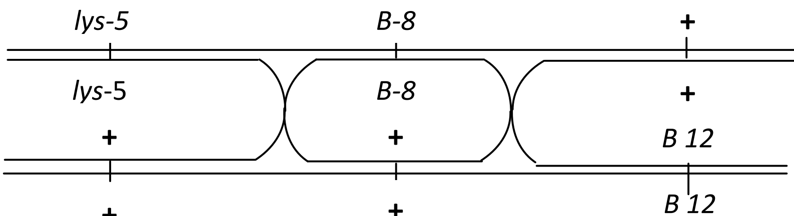
Table 75. Total no of germinating and growing spores on SM + lysine plate from the cross "*lys-5* + *B8* ext 8A x *B12a*"

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
lys-5 + *B8* ext *8A* x *B12a*	664	3	667

Analysis of growing spores into *lys-5* and wild type.



Order of the *trpB12* and *trpB8* with respect to *lys*-5



$$\text{Distance} = \frac{(1 + 2) \times 2}{667} \times 100 = 0.89 \text{ Centimorgan}$$

Fig. 37.order and distance of *trpB8* and *B12*

Here, 1 Centimorgan = 0.30 Centimeter

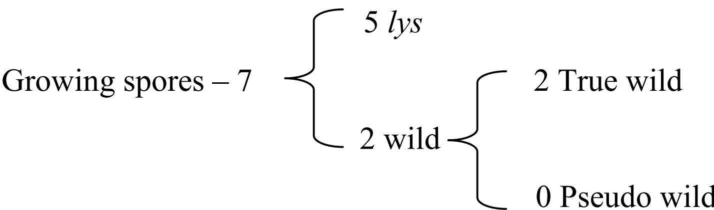
12.6 Determination of order and distance of *trp B22a*and *B8* ext 8A with respect to *lys-5*

Designation of the cross: *lys-5* + *B8* ext 8A x *B22a*

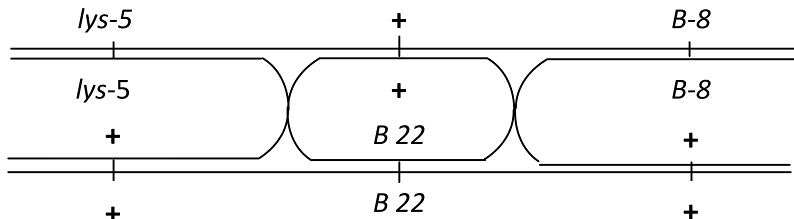
Table 76. Total no of germinating and growing spores on SM + lysine plate from the cross "*lys-5* + *B8* ext 8A x *B22a*"

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
lys-5 + *B8* ext *8A* x *B22a*	590	7	597

Analysis of growing spores into *lys-5* and wild type.



Order of the *trpB22* and *trpB8* with respect to *lys*-5



$$\text{Distance} = \frac{(5 + 2) \times 2}{597} \times 100 = 2.345 \text{ Centimorgan}$$

Fig. 38.order and distance of *trpB22* and *B8*

Here, 1 Centimorgan = 0.30 Centimeter

12.7 Determination of order and distance of *trp B28a*and *B8* ext 8A with respect to *lys-5*

Designation of the cross: *lys-5* + *B8* ext 8A x *B28a*

Table 77. Total no of germinating and growing spores on SM + lysine plate from the cross"*lys-5* + *B8* ext 8A x *B28a*"

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
lys-5 + *B8* ext *8A* x *B28a*	680	0	680

Analysis of growing spores into *lys-5* and wild type.

Growing spores – 0 { 0 *lys* / 0 wild

Order of the *trpB28* and *trpB8* with respect to *lys*-5

$$\text{Distance} = \frac{(0 + 0) \times 2}{680} \times 100 = 0.00 \text{ Centimorgan}$$

B 28
lys-5 *B 8*

Fig. 39.order and distance of *trpB28* and *B8*

12.8 Determination of order and distance of *trp B38a*and *B8* ext 8A with respect to *lys-5*

Designation of the cross: *lys-5* + *B8* ext 8A x *B38a*

Table 78. Total no of germinating and growing spores on SM + lysine plate from the cross"*lys-5* + *B8* ext 8A x *B38a*"

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
lys-5 + *B8* ext *8A*x *B38a*	770	0	770

Analysis of growing spores into *lys-5* and wild type.

Growing spores – 0 { 0 *lys*, 0 wild }

Order of the *trpB38* and *trpB8* with respect to *lys*-5

$$\text{Distance} = \frac{(0 + 0) \times 2}{770} \times 100 = 0.00 \text{ Centimorgan}$$

B 38
B 8
lys-5

Fig. 40.Order and distance of *trpB38* and *B8*

12.9 Determination of order and distance of *trp B42a*and *B8* ext 8A with respect to *lys-5*

Designation of the cross: *lys-5* + *B8* ext 8A x *B42a*

Table 79. Total no of germinating and growing spores on SM+ lysine plate from the cross "*lys-5* + *B8* ext 8A x *B42a*"

Name of the crosses	Number of germinating spores	Number of growing spores	Number of total spores
lys-5 + *B8* ext *8A* x *B42a*	759	0	759

Analysis of growing spores into *lys-5* and wild type.

Growing spores – 0 { 0 *lys*, 0 wild }

Order of the *trpB42* and *trpB8* with respect to *lys*-5

$$\text{Distance} = \frac{(0 + 0) \times 2}{759} \times 100 = 0.00 \text{ Centimorgan}$$

B 42
lys-5 *B 8*

Fig. 41.Order and distance of *trpB42* and *B8*

Table 80. Order and distances of mutants of linkage group III and VI

Name of the cross	Linkage group of markers	Number of growing spores	Number of germinating spores	Total number of spores	Marker recombinant	wilds	Order and distance
*leu*1+B21 ext 4A x B13a	III	69	761	830	22	47	*leu-1* — *B13* — *B21*; 10.12
*leu*1+B21 ext 4A x B41a	III	20	548	568	8	12	*leu-1* — *B41* — *B21*; 5.281
*leu*1+B21 ext 4A x B51a	III	10	414	424	6	4	*leu-1* — *B51* — *B21*; 4.71
*lys*5+B8 ext 8A x B12a	VI	3	664	667	1	2	*lys-5* — *B8* — *B12*; 0.89
*lys*5+B8 ext 8A x B22a	VI	7	590	597	5	2	*lys-5* — *B22* — *B8*; 2.345
*lys*5+B8 ext 8A x B28a	VI	0	680	680	0	0	*lys-5* — *B28* *B8*
*lys*5+B8 ext 8A x B38a	VI	0	770	770	0	0	*lys-5* — *B38* *B8*
*lys*5+B8 ext 8A x B42a	VI	0	759	759	0	0	*lys-5* — *B42* *B8*

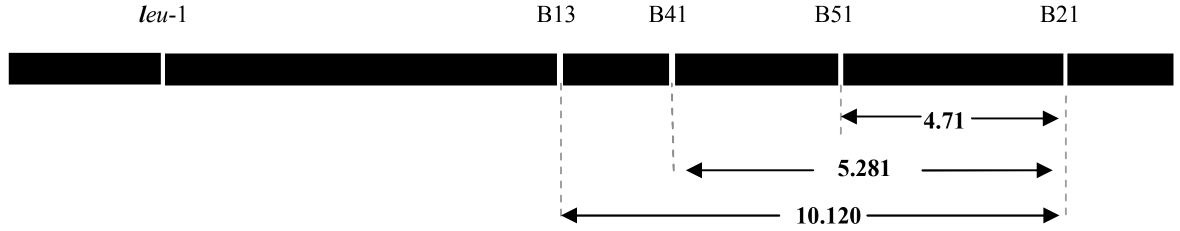


Fig. 42. Fine structure map of *trp*-1 mutants showing order and distances of *trp*-1 mutants.

Here, 1 Centimorgan = 0.30 Centimeter

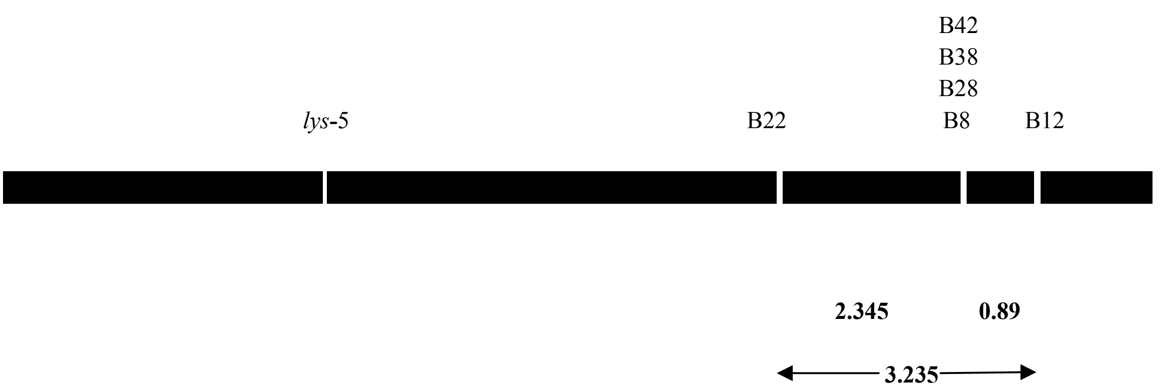


Fig. 43. Fine structure map of *trp*-2 locusshowing order and distances of *trp*-2 mutants.

Here, 1 Centimorgan = 0.90 Centimeter

13.1 CROSS FEEDING TEST OF *tryptophan* AUXOTROPHS

Vogel's Minimal medium supplemented with 1 mg tryptophan for 100 ml media were used for cross-feeding test. Culture filtrates were prepared from each culture and then mutant inoculated in each filtrate as mentioned under media and methods. Observations on growth of mutants in each culture filtrate were taken after 24, 48, 72 and 168 hours as shown in Fig. 44.

Fig. 44. Result of cross-feeding test of *tryptophan* mutants.

← *tryptophan* mutants →

Fill trade of *tryptophan* mutants

	B5	B8	B12	B14	B22	B28	B38	B42	B55	B11	B13	B17	B41	B51	B54	B21	B33
B5	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B8	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B12	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B14	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B22	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B28	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B38	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B42	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B55	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
B11	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○
B13	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○
B17	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○
B41	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○
B51	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○
B54	●	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○
B21	●	●	●	●	●	●	●	●	●	⊙	⊙	⊙	⊙	⊙	⊙	○	○
B33	●	●	●	●	●	●	●	●	●	⊙	⊙	⊙	⊙	⊙	⊙	○	○

Notes. ○ = no growth, ⊙ = mycelial growth, ● = conidial growth.

14.1 Classification of mutants on the basis of cross-feeding test.

The mutants were classified into three groups, Group-A, Group-B and Group-C. Nine anthracitic utilizing mutants belong to Group-A (B5a, B8a, B12a, B14a, B22a, B28a, B38a, B42, B55a). Six indole utilizing mutants belong to Group-B (B11a, B13a, B17a, B41a, B51a, B54a). Two trytophan utilizing mutants belong to group-C (B21a, B33a).

Fig. 45. Classification of tryptophan mutants on the basis of cross-feeding test.

Filtrate of Mutants	Mutant of		
	Group-A	Group-B	Group-C
Group-A	○	○	○
Group-B	●	○	○
Group-C	●	⊙	○

DISCUSSION

Fresh conidia of Ema of *Neurospora crassa* were treated with 1.16% concentration of Formaldehyde (HCHO) solution for ten minutes and produced four types of morphological mutants (*albino, fluffy, ropy* and *vigorous*).

Treating of conidia with 3% concentration of Ethyl-methane sulphonate (EMS) for twelve hours produced five types of morphological mutants (*albino, conidial-band, buff, fluffy* and *ropy*).

UV irradiation of Ema at a wave length of 254 nm for ninety seconds produced six types of morphological mutants (*albino, buff, conidial-band, fluffy, ropy* and *vigorous*) and three types of bio-chemical mutants *tryptophan, leucine* and *arginine*.

The mutants *albino, fluffy* and *ropy* are common in all of the three mutagens. The highest frequency of the group A (*albino*) was 36.486% and the lowest frequency of the group D (*fluffy*) was 9.459%. This variation in the frequency can easily be explained by assuming that few loci are easily mutable than the others. This observation is in keeping with earlier investigation by Dutta (1982) and Chowdhury (1974).

Mozmader and Haque (1995) obtained four groups (*checked, conidial-band, ropy and cauliflower)* and Keya (2009) found five groups of morphological mutants (*checked, conidial-band, ropy, fluffy* and *mat*). Rahim (2001) treated formaldehyde of different concentrations and found eight different groups of mutants which are almost similar with that of the author.

EMS was used as mutagen on *Schizophyllum commune, Rhizobium metialoti, Neurospora crassa, Saccharomyces cerevisiae, Drosophilla melanogaster* : Raper *et al.* (1965), Mikaelson *et al.* (1971), Kaul and Bhan (1977), Gree and Well-man (1980), Miasniankina *et al.* (1983), Cid *et al.* (1994), Kraepiel *et al.* (1994) and Rahim (2001).

On the whole the author obtained six groups of morphological mutants (*albino, buff, conidial-band, fluffy, ropy* and *vigorous*) by using HCHO, EMS and UV ray. One mutant of each morphological group was selected for further study. The studies included

germination of conidia, radial growth, linear growth, weight of dry mycelia, fertility and mating type. The seventeen *tryptophan* mutants were also selected for detailed genetical studies like complementation, linkage and fine structure (Table 15, 32, 66 & 80 and Fig. 8, 10, 16, 44 & 45).

On the basis of their specific growth requirements physiological diversity was detected amongst the 17 *tryptophan* auxotrophs. The mutant fall into three categories namely anthranilic acid requiring mutants, indole requiring mutants and tryptophan requiring mutants. Nine anthranilic acid mutants are (B5, B8, B12, B14, B22, B28, B38, B42 and B55). Six indole requiring mutants are (B11, B13, B17, B41, B51 and B54). Two tryptophan requiring mutants are B21 and B33. The result is in keeping with the findings of Ahmad and Catcheside (1960). But differs with the findings of Suyama *et al.* (1964), Ahmad *et al*. (1964) and Ahmad and Islam (1969). The mutants of Ahmad and Catcheside belonged to four genetic loci; *trp*-1*,* *trp*-2, *trp*-3 and *trp*-4. Whereas the mutants induced by the author fall into five genetic loci; *trp*-1*,* *trp*-2, *trp*-3, *trp*-4 and *trp*-5.

Time required for germination of conidia of some selected mutants and Ema was variable. Wild type Ema and *vigorous* mutant germinated after 5 hours but *albino* mutant germinated after 10 hours (Table 9).

Comparative study of radial growth of Ema and selected mutants showed variation among themselves. Growth measured after 3 hours interval up to 24 hours. The maximum growth (full growth) obtained in the mutant *vigorous* and Ema within 21 hours and minimum growth found in mutant *albino* within the same period of time (Table 10).

Growth on race tube (Ryan *et. al* 1943) is also helpful for accurate measurement of liner growth. After 96 hours the linear growth of Ema was 29.0 cm. The maximum growth of mutant (*vigorous*) was 32.3 cm and the minimum growth in case of mutant (*albino*) was 8.5 cm (Table 11 and Fig. 4).

The author studied dry mycelial weight of some selected mutants and Ema. After 72 hours dry mycelial weight of Ema was 0.305g. The highest mycelial weight obtained in (*vigorous*) mutant was 0.33g and lowest weight obtained in case of (*albino*) was 0.12g (Table 12, Fig. 7).

The fertility and mating types are of 17 *tryptophan* mutants were checked by crossing them with EmA and Ema. B5, B8, B12, B14, B21, B28, B33, B38, B51, B54 and B55 mutants were found fertile with EmA and their mating types are “a”. But mutant B11, B13, B17, B22, B41 and B42 were found fertile with Ema and their mating types are “A”. Their mating type was changed during mutation. Similar observation was made by Ahmad *et al.* (1972). Different mutants behaved differently some are good fertile and some are fertile in their fertility. No sterile crosses were found. Perithecia were formed in 8-12 days and spores were shed after 18-22 days. In case of good fertile crosses perithecia were formed after 8 days and spore shed after 18 days (Table 13).

Complementation test of all the seventeen *tryptophan* mutants were made with all the *tryptophan* standard markers- *trp*-1 (10575), *trp*-2 (75001), *trp*-3 (C83), *trp*-4 (Y2198) and *trp*-5 (A420). It was found that four mutants (B11, B17, B41 and B51) belong to *trp*-1. Seven mutants (B8, B12, B14, B22, B28, B38 and B42) belong to *trp*-2, two mutants (B21 and B33) belong to *trp*-3, two mutants (B13 and B54) belong to *trp*-4 and two mutants (B5 and B55) belong to *trp*-5 (Fig. 8 and Table 14).

Interallelic complementation studies of four *trp*-1 auxotrophs reveal two heterocaryon groups (I and II) and two complons A and B (Fig.10).

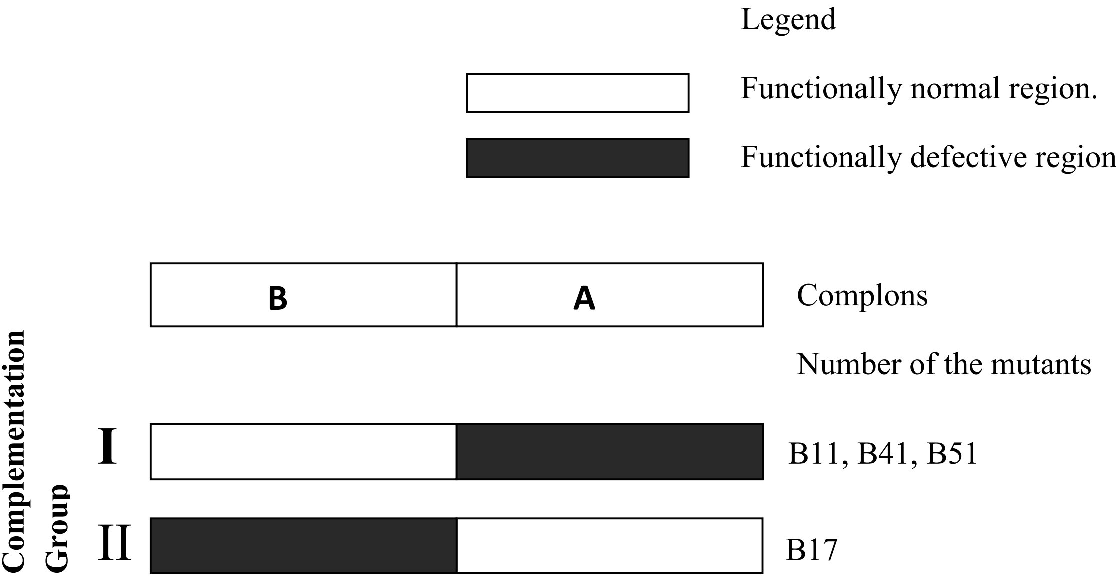


Fig.10. Complementation map of four *trp-1* mutants.

Interallelic complementation studies of seven *trp*-2 auxotrophs reveal three complementation groups (I, II and III) and two complons A and B (Fig.12).

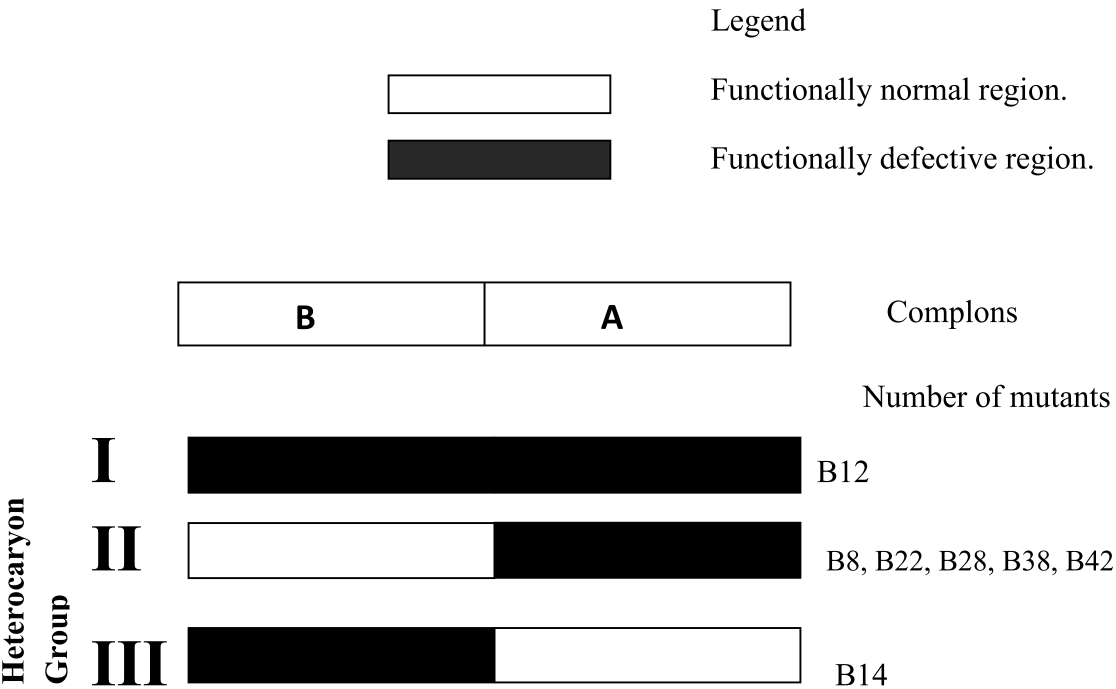


Fig.12. Complementation map of seven *trp-2* mutants.

Auxotrophs of *trp*-3, *trp*-4 and *trp*-5 do not show any interallelic complementation and no heterocaryon grouping among themselves (Fig. 13-15).

Complementation map of locus *trp*-1 and *trp*-2 is linear and continuous. Catcheside (1964), Lutfor and Mozmader (1999) also have got linear complementation map in *trp*-1 and *arginine* mutants. Whereas the complementation map of *trp*-3 (Ahmad and Islam 1969), *lys*-5 (Ahmad *et al.* 1981) and *ade*-8 (Kapular and Bernstein 1963) show discontinuous defects in the case of a few mutants. These differences in the complementation maps for *trp*-1, *trp*-2 as compared to *trp*-3, *lys*-5 and *ade*-8 may follow from differences in the folding of the respective enzyme molecules, the enzyme specified by *trp*-3, *lys*-5 and *ade*-8 being possibly folded in a more complex manner than the enzyme synthesized by *trp*-1 and *trp*-2.

Linkage studies of seventeen *tryptophan* auxotrophs with standard markers representatives of all the seven linkage groups revealed that *trp*- B13a, *trp*-B21a, *trp*-B41a and *trp*-B51a are linked with *leu*-1 (33757) of linkage group III; *trp*-B8a, *trp*-B12a, *trp*-B14a, *trp*-B22a, *trp*-B28a, *trp*-B38a and *trp*- B42a are linked with *trp*-2 (75001) of linkage group VI; *trp*-B17a, and *trp*-B54a are linked with *arg*-2 (33442) of linkage group IV; *trp*-B-5 and *trp*-B-55a are linked with *leu*-5 {45208(t)} of linked group V; *trp*-B11a and *trp*-B33a are linked with *leu*-3 (R156) of linkage group I and *arg* -5 (27947) of linkage group II respectively (Table 66).

Later on, few mutants were found to grow on minimal medium. Mutants have occasionally shown a tendency to become or start growing. It seems that this follows through mutation which restores the capacity to synthesize the enzyme lacking in the mutants as a result of the damage done to the original gene by the ultraviolet rays (Giles 1951, Kolmark and Westergaard 1949). Since mutation would change only such nuclei as have come to exit as a result of multiplication of the mutants nucleus each culture which shows reversion in the above manner is expected to carry some original mutant nuclei which have not undergone mutation.

The author took a much more thorough and detailed study of the order as well as distances of these ten non growing mutants through triple point interallelic crosses. The *tryptophan* mutants (anthranilic acid, indole and tryptophan) were crossed with respective double mutants and their order and distances were found (Table 80).

Triple point inter allelic crosses of *trp*-1 mutant B13 with bouble mutant (*B21+leu-1*) and *trp*-1 mutant B41 with (*B21+leu-1*) gave high percentage of prototroph frequencies (Table 71). The formation of a abnormally high proportion of prototrophs in interallelic crosses has been assigned to many causes :-

The formation of pseudo-wilds are due to the recovery of n-1 spores, (Ahmad *et al.* 1969 and Mitchell *et al.* 1952).

The formation of a normal locus as a result of unequal crossing over (Bausum and Wagner 1965, DeMerec 1962, Magni and Von Borstel 1962 and Mitchell 1955a).

The increase in frequency of recombination due to the presence of genes which accelerate recombination (Catcheside *et al*. 1964, Jha 1967, Smith 1966, 1971 and 1973a and b). Chalmers and Seale 1971).

The presence of suppressor and super suppressors (Chalmers and Seale 1971, Yourno and Suskind 1964).

The presence of genes which accelerate reversion (Demerec 1963, Mortimer and Fogel 1974 and Stadler 1959).

The presence of double crossing overs; (Stadler 1956 and 1973).

The effect of flanking markers (Fincham 1966, Thomas and Catcheside 1969).

The data presented here in the preceding pages showed that the high member of prototrophs formed in the interallelic crosses by few mutants of locus *trp*-1 followed from the formation of pseudo wilds. But this formation of a high proportion of prototrophs by other mutants of *trp*-1 locus can not be attributed to the formation of pseudo wild only. It seems that the recombination phenomenon is not governed by a single process or factor but by many factors. (Case and Giles 1958, Catcheside 1966, Foss *et al*. 1993a, Irelan *et al*. 1994, Perkins *et al*. 1992, Rockmill *et al*. 1995, Simchen *et al*. 1969, Sobell 1972 and 1974).

Fine structure map of *trp*-1 (indole utilizing) mutants with *leu*-1 (33757) as a marker comprises about 10.120 Centimorgan (Fig. 42).

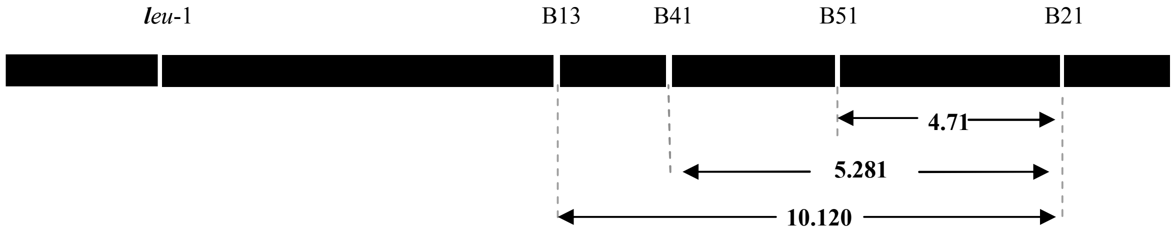


Fig. 42. Fine structure map of *trp*-1 mutants showing order and distances of *trp*-1 mutants.

Here, 1 Centimorgan = 0.30 Centimeter

The indole mutant B13 of linkage group III occupies the extreme left position and B21 occupied the extreme right position and their distance is about 10 Centimorgan.

Fine structure map of *trp*-2 (anthranilic acid utilizing) mutants with *lys* -5 (DS 6-85) comprises 3.235 Centimorgan (Fig. 43).

Fig. 43. Fine structure map of *trp*-2 locus showing order and distances of *trp*-2 mutants.

Here, 1 Centimorgan = 0.90 Centimeter

The length occupied by the anthranilic acid mutant of *trp*-2 locus is about 3.325 Centimorgan. The anthranilic acid mutant B12 occupied the extreme right position and B22 occupies the extreme left position and distances between B12 and B22 is about 3 Centimorgan.

It is further seen that few mutants of *trp*-2 are clustered in the same position as *trp*-2 B8. The reason occupied by *trp*-2 B8 and other three mutants (*trp*-2 B28, *trp*-2 B38 and *trp*-2 B42) seem to be easily mutated reason of the locus i.e hot-spot (Fig. 43). The results is in keeping with the findings of Ahmad *et al*. (1969) in *trp*-3 locus and Ahmad *et al*. (1981) in *lys*-5 mutants but varies from the findings of Ahmad *et al*. (1979) in *lys*-3 mutants where no such hot-spot was detected.

The absence of any recombinant of *trp*-2 mutants can easily be explained by postulating defects in the same functional sub units. Mutants for *trp*-1 and *trp*-2 seem to have defects in two functional sub units and mutants of *trp*-3, *trp*-4 and *trp*-5 each has defect only in one functional unit (Fig.10, 12-15).

Considering the map length occupied by some other loci in *Neurospora crassa* it is seen that trp-3 comprises 0.322 map unit (Ahmad *et al.* 1963) Pyrimidine -3 comprises 0.2 map units (Woodward 1962), Pantothanic -2 comprises about 0.338 map units (Case and Giles 1960), and three isoleucine and valine together occupy a segment of not more than four map units (Wagner, Somers and Barquist 1960). *leu*-2 comprises about 1.08 (Ahmad *et al*. 1976), *lys*-5 comprises about 1.32 map units (Ahmad *et al*. 1981), *lys*-4 comprises about 5.7 map units (Ahmad et al 1979a and b). The length occupied by *trp*-1 locus comprises 10.120 Centimorgan which is higher than *lys*-4 locus. The length occupied by *trp*-2 therefore compares favorably with that of reported for the *iv*.

An additional point of interest is the possible relationship between the complementation maps presented here and recombination maps of the *trp*-1and *trp*-2 locus (Fig. 10, 12, 42, 43) Since in these maps, mutant strains can be arranged in a linear sequence. It is of interest to see that there is no close correlation between the placements of alleles in each of the map.

The problem of relation between genetic and complementation map has been examined in considerable detail at the Pan-2 locus in *Neurospora crassa* by (Case and Giles 1960). They concluded that there is a good over all correspondence between the two maps although there are instances on their maps in which the placement of alleles is not identical. In *trp*-3 a similar situation for the locus has been noticed in which complementation can occur between two alleles and yet no genetic recombination between these alleles has been detected. On that basis they indicated that the complementation is not necessarily a reflection of the genetic map.

Cross-feeding test of seventeen *tryptophan* mutants were done. Culture filtrates were prepared from each cultures and then each mutant was inoculated in each filtrate as mentioned under media and method. Observations on growth of mutants in each culture

filtrate were taken after 24, 48, 72 and 168 hours. The mutants were classified into three groups. Group-A, group-B and group-C. Nine anthranilic acid utilizing mutants (B5a, B8a, B12a, B14a, B22a, B28a, B38a, B42a and B55a) belong to group-A. Six indole utilizing mutants (B11a, B13a, B17a, B41a, B51a and B54a) belong to group-B. Two *tryptophan* utilizing mutants (B21a and B33a) belong to group-C.

Group-A mutants grow in the filtrate of group-B and group-C mutants. Group-B mutants grow poorly in the filtrate of group-C mutants only. Group-C mutants do not grow in the filtrate of either group-A and group-B mutants (Fig. 44 & 45).

The genetical and biochemical studies of *histidine* requiring mutants of *Neurospora crassa* by Webber (1960), Webber and Case (1960) and Mozmader (1996) also revealed evidence concerning heterogeneity of *his*-3 and *trp*-1 mutants respectively. It can be concluded that on the basis of cross-feeding test *tryptophan* auxotrophs can be classified in to three classes only and not in to six biochemical classes as shown by De Moss *et al.* (1967).

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